Mycologia, 83(3), 1991, pp. 296–302.

MEDIA FOR SELECTIVE ISOLATION OF HYMENOMYCETES

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ABSTRACT

The growth rates of 32 hymenomycetes and non-hymenomycetes were compared on five media that have been devised for isolation of hymenomycetes. A medium with 2 mg/L benomyl permitted virtually uninhibited growth of hymenomycetes while strongly inhibiting many non-hymenomycetes, but members of the Mucorales and several other species still grew well. Testing of various combinations and concentrations of ingredients led to the use of 2 mg/L benomyl and 2 mg/L dichloran as antifungal ingredients. This was tested in isolations from various field materials and in every case performed as well as or better than other commonly used media.

Key Words: hymenomycetes, selective media, benomyl, orthophenylphenol

Partly because of their unique ability to rapidly degrade wood, hymenomycetes are an economically and ecologically important group of fungi. They are among the most damaging pathogens of woody plants, cause losses of wood products in service, and probably decompose the majority of the fixed carbon in terrestrial ecosystems. There is also a growing interest in biotechnological application of these fungi in the wood-using industries.

Although many media have been devised for the semi-selective isolation of hymenomycetes (**1–12**), no comparisons have been reported that would allow one to rationally choose among them.

o-Phenylphenol was recommended for general isolation of basidiomycetes (**9**), and has been used more recently for isolating *Armillaria* species (**7**, **13**). Kuhlman and Hendrix (**4**) felt that it was too inhibitory to *Heterobasidion annosum* (Fr.) Bref. and devised a medium using peptone as a carbon source with pentachloronitrobenzene (PCNB) as the main antifungal ingredient. Vaartaja (**12**), apparently independently, combined PCNB and o-phenylphenol along with the antifungal antibiotic nystatin in a medium that was used for isolating a variety of basidiomycetes. An acidified medium containing o-phenylphenol along with copper-chrome-arsenate (a wood preservative) was devised for isolation of *Phaeolus schweinitzii* (Fr.) Pat. from soil (**11**). However, it was intentionally inhibitory to other hymenomycetes.

Benomyl has been used at 15 mg/L for isolating *Armillaria* species (**6**). For general isolation of basidiomycetes, it has been used at 5 mg/L (**10**) and, along with dichloran and phenol, at 8 mg/L (**2**). Maloy (**5**) showed that at 5 mg/L and above, benomyl was inhibitory to some hymenomycetes, but at 1 mg/L it inhibited several deuteromycetes and had little or no effect on a large number of hymenomycetes. It has been effectively used at 2 mg/L (**8**).

The objective of this study was to determine which is the best isolation medium for hymenomycetes. Combinations and concentrations of selective ingredients were explored in an effort to improve described media.

MATERIALS AND METHODS

Cultures.—Wood-decay hymenomycetes were chosen for testing based on their economic importance and taxonomic diversity (TABLE I). Non-hymenomycetes were chosen to represent important wood-inhabiting groups and in particular those that commonly interfere with isolation of hymenomycetes on selective media.

Media.—In some original formulae for media, the chemicals used or methods of sterilization are ambiguous. For clarity and convenience, the formulae that I used are described together here. Media were autoclaved for 30 min and cooled to about 50 C before any post-autoclave ingredients were added. Twenty-five ml of medium was dispensed in 90-mm-diam Petri plates.

BDP (**2**): One liter of potato dextrose agar (Difco) prepared according to label directions, plus (added after autoclaving) 5 ml of a stock solution containing 1 g phenol, 0.32 g Benlate 50% W.P. (50% benomyl), and 0.16 g dichloran

TABLE I

ISOLATES USED, THEIR TAXONOMIC AFFINITIES, AND THEIR GROWTH RATES ON MEA

	Fungus	Isolate No.	Growth on MEA, mm/wk
Mucorales			
Mortierellaceae	*Mortierella isabellina* Oud. & Koning	P62[a]	25
	M. spinosa Linn.	N11	53
Mucoraceae	*Gongronella* sp.	P123[a]	24
	Mucor hiemalis Wehmer	158[a]	82
	M. ramannianus[b] Möller	N10	31
	Rhizopus arrhizus[b] Fischer	N12	175
Ascomycetes	*Daldinia concentrica* (Bolt.:Fr.) Ces. & DeNot.	N7[c]	70
	Ophiostoma minus (Hedgc.) Syd. & P. Syd.	N4[d]	19
	Xylaria polymorpha (Pers. ex Mér.) Grev.	N5	22
Deuteromycetes	*Alternaria alternata* (Fr.) Keissler	CAR27A[a]	40
	Aspergillus niger v. Tiegh.	CAR32[a]	31
	Fusarium negundi Sherb.	N6	32
	Penicillium diversum Raper & Fennell	D16[a]	13
	Phialocephala dimorphospora Kendrick	P109[a]	12
	Trichoderma harzianum Rifai	EP22[a]	105
	T. koningii Oud.	P947[a]	137
Agaricales	*Armillaria calvescens* Berube & Dessureault	80	3
	A. gemina Berube & Dessureault	64	5
	A. gallica Marxm. & Romagn.	97	9
	A. mellea (Vahl.:Fr.) Kummer	312	4.5
	A. ostoyae (Romagn.) Herink	29	3.5
	Lentinus edodes (Burk.) Sing.	301[e]	22
Aphyllophorales			
Corticiaceae s.l.	*Coniophora puteana* (Schum.:Fr.) Karst.	61	34
	Merulius tremellosus Fr.	63[f]	41
	Serpula lacrymans (Wulf.:Fr.) Schroet.	28	2.5
Stereaceae	*Stereum subtomentosum* Pouzar	5	24
Polyporaceae s.l.	*Ganoderma tsugae* Murr.	58	11
	Heterobasidion annosum (Fr.) Bref.	31	47
	H. annosum	41	33
	Perenniporia subacida (Pk.) Donk	2	32
	Phellinus pini (Thore.:Fr.) A. Ames	4	11
	Trametes versicolor (L.:Fr.) Pilát	1	45

[a] Provided by C. J. K. Wang; her numbers used.
[b] Sensu Zycha and Siepmann (**14**).
[c] Provided by K. E. Hammell.
[d] Provided by T. C. Harrington; his no. C189.
[e] Purchased from the culture collection of Pennsylvania State University; their no. WC 305.
[f] Provided by R. A. Blanchette; his no. 23.

(2,6-dichloro-4-nitroaniline) per 100 ml 50% ethanol. The stock solution was refrigerated for up to two weeks.

MEA: 15 g dried malt extract (Difco) and 15 g agar per L of water.

BSMA (**8**): MEA plus 10 ml Benlate stock (40 mg/100 ml H_2O) and (added after autoclaving) 100 mg streptomycin sulfate. The Benlate did not completely dissolve in the stock solution or medium.

OPP (**7**): 30 g malt extract and 15 g agar per L of water, plus (added after autoclaving) 6.6 ml 2.5 N (18.7%) lactic acid and 4 ml 1% Na-OPP (2-phenylphenol, sodium salt tetrahydrate).

PPP (**3**, **4**): 5 g Bacto peptone, (Difco) 20 g agar, 0.25 g $MgSO_4 \cdot 7H_2O$, 0.5 g KH_2PO_4, 0.2 g pentachloronitrobenzene (PCNB) and 0.13 g sodium deoxycholate per L of water, plus (added after autoclaving), 50 mg penicillin G, 1.3 ml 85% lactic acid, and 20 ml 95% ethanol.

PON (**12**): 2 g sucrose, 10 mg PCNB, 10 mg Na-OPP, and 12 g agar per L of water, plus (added after autoclaving) 50 mg streptomycin sulfate and 8 mg nystatin (mycostatin).

TABLE II

COMPARISON OF ANTIFUNGAL INGREDIENTS AND CONCENTRATIONS IN THE MEDIA TESTED

Ingredients	Media					
	Concentration (mg/L)					
	BDP	BSMA	OPP[a]	PON	PPP[a]	BDS
benomyl	8	2	—	—	—	2
dichloran	8	—	—	—	—	2
phenol	50	—	—	—	—	—
Na-OPP	—	—	40	10	—	—
PCNB	—	—	—	10	200	—
nystatin	—	—	—	8	—	—

[a] Acidified.

Concentrations of the antifungal ingredients are summarized in TABLE II. pH was measured on solidified media using a flat-surface pH electrode. Results were: MEA, 5.7; BSMA, 5.5; BDP, 5.5; PPP, 4.2; OPP, 3.8; PON, 6.2.

Based on results with those published media, several new combinations and concentrations of antifungal ingredients were tested. In one experiment, MEA was amended with 2 mg/L benomyl (added as in BSMA, above), 4 mg/L dichloran (added as a stock solution of 10 mg in 50 ml of 50% ethanol), 2 mg/L benomyl plus 4 mg/L dichloran, or 2 mg/L benomyl plus 2 mg/L dichloran. It is known that benomyl retains activity after autoclaving (**5**). A preliminary experiment showed that autoclaving also had no effect on the antifungal activity of dichloran (data not shown). In another experiment, MEA plus 2 mg/L benomyl plus 2 mg/L dichloran was amended with 0, 10 or 50 mg/L PCNB before autoclaving. Finally, BDS (see Results and Discussion) was amended with 0, 1, 10 or 50 mg/L cycloheximide, added as an aqueous stock solution after autoclaving.

Growth tests.—Media were inoculated with 4-mm-diam plugs of mycelium taken from the edge of 3- to 4-wk-old colonies. Plugs were placed near the edge of the plate. Plates were incubated in the dark at 25 C ± 0.1 C, except for *Serpula lacrymans,* which was incubated at 20 C (its maximum temperature for growth is about 25 C). The longest colony radius was measured after 3 or 4 da, at 1 wk, and at 2 wk. Where rhizomorphs grew beyond the mycelial margins, these were included in the measurement. Recognizing that colony growth may not be linear with time for all fungi, the longest possible growth period was used to calculate radial growth in mm/wk. Thus, for very fast fungi, which covered the plate in 4 da, growth rate was calculated from 3 da growth; for the slowest fungi it was calculated from 2 wk growth.

Isolations from field material.—To further discriminate among media that performed reasonably well in laboratory tests, several series of parallel isolations were made. Several samples of white and brown rots, mycelial cords of an unknown basidiomycete, and rhizomorphs of *Tricholomopsis platyphylla* (Pers.: Fr.) Sing. were used. The decayed wood was split to expose a clean surface before chips were removed. Cords and rhizomorphs were soaked for 1 min in an aqueous solution of 10% bleach/10% ethanol before isolation. Chips of wood or segments of cords or rhizomorphs were subdivided and one portion put on each medium.

RESULTS

Relative to growth on MEA, PON greatly inhibited almost all the fungi tested (FIG. 1). PPP moderately inhibited most fungi, with no apparent selectivity for hymenomycetes. Several *Armillaria* species grew well on PPP because rhizomorph growth was stimulated. However, fast-growing non-hymenomycetes were only partially inhibited on PPP. OPP strongly inhibited most fungi, but several *Armillaria* spp. and a few other species grew fairly well. OPP inhibited the Mucorales, but some deuteromycetes were only partially inhibited. BSMA permitted excellent growth of hymenomycetes and more or less completely inhibited some non-hymenomycetes, including the fast-growing and ubiquitous *Trichoderma* spp. However, it was not effective against the Mucorales and several deuteromycetes. BDP was additionally effective against *Fusarium negundi* Sherb., but strongly inhibited a few hymenomycetes.

Because the media with benomyl (BDP and BSMA; FIG. 1) had performed well, benomyl was tested along with the amendments dichloran, PCNB and cycloheximide on a subsample of the fungi. Dichloran alone was not effective against *Trichoderma* spp., but benomyl was (FIG. 2). However, dichloran, unlike benomyl, was effective against *Mucor hiemalis* Wehmer, and it did not inhibit the tested hymenomycetes. The combination with 2 mg/L dichloran was more effective than that with 4 mg/L. Therefore, BSMA

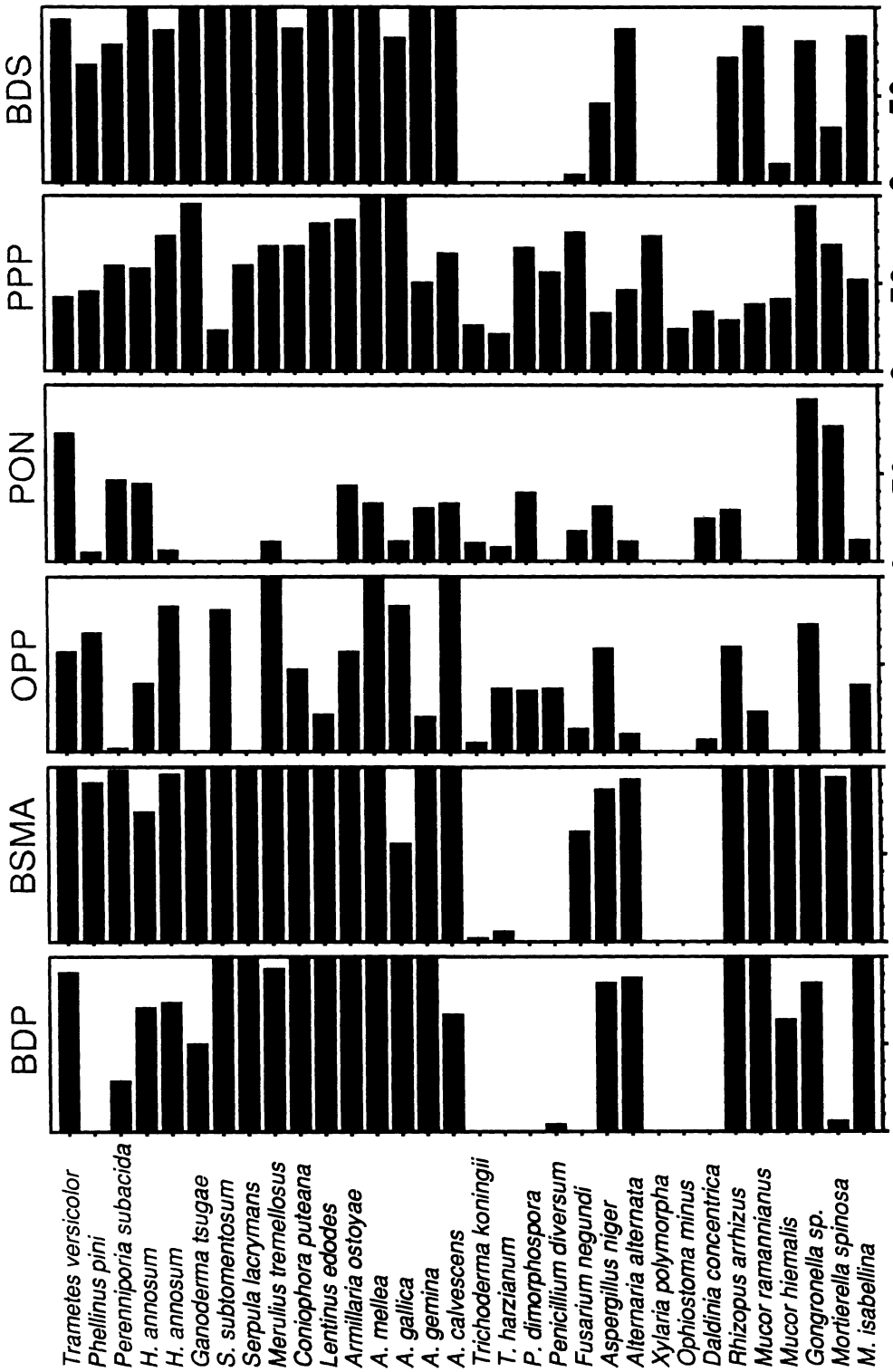

FIG. 1. Growth of test fungi (TABLE 1) on various semi-selective media relative to growth on MEA. Bars are truncated above 100%.

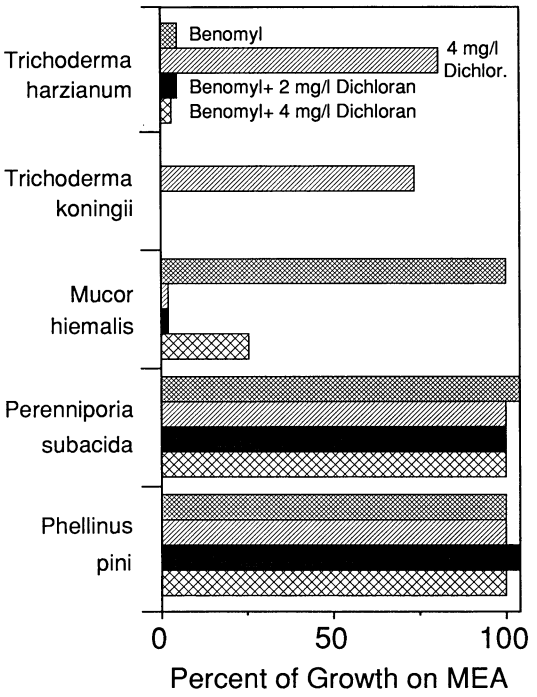

FIG. 2. Effect of benomyl and dichloran alone and together on growth of five fungi. Benomyl was used at 2 mg/L. The basal medium was MEA.

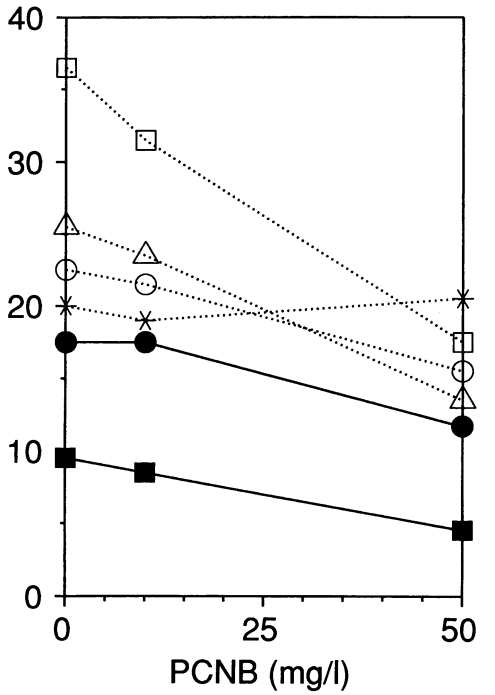

FIG. 3. Effect of PCNB on growth of two hymenomycetes (solid lines) and four non-hymenomycetes that are not effectively inhibited by benomyl and dichloran (dashed lines). The basal medium was MEA with benomyl and dichloran at 2 mg/L each. □ = *Alternaria alternata*, △ = *Mucor ramannianus*, ○ = *Mortierella isabellina*, * = *Gongronella* sp., ● = *Heterobasidion annosum*, ■ = *Phellinus pini*.

was prepared with the addition of 2 mg/L dichloran (BDS) and tested against all the fungi. Although several Mucorales and deuteromycetes were still relatively uninhibited, *Fusarium negundi* and *Mucor hiemalis* (one of the fastest-growing fungi tested) were inhibited at no cost in hymenomycete growth (FIG. 1). Also, the fast-growing *Trichoderma* spp. were more inhibited than by BSMA alone.

Although the media containing PCNB did not perform well (PON and PPP; FIG. 1), it was tried at several concentrations along with benomyl and dichloran. It was at least as inhibitory to the hymenomycetes as it was to the non-hymenomycetes that were not inhibited by benomyl and dichloran alone (FIG. 3).

Cycloheximide was also added (after autoclaving) at 0, 1, 10 and 50 mg/L in combination with BDS, but it prevented growth of most basidiomycetes at 10 mg/L, at which concentration non-hymenomycetes were virtually uninhibited (data not shown).

The media that showed promise in laboratory experiments were compared in isolations from field samples. In one series, isolations were made from Norway spruce [*Picea abies* (L.) Karst.] stumps cut 13 years before sampling with advanced white rot by *Heterobasidion annosum*, sugar maple (*Acer saccharum* Marsh.) stumps cut 5 years before sampling with mostly advanced white rot by *Armillaria* sp., a dead white pine (*Pinus strobus* L.) with mixed decay containing *Hyphoderma praetermissum* (Karst.) Erikss. & Strid, oak (*Quercus* sp.) stumps with brown rot by *Daedalea quercina* Fr., and a Norway spruce log with brown rot by *Fomitopsis pinicola* (Swartz : Fr.) Karst. In another, smaller series, *Tricholomopsis platyphylla* was isolated from both rhizomorphs and decay in sugar maple stumps, and an unidentified basidiomycete was isolated from mycelial cords.

With *H. annosum* and *Armillaria* sp., isolation efficiency was similar for all media, but BDS performed marginally better than the others (TABLE III). *H. praetermissum* was not isolated with PPP or OPP. With the brown rots, PPP was distinctly less effective than other media. OPP

TABLE III

EFFICIENCY OF ISOLATION OF SEVEN HYMENOMYCETES ON FIVE SEMI-SELECTIVE MEDIA

Fungi	Media				
	Percent of total chips				
	PPP	BSMA	BDP	BDS	OPP
Armillaria sp.[a] (6; 60)[b]	22	18	15	23	22
Heterobasidion annosum[a] (9; 60)	40	48	42	50	43
Hyphoderma praetermissum (1; 20)	0	35	22	35	0
Daedalea quercina (1; 8)	25	75	88	100	100
Fomitopsis pinicola (1; 32)	50	84	75	91	66
Tricholomopsis platyphylla (3; 12)	NT[c]	100	100	100	0
Unidentified mycelial cords (2; 8)	NT	75	63	88	0

[a] Samples of *Armillaria* sp. and *H. annosum* were from stumps with very advanced, old decay, material that generally presents a challenge in isolation.

[b] The number of distinct samples is followed by the total number of chips (or cord segments) per medium.

[c] NT = not tested.

worked well for *D. quercina* but less so for *F. pinicola*. In the second series of isolations, neither *T. platyphylla* nor the unidentified basidiomycete was isolated with OPP; BSMA, BDP and BDS worked fairly well.

Besides isolation efficiency, other factors are important in selecting a medium. Therefore, the following observations are offered.

The most fast-growing contaminants occurred on PPP. Thus, frequent inspections were necessary to rescue a desired colony from a contaminant growing from another chip in the same plate. This also occurred, but was generally less of a problem, with BSMA, BDP and BDS. It rarely occurred on OPP. The types of contaminants differed among the media. On PPP, *Trichoderma* sp. was a common contaminant. On the benomyl media, yeasts, bacteria, other basidiomycetes and occasionally members of the Mucorales were the most frequent contaminants. Except for BDP, only other basidiomycetes and Mucorales threatened neighboring chips on the same plate. On BDP, yeast growth was very heavy and sometimes spread across plates.

OPP resulted in slower growth of target fungi than the other media. It was sometimes necessary to subculture on MEA to verify identity of isolates on OPP. BDP generally had the most luxuriant growth of hymenomycetes. However, in some cases, they were at first inhibited, producing aerial mycelium on chips but not growing on the agar.

DISCUSSION

For general isolation of hymenomycetes, benomyl is the closest to an ideal ingredient. As Maloy (**5**) showed, it is best used at concentrations below 5 ppm. The present results show that a good number of non-hymenomycetes grow well on BSMA, but that is because these species were chosen to represent the most troublesome fungi. Although Heterobasidiomycetes were not explicitly tested, I have easily isolated a jelly fungus, *Dacrymyces stillatus* Nees : Fr., on BSMA. Although laboratory tests suggested that benomyl plus dichloran provided only an incremental improvement in selectivity over benomyl alone, the field isolations showed a clear difference with some materials. BDS was the best among the media tested.

For isolation of specific hymenomycetes, other media may also serve well. For example, although some non-hymenomycetes grew somewhat slower on BDS than on BDP, other problem fungi not tested may be better inhibited by BDP. If the target hymenomycete is one of those that grow well on it, BDP would then be a more suitable medium. A richer medium than the others, BDP leads to more luxuriant characteristic growth of hymenomycetes that can aid in rapid identification. A negative side of the richness is heavy growth of yeasts.

The use of OPP is a special case. Although o-phenylphenol was originally suggested for general isolation of basidiomycetes (**9**), the medium tested here and a similar one tested by Whitney *et al.* (**12**) are clearly inappropriate for all but a few. The pH seems to be within the range for growth of most wood-decay fungi, so the inhibition is apparently due to Na-OPP itself. OPP inhibits many non-hymenomycetes as well as or better than does BDS, but several, notably the

fast-growing *Trichoderma harzianum,* grow faster on OPP. Although some *Armillaria* species grew well on OPP, others were inhibited, in one case severely. OPP does have an advantage in that isolation plates can be untended for weeks with less chance of being overgrown than with other media. On the other hand, most hymenomycetes also grow more slowly on OPP than on other media. Whitney *et al.* (**12**) isolated fewer hymenomycetes other than *Armillaria* sp. on an o-phenylphenol medium than on MEA.

It should also be noted that there are many forms of media based on o-phenylphenol in the literature. Some workers use the sodium salt (**7**); others apparently use the acid form (**9**, **12**), which is relatively insoluble. Some media are acidified (**7**), combined with peptone (**9**) or used with reduced levels of malt extract (**13**).

Although PCNB is reportedly effective against the Mucorales, the media containing PCNB did not perform well. The dose-response experiment showed that it was not useful in combination with benomyl and dichloran. Harrington *et al.* (**1**) found that 150 ppm PCNB, when added to an acidified benomyl medium, made little difference in the isolation of hymenomycetes from Douglas-fir beetles.

Not addressed explicitly in this study are comparisons among anti-bacterial agents. Generally, bacteria have not been a serious problem on either BSMA or BDS. Although streptomycin is commonly used with benomyl, other antibiotics may work better with particular field materials. Acidification has also been used to inhibit growth of bacteria, but it is not recommended for benomyl-dichloran media without carefully investigating the effect on antifungal activity. In preliminary investigations, a medium made with 2 ppm benomyl and 200 ppm PCNB and acidified to about pH 3 was less effective against *Trichoderma* spp. than was BSMA (data not shown).

In conclusion, BDS is recommended for general isolation of hymenomycetes. A convenient stock solution is prepared by dissolving 40 mg Benlate 50% W.P. in 50 ml of warm 95% ethanol, diluting to 100 ml with water, and adding 20 mg dichloran. The stock solution can be refrigerated for at least several months without loss of activity. Ten ml of the stock solution is added to one L MEA before autoclaving, after which 100 mg streptomycin is added.

ACKNOWLEDGMENTS

This project was supported by the USDA via a McIntire-Stennis grant. L. M. Dann provided excellent technical assistance. Some of the isolates were kindly provided by R. A. Blanchette, T. C. Harrington and C. J. K. Wang. T. C. Harrington reviewed the manuscript and provided helpful advice. C. J. K. Wang assisted in the identification of some isolates.

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Accepted for publication January 14, 1991