SELECTIVE ISOLATION OF THE ENTOMOPATHOGENIC FUNGI *BEAUVERIA BASSIANA* AND *METARHIZIUM ANISOPLIAE* FROM AN ARTIFICIAL POTTING MEDIUM

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ABSTRACT

A basal medium consisting of oatmeal agar was superior to seven other media for isolation of *Metarhizium anisopliae* and *Beauveria bassiana* from an artificial potting medium. A rate of 0.55 g/l of dodine and 5 mg/l of chlortetracycline allowed optimum recovery of *B. bassiana* alone. Decreasing the amount of dodine to 0.46 g/l and adding 0.38 g benomyl allowed recovery of both entomopathogens at high frequencies in the abscence of most other fungi. Addition of 10 mg/l crystal violet aided in visibility of the target fungi without affecting recovery frequencies. Exposure of culture plates to as little as 2 μmol $m^{-2}s^{-1}$ light delayed observation of colonies of both entomopathogens up to 7 days compared to cultures incubated in the dark.

RESUMEN

Un medio básico consistente de agar de avena fue superior a otros 7 medios para aislar a *Metarhizium anisoplia* y *Beauveria bassiana* de un medio de siembra artificial. Una razón de 0.55 g/l de dodine y 5 mg/l de clorotetraciclina permitió una recuperación de *B. bassiana* solamente. Disminuyendo la cantidad de dodine a 0.46 g/l y añadiendo 0.38 g de benomil permitió la recuperación de ambos entomopatógenos en frecuencias altas en la ausencia de la majoría de otros hongos. Añadiendo 10 mg/l de cristales de violeta ayudó la visibilidad del hongo sin afectar las frecuencias de su recobro. Exponiendo platos de cultura a niveles tan bajo de luz como 2 umol $m^{-2}s^{-1}$, demoró la observación de colonias de ambos entomopatógenos hasta 7 días en comparación con culturas incubadas en la oscuridad.

With increased concern over environmental impacts of chemical pesticides, considerable research has focused on biological pesticides. Entomopathogenic fungi such as *Beauveria bassiana* (Bals.) Vuill. and *Metarhizium anisopliae* (Metschn.) Sorokin have been investigated extensively over the past 10 to 15 years (Ferron 1981, Roberts and Campbell 1977). Interest in commercial application of these biological pesticides has led to development of information regarding their biology (Barnes et al. 1975, Campbell et al. 1983), ecology (Doberski and Tribe 1980), pathology (Doberski 1981, Gardner and Noblet 1978), and effects of chemical pesticides (Olmert and Kenneth 1974) under a wide range of conditions. Both bioassay with a susceptible insect host and direct isolation from soil, insect, or plant tissue have been utilized to evaluate reactions of these fungi to various conditions. Direct isolation of conidia of *B. bassiana* and *M. anisopliae* have primarily involved use of semi-selective culture media. One of the earliest media was based on one used for general isolation of soil fungi and contained glucose, oxgall, and peptone with rose bengal, chloramphenicol, and cycloheximide as antibiotics (Veen and Ferron 1966). Doberski and Tribe (1980) developed a medium based on similar ingredients with crystal violet substituted for rose bengal. This medium was used to isolate

B. bassiana and *M. anisopliae* from elm bark and soil. A medium with V-8 juice, glucose, yeast extract, and oxgall as the nutrient base amended with cycloheximide, streptomycin sulfate, and tetracycline was developed for general isolation of *B. tenella* from soils (Joussier and Catroux 1976). Sabouraud-dextrose medium and oxgall was used as another basal medium amended with penicillin G, streptomycin sulfate, oxytetracycline, cycloheximide, and binapacryl (Morocide®, a fungicide). This medium was developed for use in isolations from field soil maintained under laboratory conditions (Lingg and Donaldson 1981). More recently, Beilharz et al. (1982) discovered that dodine (n-dodecylguanidine acetate) selectively inhibited some soil fungi other than *B. bassiana* and *M.anisopliae* when added at the rate of 1.0 g formulated (Cyprex 65WP, American Cyanamid) product per liter. Each of these media was developed in response to a need by researchers to evaluate effects of various treatments on survival of these entomopathogens. The present paper describes a modification of one of these media (Beilharz *et al.* 1982) to maximize recovery of either *B. bassiana* or *M. anisopliae* from an artificial potting medium.

Materials and Methods

Preparation of the Basal Medium

The basal medium used for most trials was oatmeal agar prepared from 20 g Gerber Oatmeal Cereal For Baby (Gerber Products Co., MFR, Fremont, MI 49412) autoclaved for 20 min with 1 l of deionized water. The suspension was immediately filtered twice through four layers of cheesecloth, and the volume of filtrate adjusted to 1 l with deionized water. Agar (20 g/l) was ground in a mortar and pestle with various amounts of dodine and added to the oatmeal filtrate while stirring. The final medium was autoclaved for 20 min and cooled to 48°C prior to adding chlortetracycline (5 mg/l). One l of medium was used to pour 50 plates, (90 × 15 mm).

Preparation of the Fungi

An experimental wettable powder formulation of *Beauveria bassiana* (ABG 6112, Abbott Laboratories, North Chicago, IL) and a formulation of *Metarhizium anisopliae* (70-25 Mycogen Corporation, San Diego, CA) were used in all trials. A potting medium consisting of 3 parts Canadian peat and one part builder's sand was amended with 4 kg ground dolomitic limestone and 1 kg Micromax (micronutrient source from Sierra Chemical Co., Milpitas, CA) per m^3 and steam-treated at 95°C for ca. 1 hr. *Beauveria bassiana* or *M. anisopliae* or both were added to 500 g of the potting medium at the rate of 0.5 g formulation and mixed thoroughly by hand. The medium was used the same day it was prepared.

Dilution Plating Method and Incubation

Ten g samples of the potting medium-conidial preparation were added to 100 ml of sterilized deionized water (SDW) and placed on a rotary action shaker for 15 min. Serial dilutions were made with SDW, and 0.5 ml aliquots of the 10^{-3} and 10^{-4} dilutions were spread on the surface of five plates of each medium. Plates for all trials except those concerning light levels were incubated at 25 to 27°C with 10 μmol $m^{-2}s^{-1}$ light (Sylvania, Gro-lux, fluorescent bulbs) for 16 hr daily. Colony counts were determined at various times depending upon the trial. Specific trials with media and incubation conditions are described below.

Comparison of Oatmeal-Dodine Medium With Other Isolation Media

The oatmeal-dodine medium was compared to several media developed by others as well as basal media amended with dodine (variable rates) and chlortetracycline (5 mg/l). The following media were tested at least twice: 1) Lingg and Donaldson basal medium (1981) with dodine (0.62 g/l) and chlortetracycline amendments: 2) Doberski and Tribe (1980); 3) Martin's RB-M2 medium (Tuite, 1969); 4) Sabouraud's Dextrose agar (Difco) amended with 5 g per liter of yeast extract, dodine (0.62 g/l) and chlortetracycline; 5) Czapek's-Dox medium (Difco) amended with dodine (0.58 or 0.62 g/l) and chlortetracycline; 6) Pasteur's-MC sporulation medium (0.36 g KH_2PO_4, 1.05 g $NaHPO_4$ $7H_2O$, 0.6 g $MgSO_4$, 1.0 g KCl, 10.0 g dextrose, 0.7 g NH_4NO_3, 5.0 g yeast extract, and 20.0 g agar per liter) amended with dodine (0.58 or 0.62 g/l) and chlortetracycline; and 7) Potato Dextrose agar medium (Difco) amended with dodine (0.62 g/l) and chlortetracycline.

Effect of Dodine Rate on Recovery of Fungi

Dodine rates were varied from 0.16 to 0.62 g per liter using oatmeal agar as the basal medium. The following rates were used in at least one of five tests: 1) 0.65, 0.49, 0.32, and 0.16 g/l; 2) 0.65, 0.58, 0.52, and 0.46 g/l; 3) 0.52, 0.49, 0.46, and 0.39 g/l; 4) 0.52, 0.49, 0.46, and 0.39 g/l; and 5) 0.65, 0.62, 0.58, 0.55, 0.52, 0.49, 0.46, and 0.39 g/l.

Effect of Benomyl Rate on Recovery of Fungi

Benomyl (Benlate 50WP, E. I. Dupont Nemours, Inc., Wilmington, DE) is known to inhibit *in vitro* growth of both *B. bassiana* and *M. anisopliae* (Olmert and Kenneth 1974, Tedders 1981) at rates used to treat plants. The effect of very low rates on recovery of these two entomopathogens was tested. Benomyl (prepared as a stock suspension in SDW) was added to the basal medium prior to autoclaving. The following rates were used in three tests: 0, 0.25, 0.5, and 1.0 mg/l. Two additional tests were performed using 0, 0.25, 0.38 and 0.5 mg benomyl/l.

Effect of Crystal Violet Concentration on Recovery of Fungi

The medium of Doberski and Tribe (1980) contains 10 ppm crystal violet. When this rate of crystal violet was added to the oatmeal medium, *M. anisopliae* was not recovered, although *B. bassiana* recovery was unaffected in a preliminary trial. Various rates of crystal violet were therefore tested for a differential effect on recovery of the two fungi. Rates from 0 to 20 ppm were included: (Test 1) 0, 2, 10 and 20 mg/l (Test 2) 2.5, 5.0, 7.5 and 10 mg/l, and (Test 3) 0, 5, 10 and 15 mg/l.

Effect of Light Level During Incubation on Recovery of Fungi

Two types of tests were performed to evaluate the effect of light level on recovery of *B. bassiana* and *M. anisopliae* on oatmeal medium amended with 0.46 g dodine/l. In the first test, 25 plates were treated with 0.5 ml of a 10^{-4} soil dilution of either *M. anisopliae* or *B. bassiana*. Plates were arranged in five stacks of five plates each with the bottom plate in each stack wrapped in foil for each fungus. Stacks were incubated with 10 μmol $m^{-2}s^{-1}$ fluorescent light and colony counts recorded as soon as possible. The second type of test was performed with plates in a single layer under four light levels (10 plates per treatment): 1) wrapped in foil; 2) 2 μmol m^{-2} s^{-1}; 3) 5 μmol m^{-2} s^{-1}; and 4) 10 μmol m^{-2} s^{-1}. The second type of test was performed twice.

All data were analyzed for variance using the F test for significance.

Results and Discussion

Comparison of Oatmeal-Dodine Medium With Other Isolation Media

The mean number of colonies per plate was significantly affected by the medium used for isolation. *Beauveria bassiana* was recovered in high frequencies on oatmeal agar and Sabouraud medium amended with yeast extract, but not from other culture media utilized. *Metarhizium anisopliae* was recovered only from oatmeal agar medium. Mean recovery per plate for *B. bassiana* on oatmeal medium ranged from 29 to 92 colonies (10^{-4} dilution rate), while recovery for *M. anisopliae* ranged from 17 to 45 (10^{-4} dilution rate). These recovery frequencies relate to a percent recovery between 50 and 80 for the majority of trials.

Effect of Dodine Rate on Recovery of Fungi

An optimum rate of dodine was identified for isolation of each target fungus. The rate of 0.62 g/l dodine significantly reduced recovery of *M. anisopliae* (Table 1). Decreasing the rate/l to 0.55 g allowed a high recovery of *B. bassiana*, but was still too high for recovery of *M. anisopliae* which grew out only when the rate was decreased to 0.46 g/l. Rates of dodine from 0.32 to 0.62 generally did not influence recovery of *B. bassiana* (Table 1). Rates of dodine lower than 0.32 allowed good recoveries of the target fungi, but proved to be too low to inhibit germination and growth of several saprophytic soil fungi such as *Trichoderma* and *Rhizopus* spp. (data not included).

TABLE 1 Effect of various rates of dodine on recovery of *Beauveria bassiana* and *Metarhizium anisopliae* from an artificial potting medium.

	Mean recovery at 10^{-4} dilution									
	Beauveria bassiana (Tests)					*Metarhizium anisopliae* (Tests)				
Dodine g/l	1	2	3	4	5	1	2	3	4	5
0.32	19.2[a]	NT[b]	NT	NT	NT	193.0	NT	NT	NT	NT
0.39	NT	NT	10.2	17.4	21.0	NT	NT	20.2	8.2	19.6
0.46	NT	22.6	8.2	22.2	16.6	NT	67.8	25.2	10.8	21.0
0.49	25.0	NT	10.2	19.6	23.8	23.6	NT	0.8	0	16.0
0.52	NT	20.2	9.6	11.0	15.8	NT	4.0	7.6	0	5.8
0.55	NT	NT	NT	NT	19.2	NT	NT	NT	NT	5.2
0.58	NT	18.6	NT	NT	13.8	NT	0.8	NT	NT	4.0
0.62	NT	NT	NT	NT	13.4	NT	NT	NT	NT	2.6
0.65	16.4	0.4	NT	NT	0	0.4	0	NT	NT	0
Significance[c]	**	**	ns	ns	**	**	**	**	**	**

[a]Mean number of colonies for five replicate plates for each test.
[b]NT = Not tested.
[c]Significance level of the F test denoted as follows: ns = not significant, and ** $P<0.01$.

Effect of Benomyl on Recovery of Fungi

A rate of 0.25 mg/l of benomyl did not affect recovery of either fungus significantly while a rate of 0.38 mg/l reduced recovery of *B. bassiana* to undetectable levels, but did not affect recovery of *M. anisopliae*. Rates of 0.5 to 1.0 mg/l significantly decreased recovery frequencies of both (Table 2). Tests were performed using the oatmeal-dodine medium with dodine used at 0.46 g/l to allow recovery of *M. anisopliae*. Since this rate does allow occasional colonies of *Trichoderma* spp. to develop, the effect of low rates of benomyl proved beneficial in eliminating these contaminants. A rate of 0.38 mg/l of benomyl was chosen for addition to oatmeal-dodine medium when the 0.46 g/l rate of dodine was employed to eliminate saprophytes and select *M. anisopliae* over *B. bassiana*.

Effect of Crystal Violet Concentration on Recovery of Fungi

Although a preliminary test showed differential effects of crystal violet concentration on recovery of the entomopathogens, the results were not repeated in any subsequent test. Rates up to 10 mg/l did not affect recovery of either fungus, while those above 10 mg/l reduced recovery of the two entomopathogens similarly. Because oatmeal agar is an opaque, white medium and both organisms initially produce white to creamy colored colonies, the addition of 10 mg/l of crystal violet greatly improved colony visibility.

Effect of Light Level During Incubation on Recovery of Fungi

When plates of either *B. bassiana* or *M. anisopliae* were incubated in stacks under 10 μmol m^{-2} s^{-1} light, the recovery frequency and the number of days required for incubation were affected by their position in the stack (data not included in a table). Plates on the bottom of the stacks, which were wrapped in foil, could be counted two

TABLE 2. Effect of various rates of benomyl on recovery of *Beauveria bassiana* and *Metarhizium anisopliae* from an artificial potting medium.

	Mean recovery at 10^{-4} dilution							
	Beauveria bassiana (Tests)				*Metarhizium anisopliae* (Tests)			
Benomyl (mg/l)	1	2	3	4	1	2	3	4
0	12.2[a]	13.5	25.8	27.0	7.6	3.4	30.4	63.6
0.25	13.4	14.2	21.8	22.4	9.0	4.8	6.0	27.8
0.38	NT[b]	NT	0	0	NT	NT	9.7	20.2
0.50	0	0	0	0	10.1	0	0	0
1.00	0	0	NT	NT	0	0	NT	NT
Significance[c]	**	**	**	**	**	**	**	**

[a]Mean number of colonies for five replicate plates for each test.
[b]NT = Not tested.
[c]Significante level of the F test denoted as follows: ** $P<0.01$.

to five days earlier than those on the top of the stack. In addition, recovery frequencies of both target fungi were reduced up to 60% when incubated on the top of the stack compared to the foil wrapped plates. *B. bassiana* plates at the top of the stack yielded a mean of nine colonies/plate while those at the bottom yielded a mean of 28. Similarly, the number of *M. anisopliae* colonies was only 48 on the top of the stack compared to a mean of 115 at the bottom of the stack. Exposure of plates to various levels of light during incubation demonstrated that even 2 μmol m^{-2} s^{-1} light reduced recovery frequencies of the target fungi in some tests (Table 3). As in the previous test, colony counts for plates wrapped in foil were made approximately two to seven days earlier than plates in 10 μmol m^{-2} s^{-1} for *B. bassiana* and *M. anisopliae* respectively.

From these studies it can be concluded that a basal medium of oatmeal agar supports the highest recovery of both *B. bassiana* and *M. anisopliae* from artificial potting medium. Comparisons to most of the media developed by other researchers suggest that a single medium may not be effective under all the conditions encountered in research on these entomopathogens. It should be remembered, however, that there is a certain amount of natural variability of isolates of fungi and that our media may not be adequate for recovery of naturally occurring *B. bassiana* or *M. anisopliae*.

The effects of both dodine and benomyl on the recovery of these entomopathogens have led to the development of two selective media. Selective isolation of *B. bassiana* can occur if dodine is incorporated in the oatmeal medium at the rate of 0.55 g/l. Higher rates of dodine generally do not decrease recovery of the *B. bassiana*, but the previously used rate of 0.65 g/l significantly extends the period of time needed to incubate the plates. A much lower rate of dodine (0.46 g/l) must be used to allow high recoveries of *M. anisopliae*. Since this rate of dodine also allows good recovery of *B. bassiana* and certain soil saprophytes, the addition of benomyl to the medium at the rate of 0.38 g/l inhibits germination of these organisms and allows selective isolation of *M. anisopliae*. Decreasing the benomyl rate to 0.25 ppm will allow high recovery frequencies of both target fungi while keeping recovery of other soil fungi to a minimum.

A natural tendency of many researchers is to incubate culture plates under some level of artificial or natural light. Some effects of light on *B. bassiana* and *M. anisopliae* have been studied (Alves et al. 1979 1980, Zimmerman 1982). These studies do not

TABLE 3. EFFECT OF INCUBATION LIGHT LEVEL ON RECOVERY OF *BEAUVERIA BASSIANA* AND *METARHIZIUM ANISOPLIAE* FROM AN ARTIFICIAL POTTING MEDIUM.

	Mean recovery at 10^{-4} dilution					
	Beauveria bassiana (Tests)			*Metarhizium anisopliae* (Tests)		
Light level μmol m^{-2} s^{-1}	1	2	3	1	2	3
0 (in foil)	16.5[a]	34.4	41.4	30.2	10.4	23.4
2	11.6	33.0	24.0	30.0	8.6	22.3
5	9.8	30.0	25.6	22.2	13.0	20.9
10	8.4	25.4	32.8	17.0	10.0	18.2
Significance[b]	ns	ns	**	**	ns	**

[a]Mean number of colonies for five replicate plates for each test.
[b]Significance of the F test denoted as follows: ns = not significant, and ** P>0.01.

report the effects of relatively low light levels (10 μmol m^{-2} s^{-1}) during incubation of isolation plates. In the case of *B. bassiana*, light increases the time needed for recovery plates by 2 to 5 days. Incubation of *M. anisopliae* in as little as 10 μmol m^{-2} s^{-1} not only increases the incubation time, but also results in a significant decrease in recovery frequencies compared to plates incubated in the dark.

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MONITORING POPULATIONS OF *LIRIOMYZA TRIFOLII* (DIPTERA: AGROMYZIDAE) IN CELERY WITH PUPAL COUNTS

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ABSTRACT

Populations of *Liriomyza trifolii* (Burgess) were monitored in 16 commercial fields of celery during 1985 by taking 10 sets of 10 leaflet samples per field and counting puparia after 3, 7, and 14 days. Three indices of dispersion showed that the population had an aggregated distribution. Sample size for 3-day pupae counts were calculated to be 7 sets of 10 leaflets for 25% precision at densities of 5 or more puparia per 10 leaflets. The proposed sampling scheme requires between 30 and 45 minutes total sampling time per field and will allow the grower to monitor *L. trifolii* population trends and evaluate previous control actions. Sequential sampling plans may further reduce sampling time.

RESUMEN

Las poblaciones de *Liriomyza trifolii* (Burgess) se esudiaron en 16 parcelas comerciales de apio durante 1985. Diez grupos de muestras de diez hojas se obtuvieron de cada parcela, contandose las crisálidas después de 3, 7 y 14 días. Tres índices de dispersión montraron que la poblacion tenía una distribucion aglomerada. Se determinó que el tamaño de la muestra para el conteo de crisálidas despues de 3 días debe ser 7 grupos de 10 hojas para obtener una precisión de 25% en densidades de 5 o más crisálidas por cada 10 hojas. El sistema de muestreo propuesto requiere entre 30 y 45 minutos de tiempo para el muestreo de cada parcela y permitirá al productor el estudiar las tendencias de las poblaciones de *L. trifolii* y evaluar acciones de control previas. Planes de muestreo de secuencia pueden reducir aún más el tiempo de muestreo.