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CTC medium: A novel dodine-free selective medium for isolating entomopathogenic fungi, especially *Metarhizium acridum*, from soil

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ABSTRACT

The selective media most commonly used for isolating hyphomycetous species of entomopathogenic fungi from non-sterile substrates rely on *N*-dodecylguanidine monoacetate (dodine) as the selective fungicide. Although these media are effective for isolating many species of *Metarhizium* and *Beauveria* from soil, they are inefficient media for isolation of an important *Metarhizium* species, *Metarhizium acridum*, from non-sterile soil. Our current study was directed to formulating a dodine-free selective medium that is efficient for isolating naturally occurring *Beauveria* spp. and *Metarhizium* spp., especially *M. acridum*, from soil. The selective medium (designated CTC medium) consists of potato dextrose agar plus yeast extract (PDAY) supplemented with chloramphenicol, thiabendazole and cycloheximide. In comparisons with selective media previously reported in the literature, the CTC medium afforded colonies that were larger and had both earlier and more abundant conidiation of entomopathogenic fungi, features which greatly facilitated identification of the emerging entomopathogenic fungi. In addition to efficient re-isolation of *M. acridum*, this medium also is an effective tool for selective isolation of *Metarhizium brunneum*, *Metarhizium robertsii*, *Beauveria bassiana* and *Beauveria brongniartii* from non-sterile field-collected soil samples inoculated (spiked) with fresh conidia in the laboratory.

1. Introduction

The use of entomopathogenic fungi for biological control of arthropods dates back to the 1880s (Krassilstschik, 1888), and there are current examples worldwide of successful fungus-based insect control programs (Roberts and St. Leger, 2004; Shah and Pell, 2003). *Metarhizium anisopliae* sensu lato (s.l.) (Metschnikoff) Sorokin and *Beauveria bassiana* (Balsamo) Vuillemin are the fungi most commonly employed for pest control. Another *Metarhizium* species, *Metarhizium acridum* (Driver and Milner) Bischoff, Rehner and Humber was found to be an effective pathogen of locusts and grasshoppers (Acrididae), and it is quite host-specific to these and closely related insects. Two *M. acridum* (one African and one Australian) have been developed as commercial products for biological control of this insect group (Lomer et al., 2001).

Fungal species tend to occur in nature as mixtures of strains with widely diverse physiological traits. Exploration of this rich diversity by isolating new fungal isolates from the field is expected to yield strains with attributes superior to those currently available for biological pest control. The methods for these searches are still under development. Conidia produced on cadavers of insects killed in nature by entomopathogenic fungi are routinely deposited on or in nearby soil. Pathogenic fungi, however, are a distinct minority amidst a myriad of diverse microorganisms in soil (Tsao, 1970), and this probably is especially true for entomopathogenic fungi. The isolation of entomopathogenic fungi from soil is often difficult, and requires "finely tuned" methods to maximize success. Two of the most commonly employed methods are: (1) baiting the environment with a susceptible insect host (Zimmermann, 1986) or (2) using specific selective media containing chemicals that preclude or reduce the growth of contaminants (Beilharz et al., 1982; Doberski and Tribe, 1980; Joussier and Catroux, 1976; Liu et al., 1993; Veen and Ferron, 1966).

In 1966, Veen and Ferron developed a selective medium that contained oxgall, chloramphenicol and cycloheximidine (Actidione) for the isolation of two entomopathogenic fungi, *Beauveria brongniartii* (Saccardo) Petch and *M. anisopliae* s.l. In 1982, the fungicide dodine (*N*-dodecylguanidine monoacetate) was successfully incorporated into a culture medium for isolation of *M. anisopliae* s.l. from soil (Beilharz et al., 1982). This report led to the development of a widely utilized dodine-based selective medium for isolation of *B. bassiana* and *M. anisopliae* s.l. (Chase et al., 1986). Later, the addition of low dodine concentrations (10 and 50 μg/ml) to Veen's

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medium was found to reduce the number of contaminants by over 90% and to improve the isolation of *M. anisopliae* s.l. from soil (Liu et al., 1993).

Recently, potato dextrose agar enriched with yeast extract (PDAY), when supplemented with dodine and gentamicin, proved to be a very efficient substrate for selecting several entomopathogenic fungi from contaminated environments (Rangel et al., 2010). Nevertheless, this study also demonstrated that *M. acridum* is more susceptible to dodine than other *Metarhizium* spp.; i.e., it grows and sporulates poorly on PDAY containing very low dodine concentrations. *M. acridum* is an Orthoptera-host-specific fungus (Alston et al., 2005; Bridge et al., 1997; Goettel and Jaronski, 1997; Milner et al., 2002; Peveling and Demba, 1997) that has been isolated in hot climates and deserts in Australia, Thailand, Brazil, Mexico, Senegal, Benin, Tanzania, Madagascar and Niger. In fact, *M. acridum* isolates from Africa and Australia are currently registered for commercial use in their respective continents. The importation of these products, however, currently is not permitted by some countries, e.g., USA, for which *M. acridum* is an exotic species. The isolation of native *M. acridum* should facilitate importation of exotic commercial isolates into the restrictive nations, and/or newly discovered native *M. acridum* isolates could be developed as novel domestic products for biological control of orthopteran pests. With these goals in mind, we are conducting an energetic search for native USA *M. acridum* isolates. The project, however, was severely impeded initially by the absence of an effective medium for selectively isolating *M. acridum* from non-sterile soil.

Effective selective media for the isolation of a specific fungus should contain both a nutrient source and anti-microbial agents (i.e., fungicides and antibiotics), with the latter in appropriate concentrations to allow the target-fungus to grow while impeding the growth of common contaminants (Luz et al., 2007). Although widely used for isolation of most species of entomopathogenic fungi, selective media containing dodine are not suitable for isolating *M. acridum* from non-sterile soil due to this species' high sensitivity to dodine (Rangel et al., 2010). Accordingly, a selective medium with a suitable dodine alternative was needed to efficiently search for new isolates of *M. acridum* in soil samples.

The study reported here investigates the effectiveness of several selective media with different concentrations of several anti-microbial compounds for re-isolation of *Metarhizium* spp. and *Beauveria* spp. from non-sterile soil inoculated in the laboratory with fresh conidia of several species of entomopathogenic fungi. A novel selective medium is described. It is based on modifications of previous media (Veen and Ferron, 1966; Luz et al., 2007; Rocha and Luz, 2009; Rangel et al., 2010) and is designed to maximize recovery of naturally occurring *Beauveria* spp. and *Metarhizium* spp., especially *M. acridum*, from soil. The new medium, "CTC medium", follows an uncomplicated preparation method and utilizes a simple nutritive substrate; i.e., potato dextrose agar enriched with yeast extract (PDAY), supplemented with chloramphenicol, thiabendazole and cycloheximide. The acronym "CTC" refers to these three chemicals.

2. Materials and methods

2.1. Soil samples and fungal isolates

Four soil samples were collected within the state of Utah, USA (Table 1). The sampling protocol was as follows: (1) the most obvious surface duff and growing vegetation were cleared away from an area of approximately 225 cm^2; (2) approximately 200 g of soil was taken at each site from within the top 5 cm of the surface; (3) the samples were placed in plastic bags and held at room temperature (20 ± 2 °C) until experimentation began. Soil sample physical and chemical characterization was performed by the Analytical Laboratory of Utah State University. Soil physical traits are listed in Table 2, and concentration of 19 elements in Table S1 (see Supplementary data available online).

Table 1
Collection sites of soil samples in 2009.

Soil sample	Sites (Utah, USA)	Longitude	Latitude
# 1	Piute	112°3801′W	38°4317′N
# 2	Sevier	112°2295′W	38°5126′N
# 3	Millard	112°0430′W	39°0954′N
# 4	Millard	112°0519′W	39°1151′N

Six isolates representing five fungal species (viz., *B. bassiana*, *B. brongniartii*, *M. acridum*, *Metarhizium brunneum* Petch, and *Metarhizium robertsii* Bischoff, Rehner and Humber) were investigated. Details of the isolates are given in Table 3.

2.2. Media preparation

Four selective media were prepared as follows: (1) "Dodine medium", consisting of PDAY [potato dextrose agar (Difco Laboratories, Sparks, MD, USA) supplemented with 1 g/l yeast extract (Technical; Difco Laboratories)], +0.05 g/l gentamicin (BioWhittaker, Walkersville, MD, USA), and one of four dodine (*N*-dodecylguanidine monoacetate) concentrations [0.001%, 0.002%, 0.004% or 0.006% active ingredient (A.I.)]. The commercial fungicide "Syllit®" (65% A.I.) (Chimac-Agriphar S.A., Aceto Agricultural Chemicals

Table 2
Physical characterization of soil samples.

Soil sample	pH	EC (dS/m)*	Sand (%)	Silt (%)	Clay (%)	Texture
# 1	7.3	4.18	39	41	20	Loam
# 2	7.2	2.49	48	38	14	Loam
# 3	7.3	4.08	52	37	11	Sandy Loam/Loam
# 4	7.6	1.28	36	46	18	Loam

* EC, eletrical conductivity in deci-Siemens/meter.

Table 3
Origins of fungal isolates used to compare several potential selective media for recovering conidia added to soil.

Fungal isolate*	Origin	Host	Latitude	Collection year
Metarhizium acridum				
ARSEF 324	Queensland, Australia	Orthoptera: Acrididae	19°00′S	1979
ARSEF 5628	Shelsela, Ethiopia	Orthoptera: Acrididae	12°28′S	1996
Metarhizium robertsii				
ARSEF 2575	South Carolina, USA	Coleoptera: Curculionidae	34°00′N	1988
Metarhizium brunneum				
ARSEF 5626	Pälkäne, Finland	Coleoptera: Tenebrionidae	61°20′N	1986
Beauveria bassiana				
ARSEF 252	Orono, Maine, USA	Coleoptera: Chrysomelidae	44°53′N	1978
Beauveria brongniartii				
ATCC 58798	Czechoslovakia	Diptera: Tipulidae	50°05′N	Unknown

* Culture collection: ARSEF, ARS Collection of Entomopathogenic Fungal Cultures – ARSEF, USDA, Ithaca, NY, USA; ATCC, American Type Culture Collection, Manassas, VA, USA.