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Special considerations for operating a culture collection of fastidious fungal pathogens

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INTRODUCTION

This overview draws on nearly 20 years of experience with the USDA-ARS Collection of Entomopathogenic Fungal Cultures (ARSEF), the world's largest repository for fungal pathogens of arthropods and nematodes [3]. The problems of maintaining so specialized a collection of fungal pathogens may be more acute, but they are shared by other culture collections.

'Fastidious' fungi are here taken to be taxa occurring in nature exclusively as obligatory pathogens or parasites. The great majority of pathogens in the ARSEF collection—including many that are known only from their natural hosts—grow readily on such simple and standard culture media as Sabouraud dextrose agar + 1% yeast extract. Some of the most cosmopolitan and important entomopathogens such as *Beauveria bassiana*, *Metarhizium anisopliae*, *Verticillium lecanii*, and species of *Paecilomyces* can also be isolated from soil and plant detritus as facultative saprobes and will not be considered further here.

Experience indicates that the pathogens with the narrowest host ranges are the most tightly adjusted to the biologies of their hosts and are usually the fungi whose isolation and maintenance can be most challenging. Exceptionally fastidious pathogens may consistently elude attempts, however sophisticated, to culture them. Entomopathogens such as *Coelomomyces* spp., some taxa of the Entomophthorales, and trichomycetes (endocommensals from arthropod guts, few of which have been cultured), as well as phytopathogens such as powdery mildews (Erysiphales) offer worthy challenges to ambitious biologists.

ISOLATION AND CULTURE

Most fungi grow easily from spores inoculated onto simple culture media but this generalization does not hold for many taxa that are obligatory pathogens in the ecological rather than physiological sense; these fungi often resist attempts to culture them with routine methods and media. Every successful culture of a fungus must include vegetative growth in vitro even if the culture never sporulates or displays the full range of development seen on its natural substrate. Even if vegetative cultures of pathogens may fail to sporulate when reintroduced to a susceptible host, germplasm may be preserved indefinitely and many invaluable genetic, biochemical, physiological and developmental studies can be completed with fungi that never produce their complete life cycle in culture.

Inoculum

Many mycologists have been frustrated by trying to culture fungal parasites and pathogens when spores fail altogether to germinate or germ tubes stop growing without ever establishing cultures. Manners [4] recognized such a distinct separation of germinative and vegetative growth to be routine for obligately parasitic fungi. Using identical media and physical conditions, cultures inoculated with vegetative material of fastidious fungi may thrive while those inoculated with spores always fail.

It is easier to maintain previously established vegetative growth of fastidious fungi than to obtain vegetative growth in vitro from their spores. Cultures are also obtained sooner by inoculating primary cultures with vegetative cells than with spores. A series of critical developmental events must occur for spores to germinate and, eventually, to initiate vegetative growth. Before and during spore germination, for example, the metabolism of fastidious fungal cells draws (except for water and oxygen) on internal reserves of nutrients and structural materials but during vegetative growth it must convert to the obligatory use of extracellular nutrient sources.

The unusual growth forms for some fungi can dictate the use of unconventional culture techniques. The vegetative cells of many entomopathogens in several genera of the Entomophthorales are naturally wall-less, amoeboid to more typically hypha-like protoplasts [2] that cannot be used to inoculate solid culture media.

Media

While some entomophthoraleans seem to sporulate better on rich, nutritionally complex media, less fastidious and

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facultatively saprobic entomopathogens may sporulate more freely on nutritionally weak or dilute media.

Tissue culture media—whether originally devised for insect or mammalian cells, with or without supplemental fetal bovine serum—are the routine choices of liquid media for many entomopathogenic fungi and are the *only* media able to sustain some of them. It should be noted that the undisputed benefits of using fetal bovine serum for entomopathogenic fungal culture media may depend more on effective medium buffering, increased availability of ions in chelated forms, or other physicochemical factors than on strictly nutritional bases. Many protoplastic entomophthoraleans require liquid media for their physical support even though they may not require the nutritional complexity of tissue culture media.

Tissue culture or other complex liquid media do have disadvantages: they must be filter-sterilized rather than autoclaved, are more expensive than many standard mycological media, and require the use of relatively expensive consumables such as tissue culture flasks. Highly fastidious fungi, especially those grown in liquid media, may also require the occasional use of a varied and expensive battery of antibiotics to combat contaminants.

Liquid media and vegetative inoculum remain our first and best option when attempting to isolate invertebrate pathogens that will not grow readily from asexual spores.

LONG-TERM PRESERVATION

Lyophilization (freeze-drying) is the most common method for preserving fungal cultures. Lyophilization is unsuitable, however, for cells with a high ratio of vacuolar to cytoplasmic volume (e.g. watermolds and zygomycetes such as the Entomophthorales). Cryogenic storage is the preferred long-term preservation technique for cultures of fungi that resist lyophilization, although storage of cultures in sterile distilled water or under mineral oil [5] may be acceptable for small working culture collections.

Cryogenic storage requires the use of a cryoprotectant to suppress harmful ice crystals during the freezing, maintenance, and/or thawing phase of storage [1]. There is no need to freeze all fungal cultures at a controlled rate of temperature drop of 1 °C per minute [7]; all those ARSEF cultures that can be lyophilized also survive direct plunging of vials from 4 °C into liquid nitrogen (−196 °C). Fungi that cannot tolerate lyophilization require controlled freezing rates, whether achieved by expensive electronically controlled cell freezing units, inexpensive commercial freezing blocks for use inside dewar flasks or other improvised techniques and equipment.

Cryogenic storage of cultures of highly fastidious entomophthoraleans is more problematic than for cultures of saprobes or less fastidious eukaryotic pathogens. Cultures of these relatively few fungi may be vigorous and sporulating well when frozen but when recovered from several years of liquid nitrogen storage, they may grow poorly and sporulate sparsely if at all. We have no means to assess the extent to which virulence or pathogenicity may decline during long-term cryostorage.

ALTERNATIVE USES FOR ENTOMOPATHOGENS

The ARSEF collection was begun to support basic research on fungi for potential biocontrol of insects, and this remains its primary mission. However, this collection includes many taxa that are closely related to the ergot fungus, *Claviceps purpurea*, which produces a range of pharmaceutically valuable compounds. Many of these relatives of *Claviceps* also produce toxins or other compounds that play several roles in nature and have proven pharmaceutical properties [8]. They may help to kill the host [6] or to protect mycotized cadavers against bacterial or fungal contaminants, or against destruction by mycophagous animals until the pathogen sporulates. The ARSEF collection is a global resource for the investigation of fungus-based biocontrol of invertebrate pests, but it also awaits exploitation as a potentially rich source of a diverse array of compounds that may have significant commercial value for agriculture and for medicine.

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