Manual for Mass Production of Entomopathogenic Fungi in Developing Countries with Particular Reference to *Beauveria bassiana* and *Metarhizium anisopliae*

Vladimir Gouli, Svetlana Gouli, Michael Brownbridge, Margaret Skinner & Bruce L. Parker

Entomology Research Laboratory
The University of Vermont
Burlington, VT 05405, USA

Produced with financial support from
US Agency of International Development
Bureau for Economic Growth, Agriculture and Trade
US-Israel Cooperative Development Research Program

Project Number: 415-921, Award Number: TA-MOU-99-C16-125

*Insect Pest Management with Fungi: A Mass-Production Technique for Farmers*

© 2005 The University of Vermont, Entomology Research Laboratory
Citation: Gouli, V., S. Gouli, M. Brownbridge, M. Skinner & B. L. Parker. 2005. Manual for Mass Production of Entomopathogenic Fungi in Developing Countries with Particular Reference to Beauveria bassiana and Metarhizium anisopliae. The University of Vermont, Entomology Research Laboratory, Burlington, VT, USA.

Copies of this publication can be requested from:

Bruce L. Parker, Program Leader
The University of Vermont
Entomology Research Laboratory
661 Spear Street, Burlington, VT 05405-0105 USA
Email: barker@uvm.edu
# Table of Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>TABLE OF CONTENTS</td>
<td>i</td>
</tr>
<tr>
<td>PREFACE AND ACKNOWLEDGMENTS</td>
<td>iii</td>
</tr>
<tr>
<td>CHAPTER 1. INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>CHAPTER 2. CHARACTERISTICS OF FUNGI</td>
<td>4</td>
</tr>
<tr>
<td>2.1 Classification and Morphology</td>
<td>4</td>
</tr>
<tr>
<td>2.2 Insect Infection and Biology</td>
<td>7</td>
</tr>
<tr>
<td>CHAPTER 3. MASS PRODUCTION</td>
<td>10</td>
</tr>
<tr>
<td>3.1 Basic Steps for Mass Production of Fungi</td>
<td>10</td>
</tr>
<tr>
<td>3.2 Equipment and Substrates</td>
<td>11</td>
</tr>
<tr>
<td>3.3 Cultivation of Fungi</td>
<td>15</td>
</tr>
<tr>
<td>3.4 Processing of Fungal Biomass</td>
<td>20</td>
</tr>
<tr>
<td>CHAPTER 4. QUALITY CONTROL</td>
<td>24</td>
</tr>
<tr>
<td>CHAPTER 5. CHEMICALS AND RAW MATERIALS SUITABLE FOR CULTIVATION OF FUNGI</td>
<td>26</td>
</tr>
<tr>
<td>5.1 Chemicals</td>
<td>26</td>
</tr>
<tr>
<td>5.2 Raw Materials</td>
<td>26</td>
</tr>
<tr>
<td>CHAPTER 6. EQUIPMENT</td>
<td>27</td>
</tr>
<tr>
<td>6.1 Routine Small Instruments and Equipment for Fungal Production</td>
<td>27</td>
</tr>
<tr>
<td>6.2 Larger Pieces of Essential Equipment</td>
<td>27</td>
</tr>
<tr>
<td>6.3 Miscellaneous Items</td>
<td>28</td>
</tr>
<tr>
<td>CHAPTER 7. MASS PRODUCTION FACILITY</td>
<td>29</td>
</tr>
<tr>
<td>7.1 Basic Facility</td>
<td>29</td>
</tr>
<tr>
<td>7.2 Additional Needs</td>
<td>29</td>
</tr>
<tr>
<td>CHAPTER 8. SAFETY AND SANITATION</td>
<td>30</td>
</tr>
<tr>
<td>8.1 General Health and Safety Guidelines</td>
<td>30</td>
</tr>
<tr>
<td>8.2 Specific Safety Issues</td>
<td>30</td>
</tr>
<tr>
<td>CHAPTER 9. CALCULATION OF PRODUCTION COSTS</td>
<td>32</td>
</tr>
<tr>
<td>CHAPTER 10. LITERATURE CITED AND KEY REFERENCES</td>
<td>33</td>
</tr>
<tr>
<td>APPENDICES</td>
<td>35</td>
</tr>
<tr>
<td>A. Calculation of Spore Concentration</td>
<td>37</td>
</tr>
<tr>
<td>B. Key Fungal Production Procedures In Pictures</td>
<td>39</td>
</tr>
</tbody>
</table>
PREFACE AND ACKNOWLEDGMENTS

In many developing countries, including Ethiopia, farmers are largely dependent on agricultural chemicals for management of insect pests. In some areas farmers are so poor they cannot afford to buy anything for pest management. New cost-efficient pest management solutions are needed for both of these cases. Indigenous locally-produced entomopathogenic (insect-killing) biological control agents offer inexpensive, non-toxic and self-sustaining means of pest management. Fungi have been largely neglected in the past as biological control agents, yet they represent a viable alternative to chemical insecticides. New discoveries in their growth characteristics and methods of formulation have stimulated renewed interest in their development. To promote use of fungi in developing nations, suitable in-country mass-production techniques must be devised that deliver viable, stable inoculum, are simple to implement and maintain, and utilize local ingredients.

This manual is the final outcome of a project to promote the use of insect-killing fungi for pest management in Ethiopia. The overall goal was to enhance local capacity to conduct research on microbial control and develop appropriate procedures for production of beneficial fungi. It was a collaboration among scientists from the University of Vermont, Entomology Research Laboratory (UVM); the Plant Protection Research Center (PPRC), Ambo, Ethiopia; the Volcani Institute and Tel Aviv University in Israel. Research was conducted collaboratively among the Ethiopian, Israeli and US scientists to evaluate methods for fungal mass production and test the efficacy of indigenous fungal isolates produced with these methods. At the close of this project, several Ethiopian scientists and technicians had been trained in insect pathology techniques and have a laboratory in which to carry out research to mass produce and further develop insect-killing fungi.

We appreciate the financial support from the US-Israel Cooperative Development Research Program, US Agency for International Development; and the University of Vermont College of Agriculture and Life Sciences. Assistance with research leading to development and testing of methods for fungal mass production was provided by Professor I. Barash, Dept. of Plant Sciences, Faculty of Life Sciences, Tel Aviv University, Tel Aviv, Israel; Dr. G. Gindin, Volcani Center, Bet Dagan, Israel; Dr. E. Bekele, Ethiopian Agricultural Research Organization (EARO), Holetta, Ethiopia; and Drs. W. Mandefro and M. Dawd, EARO, National Plant Protection Research Centre, Ambo, Ethiopia.
CHAPTER 1. INTRODUCTION

For many years, agrochemicals have been used to protect crops from invertebrate pests and diseases. These toxic pesticides have been responsible for maintaining, and in many cases increasing, agricultural productivity, and improving the quality of food and fiber. Extensive use of these materials has resulted in the development of resistance in many arthropod pests though, and strains that are resistant to different classes of insecticide or fungicide are common. In some cases, resurgences of secondary pests have occurred as a result of the indiscriminate action of the pesticides, which also eliminated natural enemies that had kept pest populations in check. These factors, combined with concerns over environmental and human safety, have provided major impetus to search for and develop cost-effective and more environmentally-safe plant protection techniques to minimize negative ecological impacts and other significant problems that can arise from extensive pesticide use. An integrated pest management (IPM) approach, where biological control agents play a major role in pest regulation, is increasingly advocated.

Several microbial control agents have been commercialized for selected target pests. For example, the milky spore disease, Bacillus popillae and B. lentimorbus were officially registered for use against scarab beetles in the U.S. in 1948. Bacillus thuringiensis (Bt) was first commercially used in the U.S. in 1958, and has since become the most widely used biopesticide in the world. The first strains discovered and used were active against lepidopteran pests, and Bt products based on the kurstaki strain, e.g., Dipel®, are frequently used to regulate caterpillar pests on ornamental, vegetable and field crops, and have become the products of choice against a number of forest defoliators. The discovery of Bt israelensis, which is active against mosquitoes and blackflies, revealed a broader range of activity, and provided another candidate for commercial development. Bt israelensis has since been extensively used in mosquito and biting fly abatement programs in the U.S., Germany and Israel, and for Africa in vector control programs. The isolation of Bt tenebrionis provided another novel strain, this time with activity against some coleopterans. Bt tenebrionis has been used against Colorado potato beetle in the U.S. and elsewhere, and in Australia to control a chrysomelid beetle in eucalyptus plantations. The protozoan, Nosema locustae was registered in the U.S. for grasshopper control in 1980. These bacterial and protozoan pathogens must be ingested to be effective, and thus arthropods with piercing-sucking mouthparts are unaffected by them.
Entomopathogenic fungi are unique in their ability to infect via the insect cuticle, although infection through the gut lining is possible. Various products based on *Beauveria bassiana* and *Metarhizium anisopliae* have been developed for use against a wide variety of pests in forest, field, and greenhouse environments, and against structural and household pests. Commercial formulations are utilized in Australia, South America, Europe, Russia and North America, as well as several African and Asian countries. However, their level of use is still very limited.

Under natural conditions fungi are responsible for significant reductions of insect pest populations and diseases, and are probably the most frequently encountered insect pathogens. In spite of this, they rarely occur at high enough levels or early enough in a pest outbreak to prevent economic damage. Environmental conditions may be manipulated to promote the incidence and impact of these pathogens, but most attempts to exploit insect-killing fungi have focused on their use through inundative releases, and their development as biopesticides. Fungi have great, but as yet underutilized, potential for use as biopesticides. Such products have many desirable traits—they leave no toxic residues on crops, are generally harmless to beneficial insects and other non-targets, and have a minimal impact on biodiversity. In fact, their host-specific nature enhances their potential role in IPM, as it preserves other natural enemies, which can contribute to the overall regulation of pests. Furthermore, they are considered safe for humans and, compared with chemical insecticides, represent a greatly reduced hazard in terms of the disposal. Many fungi are active over a range of environmental conditions year-round and are unaffected by factors such as day length, which can inhibit the performance of other natural enemies. To promote commercialization and broader utilization of these pathogens, production, stabilization and formulation methods must ensure that high-quality products with good shelf-life are made available to farmers. Studies have largely focused on the development of production techniques that optimize the yield of infective propagules (conidia, blastospores or mycelia), for obvious economic reasons. But, culture conditions also affect the virulence, longevity and ecological fitness of the resulting propagules. Thus they have to be devised with care, and various quality control procedures established to ensure that virulence and viability is maintained. Numerous fungi are relatively easy to mass-produce on inexpensive natural substrates in systems that are highly appropriate for developing countries, and mycoinsecticide industries have the potential to contribute significantly to the local economy. The following chapters outline processes and
considerations for the mass-production of entomopathogenic fungi in developing nations. The systems described require a level of technical expertise to ensure maintenance and production of pure, viable and virulent material, but these labor-intensive, low technology production techniques require relatively modest capital investment and can be readily adapted to suit local conditions and substrates.
CHAPTER 2. CHARACTERISTICS OF FUNGI

2.1. Classification and Morphology

*Beauveria bassiana* and *Metarhizium anisopliae* are filamentous, Hyphomycete fungi (Phylum Fungi Imperfecti or Deuteromycota). They reproduce by conidia that are usually formed on aerial conidiophores that develop on the surface of the arthropod host or artificial substrate.

Within the genus *Beauveria*, there are currently six recognized entomopathogenic species: *B. bassiana*, *B. brogniartii*, *B. amorpha*, *B. vermiconia*, *B. velata*, and *B. caledonica*. *B. bassiana* is one of the most commonly observed fungal pathogens on insects, and was traditionally known as the white muscardine fungus. It is also the most prominent species used in microbial control. The mycelium of the fungus is usually white but can take on a slightly yellowish color over time. The fungal hyphae are tubular, branched, partitioned filaments. Hyphal width generally ranges from 1.7 to 2.8 µm. The fungus produces one-celled conidia in whorls or dense clusters on straight, branched conidiophores, which can be single or aggregated, elongated or bottle-shaped. The conidia are globe- or egg-shaped (Fig. 1 a, b). Colonies on Sabouraud dextrose agar supplemented with yeast extract (SDAY) are commonly white, without any specific pigmentation (Fig. 2).

![Image](image.png)

**Figure 1.** *Beauveria bassiana*, a. at 200x magnification; b. at 400x magnification. Note dense clusters of round conidia.
At present, three entomopathogenic species of *Metarhizium* are recognized: *M. anisopliae*, *M. flavoviridae*, and *M. album*. Four additional species of *Metarhizium* have been described from China: *M. pingshaeme*, *M. cylindrospore*, *M. guizhousense*, and *M. taiti*. Practically all applications for management of migratory insects, pests in field crops, and household and structural pests, have involved *M. anisopliae*. The fungus forms short, simple or double-branched conidiophores which develop uninuclear, haploid phialadic conidia in chains. The conidia have an elongated oval form and range in size from 4.8-7.2 μm in length, and from 1.6-3.5 μm in width (Fig. 3). The conidia are usually green/brown in color and cover the insect cadaver. Two varieties of *M. anisopliae* exist: *M. anisopliae* var. *anisopliae* has smaller conidia (4.8-7.2 μm in length, and 1.6-3.5 μm in width); *M. anisopliae* var. *majus* has conidia that are approximately twice that size. Fungal colonies on SDAY are initially white, becoming green/yellow during sporulation (Fig. 4).
**Figure 3.** *Metarhizium anisopliae* mycelia (left); mature conidia (right).

**Figure 4.** *Metarhizium anisopliae* fungal colonies growing on SDAY at different temperatures.

**Figure 5.** Insects with visible symptoms of infection with *Beauveria bassiana*: a. western flower thrips, *Frankliniella occidentalis*; b. wax moth, *Galleria mellonella*. 
2.2. Insect Infection and Biology

Insect infection begins when conidia contact and adhere to the cuticle of a susceptible host. Conidia absorb moisture, swell and germinate, producing germ tubes that grow over the surface of the insect. If the correct biochemical cues are received, the germ tubes differentiate to form specialized infection structures or appresoria that anchor the fungus to the insect’s cuticle. Hyphae grow from the appresoria and penetrate the cuticle, using a combination of mechanical forces and enzymatic degradation. Preferred sites of infection are the inter-segmental membranes at joints in the exoskeleton. Once in the haemocoel, fungal mycelia develop throughout the host, forming numerous yeast-like hyphal bodies (often termed blastospores) in the haemolymph. The fungus rapidly colonizes all tissues and organs and death occurs as a result of this action, combined with toxicosis. Infected insects lose their appetite, and cease feeding and moving in the later stages of infection. Death usually occurs within 3-6 days under optimal conditions, although the rate of mycosis is strongly influenced by temperature; the optimal temperature for fungal infection to occur is between 24 and 30° C for most isolates. Under cool conditions, insect death may not occur until 3-4 weeks after the initial infection.
Once the fungus has colonized the insect, hyphae will emerge from the cadaver, often at joints in the cuticle (Fig. 6). If ambient conditions of temperature and humidity are appropriate, the fungus will conidiate on or close to the surface of the dead host. Conidia are frequently dispersed by wind, rain splash, or by contact with another insect – this is how epizootics may develop in a crowded insect population. If conditions are cool and/or dry, the fungus-filled cadavers remain intact and inactive until conditions favoring external growth and conidiation occur. Pathogenesis and the course of mycoses evoked by *B. bassiana* and *M. anisopliae* are essentially the same, although some *Metarhizium* strains are also known to infect insects via the digestive tract. The green conidia are very characteristic of *M. anisopliae* infection, and mycosed insects usually have a green color.

Entomopathogenic Hyphomycetes probably have the widest host spectra of all insect pathogens, and are often able to infect several developmental stages. *B. bassiana*, for example, infects all insect stages, including eggs, larvae, pupae and adults (Figs. 5 and 6). There are several notable examples of the successful utilization of this fungus as a biological control agent, where it has generally been used as a mycoinsecticide. Similarly, *M. anisopliae*, or the green muscardine fungus, also has a wide host range, and is commonly associated with soil-inhabiting insects (Fig 7). A number of commercial products based on this fungus are available, with notable successes in the regulation of soil-inhabiting chafer grubs in temperate zones to locusts in tropical and desert regions. In spite of these common associations, there is considerable variation between isolates of the same species and it is impossible to make generic assumptions about such strains, or to predict their host range simply on the basis of species.

*Figure 7. Chafer grubs infected with *M. anisopliae*; note green conidia on cadavers.*
Pathogenicity to the target pest is essential, but virulence should not be the sole criterion governing strain selection. For all entomopathogenic fungi, host infection is influenced by a variety of factors – both biotic (e.g., insect host stage, cuticular components, presence/absence of competitive microbes, insect host plant, etc.) and abiotic (e.g., temperature, humidity, ultraviolet radiation). Strains must be able to infect the host and persist in the environment in which they will be placed, and environmental competence is an important trait. In addition, if contemplating using the fungus as a biopesticide, it must readily produce conidia on artificial substrates. This manual provides procedures for the production of large quantities of conidia on inexpensive substrates for use against insect pests. The technologies and protocols described are appropriate for developing countries, and are intended to facilitate the production of high-quality materials for use in IPM programs.
CHAPTER 3. MASS PRODUCTION

3.1 Basic Steps for Mass Production of Fungi

Mass production of fungal biopesticides as a ‘cottage-industry’ in Ethiopia, and other developing countries, will rely on simple technologies utilizing locally-available raw materials for growth substrates and locally purchased equipment. In spite of this, the system must be well maintained to ensure production of high quality inoculum free from contaminants. Organization of a small industry for mass production of fungi requires that equipment, media, and other necessary items are prepared ahead of time. For optimal production of viable and stable fungal products, each stage of the production process must occur in a timely fashion and is facilitated by the advance preparation of all necessary materials. For example, the fungal inoculum used to inoculate the production media must be used soon after its preparation to ensure good viability and reduce risks of secondary contamination.

The mass production process includes the following basic steps:

- Preparation of equipment and culture media and sterilization.
- Cultivation of fungi from storage for inoculation of mass production media. The viability and purity of starter cultures is critical for success.
- Inoculation of large-scale production media under aseptic conditions.
- Harvesting and preservation of the fungal biomass.
- Disposal of the waste materials.

Each step in the process requires a scale-up in the level of inoculum required and produced, from an initial storage slant (taken from a culture collection), to solid (conidia) or liquid (blastospore) cultures to provide inoculum for the production media, through the large scale production of conidia or blastospores in solid or liquid fermentation.
3.2 Equipment and Substrates

A wide variety of non-specific equipment and growth substrates are used for mass production of fungi. Ideally, inexpensive and locally-available materials should be used. The quality and cost of the substrate selected will affect cost of production and quality of the final product. While fungi may grow on many substrates, they have specific demands in terms of C (carbon) and N (nitrogen) requirements, and the levels present in the medium affect growth rate, conidial production, viability and virulence. So, care should be taken to define the growth needs of different strains before scaling up the production process. Appropriate selection of strains is also important, and some will be better suited to mass production than others, in terms of speed of growth and conidiation, production of mycelia, and production of conidia. On solid substrates particularly, there should be minimal mycelial growth on the surface of the medium, and the fungus should conidiate readily. Once a strain has been shown to satisfy these criteria, then the principal demands are to ensure that all substrates used through the mass production process are sterile prior to inoculation and remains free of contamination throughout.

3.2.1 Preparation of jars, cultivation units, stoppers, etc.

For preparation of the initial fungal culture, simple laboratory instruments and standard glassware, etc., utilized in microbiological research can be used. For mass production, there are more specific requirements.

To prepare inoculum for mass production, the growth substrate can be held in wide-mouthed glass jars (Fig. B7). The optimal volume of the jars should be from 300 to 500 ml. The mouth should be covered with three layers of material: the first can be any fabric, but ideally one that does not absorb too much water; the second should be paper; and the third aluminum foil (Fig. B7a). The final foil cover prevents the contents of the jar from drying out while keeping the other layers dry during autoclaving (Fig. B7b). Before use, the jars must be carefully cleaned and dried.

Mass-production of fungi is most conveniently done in plastic bags. The size and type of bag can vary, but autoclavable bags measuring approx. 30 x 60 cm are the best; they are strong and may be re-used several times (Figs. B12 & B15). However, some types of plastic grocery
bags that can be purchased in local markets that also work effectively (Fig. B12). These cost significantly less but function well. Some pre-testing of their ability to withstand autoclaving must initially be done. Once identified, bags may be purchased in bulk from local suppliers. We were able to purchase 50 grocery bags in Ethiopia, for example, for about the same price as one autoclave bag from a scientific supplier. With care, these grocery bags may be used in 2-3 production cycles, although generally they will only be suitable for a single production batch. They can be used in the same manner as the ‘autoclave’ bags, and the process described below is appropriate for both. It is essential to allow good air exchange in the bag and throughout the medium. This can be achieved using a simple ‘stopper’. The mouth of the bag is passed through a metal, plastic or waxed cardboard ring, and held in place with paper tape or a rubber band (Fig. B16). The opening is then covered in the same manner as the jars—three layers, fabric, paper, foil (Fig. B17). Paper towels and dense cotton cloth cut into squares measuring approx. 20x20 cm will provide protection against entry of contaminants, but will allow free exchange of oxygen into the bags. The complete ‘unit’ can be autoclave sterilized, or the rings and paper/cloth can be sterilized separately in autoclavable bags. The production unit can then be assembled after inoculation of the grains in a laminar flow hood or other sterile area. Instead of plastic bags, large glass jars or other non-flexible containers can be used. These must have a wide neck, and may vary in volume from 1 to 3 liters. They are not ideal, however, as they occupy relatively more space, they are heavy, production media cannot be ‘mixed’ during the growth phase of fungi, and their sterilization requires more space, time, and energy.

3.2.2 Nutrient media for starter cultures

Sabouraud dextrose agar supplemented with yeast extract (SDAY) and potato dextrose agar (PDA) are suitable starter media for both fungal species. SDAY supplemented with citric acid is prepared at one-fourth the standard full rate as follows (in g/l):

- peptone 2.5 g
- dextrose 10.0 g
- yeast extract 2.5 g
- agar 15.0 g
- 0.2 ml citric acid (stock solution 50 g citric acid in 100 ml sterile distilled water)
- 1000 ml distilled water
Potato dextrose agar is prepared according to standard media instructions, and is generally available as a pre-mixed dry powder.

If standard dry ingredients for media cannot be readily obtained, a simple medium based on potatoes can be prepared. The potatoes are peeled and boiled; 200 g of cooked potato are mixed with 200 ml water and passed through a fine sieve. A blender can be used to provide a more homogenous mixture. Dextrose (20 g), agar (15 g) and more water (800 ml) are added to the potato mixture and autoclaved.

Media should be sterilized at 121°C for 30 min. and then poured into sterile Petri dishes or tubes for slants (Fig. B4a, b). In the latter case, tubes should be laid at an angle of about 30° before the medium has cooled and solidified (Fig. B 1b & B4). The medium should not extend beyond the middle of the tube to reduce the risk of contamination and lids should be loosened slightly to minimize the build-up of condensation on the surface of the medium. Ideally, tubes or Petri dishes should be allowed to dry at room temperature for 24-48h prior to use or storage. In all cases, tubes or dishes should not be opened before inoculation. Petri dishes or slants may be stored for several months at room temperature or under refrigeration, although it is preferable to prepare several smaller batches of media every few weeks. Tube caps must be tightened to prevent dehydration of the medium but Petri dishes must be sealed individually or placed inside plastic bags which are then closed; Petri dishes must be stored in an inverted position to prevent build up of condensation on the surface of the medium. Under refrigeration, condensation will build up inside the dishes over several weeks, and they should be allowed to dry at room temperature before use.
3.2.3 Substrates for production of starter cultures

The dry substrate (usually grain) is placed into glass jars (300-500 ml capacity). If the substrate contains two or more components, these should be mixed while still dry and before adding to the jars. The substrate is then mixed with water in a 1:1 (v:v) ratio. This ratio may vary depending on the substrate used. The jars are then closed with a fabric-paper lid held in place with a rubber band, which is then covered with aluminum foil. The closed jars are held in a water bath at 90°C for 1 h. The water level in the bath should reach no higher than the middle of the jars. The grain will absorb all of the water in the jar during this time and the volume of substrate in the jar will significantly increase (Fig. B6). For example, if a jar has a capacity of 500 ml, and 125 g grain:125 ml water added to it, the grain will swell to approx. double that volume and will occupy most of the jar. This is an important consideration when filling the jars, as the final volume of substrate should occupy no more than half the total volume of the jar, ideally around 30% to ensure good aeration and colonization of the medium during fermentation. Preparation of the preliminary substrate can also be done on a larger scale, in a saucepan of 10 l or more; the cooked substrate can then be distributed to the jars (Fig. B7). In both cases, the jars plus cooked medium must then be autoclaved at 121°C for 1 h. (Do not close the bags tightly during sterilization to allow steam to enter and completely sterilize the growth medium) (Fig. B12). After cooling, the substrate is ready for inoculation with B. bassiana or M. anisopliae.

3.2.4 Solid substrates for mass production

If autoclavable bags (from a supplier or locally purchased plastic grocery bags) are used, they should be prepared in a similar manner to the jars. The substrate is mixed with water at a ratio of approx. 1:1. The bags plus substrate and water are then held in a water bath at 90°C for 1.5 - 2 h. Care should be taken to prevent entry of water from the bath into the bags. The volume of substrate in the bags will increase during cooking, and the final volume should occupy no more than half the bag, ideally between 30-50%. The bags plus substrate are then autoclaved at 121°C for 1 h. After cooling, the substrate is ready for inoculation with B. bassiana or M. anisopliae.
If no other bags are readily available, disposable (non-autoclavable) polythene bags can be used. They must be purchased new and can be used only once for production. They are filled with hot substrate that has been cooked (see 2.2.3), preferably inside a laminar flow cabinet, or at least in a clean room with no air movement. Bags are closed to prevent entry of contaminants, but should be sufficiently loose to prevent build-up of condensation and to allow good aeration. The medium is allowed to cool and should be inoculated as soon as possible after preparation. A high concentration of inoculum is used to ensure rapid colonization of the grains by *B. bassiana* or *M. anisopliae*; this will prevent growth of contaminants. Substrate can be inoculated prior to closing the bags using a regular pipette or similar, or using a syringe to inject inoculum into the bags after closure. The outside of the bag should be swabbed with 70% alcohol before injection. Tops can be folded and closed with staples to allow air exchange.

3.3 Cultivation of Fungi (refer to Appendix B for photos of most of the steps described below.)

The mass production process involves several stages: initial preparation of inoculum from stored slants; cultivation of fungi in jars for production of larger quantities of inoculum; and finally, mass cultivation on grains. Each stage demands special attention to prevent contamination and ensure the quality and virulence of the final product. Work with and maintenance of the primary fungal cultures should be done in a well-equipped laboratory by personnel trained in mycology, as this work demands use of aseptic practices and specialized equipment. These laboratories provide starter cultures for the first step in the production process (jars); once inoculated, these may be distributed to satellite institutes where inoculation of the mass production substrates occurs. This approach ensures that clean inoculum with high potential for growth and with a high level of viability and insecticidal activity is provided; and that the final product will be of high quality, reducing risks of attenuation and contamination. Similar methods are used by the mushroom industry where specialized facilities produce the inoculum (spawn) which is used to inoculate the growth medium, providing mushrooms of consistent quality.
3.3.1 Primary fungal cultures

Maintaining strain quality in storage and initial stages of production is essential to the long-term success of a mass-production facility. The initial culture must be identified to species by an appropriate specialist. After a virulent isolate has been selected that fulfills all critical selection criteria (e.g., conidiates readily on artificial growth substrates, is stable after sequential sub-culturing, has the required level of performance at low/high temperature, ecological competence, etc.). The isolate to be mass-produced must have been identified according to morphological and growth characteristics, and should be characterized fully using standard molecular methods to confirm its identity. This may have to be done at an external institution, but it is important to generate a unique molecular profile for any 'commercial’ isolate. Production of stock materials for preservation should ideally be done from single spore isolates. This reduces the level of genetic variation seen in multi-spore isolates and reduces the risk of attenuation (loss of virulence and other desirable traits) over time. Multiple transfers on artificial media should be minimized. All initial work described above should be done at a research center, not a field-based mass-production facility.

Cultures prepared from the selected single spore isolate should be put into long- and short-term storage. For short-term storage (ideally no longer than 3-6 mo, 12 mo maximum), slant cultures may be used which are ideally held under refrigeration (≈4°C). A sufficient number of working slants need to be prepared according to the projected need over time. Tubes must be inoculated under strictly aseptic conditions and incubated at 22-26°C. Both B. bassiana and M. anisopliae grow well at this temperature range, although optima for growth and sporulation will vary from isolate to isolate. To allow for complete vegetative growth and sporulation of the fungi, slants should be incubated for 12-14 d before tightly closing and sealing the lids of the culture tubes (with Parafilm) to prevent drying of the medium, which can adversely affect viability. Mature slant cultures can be maintained in refrigeration for up to 6 mo, although storage times vary depending on the medium. It is preferable to use a relatively nutrient-poor agar medium, such as quarter-strength SDAY, to minimize vegetative growth, which can occur on richer media, even at low temperatures. Tubes should be periodically selected at random, and checked for presence of contaminants. Petri dish cultures should be held for no more than 1-1.5 mo, and sealed with Parafilm prior to use (Fig. B5).
For longer term storage, slant cultures can be covered with sterilized mineral oil (Fig. B2a). This produces an anaerobic environment that prevents fungal development. The oil should be sterilized twice at 121°C for 60 min (total sterilization time 120 min), and after cooling, the oil can be used. Fungal cultures can be preserved under oil for a relatively long time, even at room temperature. *B. bassiana* can be stored this way for ~2 yr, *M. anisopliae* for at least 1 yr. One such culture tube can be used to inoculate several working tubes for each mass production cycle. This approach provides the minimum number of transfers and maintains fungal virulence and good production characteristics.

### 3.3.2 Preparation of primary inoculum

To provide inoculum for the initial phase of the mass-production process, fungal cultures should be prepared Petri dishes or large culture tubes containing quarter-strength SDAY or PDA. Large tubes may be preferable as there is reduced risk of contamination. Procedures should be carried out in a laminar flow hood or equivalent sterile environment (e.g., a room or bench-top cabinet sterilized with an ultra-violet light) using aseptic techniques. All media are prepared according to standard methodologies. The starter cultures are prepared from working slants or can be established from slant cultures held under mineral oil. For each production run, a new slant should be used. It is important to minimize the number of transfers from the initial source and protocols must take this into account.

If a culture under mineral oil is used, the oil is first removed using a sterile Pasteur pipette (Fig. B2b). Then, 10 ml of a 0.01% sterile Tween 80 is added and the conidia put into suspension using the pipette or a sterile spatula (Fig. B2c). A good slant culture will provide at least 1 x 10^8 conidia/ml. It is not necessary to determine the exact conidial concentration; a suspension of *B. bassiana* conidia will be a milky-white color while one of *M. anisopliae* will have a slightly greenish hue (Fig. B3). For inoculation of Petri dishes or culture tubes, 0.1 ml of a suspension is applied to the medium using a micropipette, and spread evenly over the surface. Once inoculated, the tubes or plates are incubated at 24°C for 12-15 d, after which any cultures showing signs of poor growth or sporulation, or evidence of contamination, are rejected (Fig. B5). Cultures showing good sporulation and freedom from contamination are used to inoculate media in the second phase of production; good cultures occupy the whole surface of the medium.
and conidiation is homogenous. Contaminants are generally bacteria or fungi. Common fungal contaminants form black, yellow, green/blue or red colonies. In the case of bacterial contamination, growth of colonies is frequently accompanied by an unpleasant, rotten smell. It is also beneficial to examine samples taken from some of the plates/tubes under a microscope to further confirm the purity of the cultures. *B. bassiana* and *M. anisopliae* have specific morphological characteristics that can be readily confirmed when viewed under a microscope.

3.3.3 Inoculation of jars for preparation of seed inoculum

If culture tubes are used in step 2.3.1, 5 ml of sterile 0.01% Tween 80 is pipetted into the tube and gently agitated using a vortex mixer. The suspensions from the tubes are collected in a sterile flask or other sterile glass container (Fig. B8). If plate cultures are used, they should be flooded with 5-10 ml 0.01% Tween 80 and conidia dislodged into suspension using a bent glass rod or a spatula. The resulting suspensions are also pooled into one container.

To determine conidial concentration and viability, 0.1 ml of suspension is added to each of three tubes containing 0.9 ml sterile distilled water. Tubes are vortexed for 1 min before estimating conidial concentration using an improved Neubauer hemocytometer (see Appendix A for calculation of conidial concentration). To estimate conidial viability, 0.1 ml is transferred from each tube to separate Petri plates of quarter-strength SDAY. The suspension is spread over the surface of the medium and Petri dishes incubated at 22°C for 16 hr for *B. bassiana* and 22 hr for *M. anisopliae*. The suspension can be spread over the surface using a bent glass rod or sterile cotton-tipped swab, using a fresh swab for each plate inoculated. After this, three separate drops of lactophenol cotton blue stain are put onto each plate and covered with glass cover slips. The stain kills the fungi and allows germination rate to be calculated. To do this, make three counts of 100 conidia from each cover slip on each Petri dish (total nine counts) under a phase contrast microscope, counting the proportion of germinated and non-germinated conidia and calculating a mean value from all readings. A spore is considered to have germinated if a germ tube has emerged from the spore; swollen spores are not considered to have germinated. As a rule, freshly harvested conidia are 95-100% viable.

18
Preparation of the culture jars and sterilization of the grain has been described in section 3.23. Once cool, the grains are inoculated by syringe; 5 ml of spore suspension is added to each jar, removing the foil cover and piercing the cloth/paper layers using the syringe needle (Fig. B9). The foil cover is replaced and the contents of the jar gently mixed. Jars are incubated at 22-24°C (Fig. B10). After 4 d, the grain will be covered with fungal mycelia. At this time, it is necessary to re-mix the grain and fungi. After mixing, the foil cover is removed from the jars to allow greater aeration of the medium. After a further 3-5 d the mixing process is repeated. Mature conidia will appear on grains after 8-10 d, but may be held for 12-d to ensure complete conidiation (Fig. B11). After this period, fungi can be harvested for inoculation of bags.

3.3.4 Mass cultivation of fungi on solid substrate

To prepare inoculum from the jars, 100 ml sterile 0.01% Tween 80 is added to each jar (Fig. B14). The contents of the jars are gently mixed, taking care not to contact the cover. Conidia are released into suspension and the jars are left to stand for several minutes to allow the larger particles to settle. The supernatant suspension is then collected via a pipette. The suspension (~50 ml) is transferred to a 100 ml sterile flask, and the conidia concentration determined using previously described methods (Figs. B13, 14). Conidial concentration should be adjusted to approx. $1 \times 10^7 - 1 \times 10^8$ conidia/ml using sterile distilled water.

Ideally, the bags containing the grain should be sterilized at the end of the previous day, remaining in the autoclave overnight to ensure their sterility is maintained (Fig. B12). Bags are inoculated when the medium has totally cooled, and inoculation should be done inside a laminar flow hood. If polythene bags are used, hot grains should be added to the bags on the day of inoculation and allowed to cool completely before inoculum is applied. Inoculum is applied to the surface of the grain using a large sterile syringe, pipette or beaker, 10 ml of inoculum for each bag (Fig. B15). If the bags were ‘pre-assembled’ for sterilization, the cloth/paper/foil cover is simply replaced. If the bags were only folded for autoclaving, the open mouth of the bags is pushed through the metal, plastic or cardboard ‘ring’ and folded back over the ‘neck’ (Fig. B16). The mouth is covered with the sterile paper/cloth layers, which are held in place with a rubber band. The third foil layer is then placed over the paper/cloth cover (Fig. B18). The grains and inoculum should be carefully mixed. Bags should be placed on their sides, making sure there is
air space above the solid medium. Bags are put on wire mesh shelves inside a designated growth room (Fig. B17). Use of open shelves allows air to circulate freely around the bags, preventing localized heating of the substrate as the fungus grows. Inside the room, the ambient temperature should remain relatively stable (22-26° C) throughout. Constant light is not required. The substrate should be distributed evenly inside the bag in as thin a layer as possible to maximize the surface area for gas exchange and growth (Fig. B17).

Three to four days after inoculation, the substrate should be gently mixed again without opening the bags; any clumps of grain should be gently broken. At this time, the foil cover can be removed from the mouth of the unit to facilitate greater aeration. The surface of the grains will be almost totally colonized by the fungus at this stage in production, so the risks of contaminants growing on the substrate are minimal. The mixing process is repeated 1-2 more times, 3-4 days apart. Fungal growth and sporulation will be complete after 10-15 d, at which point the bags can be carefully opened and the material used immediately (or within 2-4 weeks if stored in a refrigerator) or air-dried for harvesting of dry conidia and longer-term storage and formulation.

3.4 Processing of the Fungal Biomass

Once harvested, the fungal biomass can be formulated in several ways to maintain the long-term viability of the conidia in storage and transportation, and to enhance stability and other desirable characteristics during mixing and application. As a rule, industrial formulations are based on dried conidia, and incorporate several components including stabilizing and wetting agents, stickers, oxygen scavengers and desiccants (dry formulations), and substances to protect them from ultraviolet radiation. Dry conidia powders harvested from grains often contain small particles of the substrate on which they were grown. These can be hygroscopic and will negatively affect the long-term viability of the powder if incorporated into the formulation. Therefore, techniques must be used that ensure the purity of conidial powders and minimize the incidence of the substrate in a dry formulation. When mass-produced on a small scale, however, fungal material is generally produced for use in the short term. Materials can be applied fresh so that complex formulations are not necessary.
3.4.1 Storage, estimation of quality and use of fresh fungal biomass

Mature fungal cultures and conidia produced in plastic bags or jars can be stored at 2-4°C for up to 4 wks without major loss of viability. At 10-12°C viability, and hence activity, declines more rapidly and material must be used within 2 wks. An estimate of conidial viability should be made by sampling the biomass prior to the preparation of a spray suspension using protocols described previously.

The wet fresh fungal biomass can be used without special processing. Grains plus fungi are placed in 0.001% Tween 80 (or a similar detergent). The ratio of biomass:Tween should be approximately 1:10. The components are mixed carefully to dislodge the conidia from the substrate, and the suspension is separated from the grains by filtering through several layers of muslin gauze or cheesecloth. On average, 1 kg of substrate will yield approx. 10 liters of suspension containing $10^{10}$ conidia/ml. For spraying, a concentration of $10^7$-$10^8$ conidia/ml is usually prepared, so one bag can yield enough conidia for 100-1000 liters of spray mixture, sufficient to treat at least 1 ha for most pests. Once prepared, though, spore suspensions should be used within 5-7 hr, especially at temperatures >25-28°C, as conidia are rapidly killed in warm water.

3.4.2 Drying, estimation of quality and use of dry conidia

For long-term storage, fungal conidia must be dried. Two methods of drying are commonly used. In the first, conidia are dried to moisture levels equivalent to the ambient conditions. In the second, conidia are dried to a specific moisture content using a de-humidifier or by placing them over moisture absorbent chemicals or gels, e.g., anhydrous calcium carbonate or silica gel.

In the first method, the fungi plus substrate are transferred from the plastic bags to plastic or (preferably) cardboard seed trays or large brown paper grocery bags (Fig. B21). Two trays are generally needed for each bag so that the substrate can be spread in a thin, even layer over the base of the trays to enhance drying. The material is mixed periodically and the moisture content and conidial viability determined. This should be done at least three times during the drying process, and within 2-4 d of its completion. To determine moisture content, raw samples are weighed and then re-weighed after drying at 80°C for 24 h; ideally, moisture content should be
<10% for long-term viability. Viability is assessed by sampling a small quantity of the substrate plus conidia, suspending the conidia in a small volume of 0.01% Tween 80 (or other wetting agent) and plating on SDAY or PDA, evaluating germination after 16-24 h, depending on the strain. The duration of the drying process depends on the ambient temperature and humidity, air circulation and ventilation. At 25-26°C during the day, ~17°C at night, drying is complete after 4-5 d.

Alternatively, a commercial de-humidifier can be used to speed up drying. If such equipment is not available, some means of drying the air, e.g., passing the air through trays of silica gel before circulating in a drying room, can be used. Conidial viability and moisture content should be determined at the start of the process, and after 6, 12 and 24 h, and again within 4-5 d of completion.

Drying the fungal material too rapidly or slowly can be harmful to conidia, so it is critical to determine viability after drying is complete. Conidial concentration can be adjusted accordingly to ensure that sufficient viable conidia are contained in a spray suspension.

If it is necessary to separate conidia from the growth substrate for formulation or long-term storage, several methods can be used. The simplest method involves placement of the dried substrate plus conidia into a fine mesh bag (~25 x 50 cm), which is held inside a plastic bag (35 x 60 cm or larger) (Fig. B23). Any large clumps are first broken apart and the entire contents are then shaken for 3-5 minutes. Conidia are separated from the grains and pass through the mesh into the plastic bag. Conidia can then be collected from the plastic bag.

Another way to harvest dry spores involves the use of a series of sieves of decreasing mesh size; sieves used in soil washing are ideal (Fig. B22). The dry fungal material is placed in the coarsest sieve (e.g., N50) at the top of the stack; 2-3 sieves of declining mesh size (N30 and smaller) are generally sufficient. The stack is enclosed within a plastic bag and shaken gently. Conidia can be collected from a solid pan attached to the bottom of the stack or sieves, or recovered directly from the bag in which the sieves are held. Pure conidia can be dried further by placing them over silica gel or another desiccant. Spore viability will be maintained at high levels for at least 12 mo if they are kept in a cool, dry place.
During separation, two-three different fractions will be obtained. The first fraction consists of pure conidia with minimal residue from the grain; the concentration of conidia will be approx. $1 \times 10^{11}/g$. The second fraction is made up of conidia with 40-50% by volume of grain residue, containing approx. $1 \times 10^{10}$ conidia/g. The third fraction is primarily larger particulate matter – grains plus mycelia and conidia – and contains no more than $1 \times 10^9$ conidia/g. All three fractions can be used for plant protection. The first fraction containing pure conidia may be used for low-volume sprays (5-40 liters/ha), formulated in oil or water (Fig. B24). In this case, the conidia suspension has to contain no less than $10^8$ conidia/ml. The second fraction may be utilized in a high volume spray program (800+ liters/ha) where coarse spray nozzles will allow passage of the larger particulate matter (substrate). The third fraction can be used for soil application to control subterranean pests; in soil, re-growth and sporulation can occur on the grains, providing higher levels of infective inoculum over time.

### 3.4.3 Safety precautions

During processing, direct contact with the fungal biomass or conidia is not desirable because it can cause an allergic reaction. The work can produce large clouds of dry conidia unless care is taken during harvesting, and can cause respiratory problems or inflammation of mucous membranes, eyes, etc., or rashes in some people. For this reason, it is necessary to work in a well-ventilated and isolated room. All personnel working in this environment must be equipped with full-face respirators and protective (cool) clothing that can be removed on exiting the facility (Fig. B25). Any substrate that is not used can be sterilized by autoclaving or placing in 5.25% bleach to kill all life spores, and then composted. All surfaces inside the facility should be regularly cleaned and sterilized with bleach to prevent build-up of spore dusts.
CHAPTER 4. QUALITY CONTROL

Quality control is an essential component of a mass production system. It is necessary to ensure the consistent supply of a stable, viable and virulent product that is free from contaminants, and of consistent concentration and purity. Quality control protocols must be followed throughout the production process, and is especially critical when fungi are actively growing, during drying, and in storage. During the active growth period, and when harvesting conidia, particular attention must be paid to the following:

1. Control of contaminants – preventing contamination of all production media
2. Control of conidia formation – providing conditions conducive to the production of large quantities of viable conidia
3. Control of moisture in the mature material – to ensure prolonged conidial survival and to prevent growth of contaminants
4. Control of viability – ensuring conidia are harvested and dried at the optimum time and are preserved and held under appropriate conditions according to need
5. Maintenance and testing of virulence – preventing attenuation of stock cultures and ensuring that protocols are followed during production/recovery to ensure production of fungi that are viable and virulent

1. Substrates should be visually checked every 1-2 d after inoculation to make sure they are not contaminated during the active growth phases. Bacteria and/or saprophytic fungi are common contaminants. Personnel who are working on fungal mass-production should become familiar with the symptoms of growth of frequently encountered species. This will ensure that contamination is detected early. Bacterial contamination may be characterized by a putrid smell coupled with abundant moisture on the inside of the jar or bag. Saprophytic fungi frequently form localized colonies which are black, blue/green or yellow. Bags or jars with these symptoms should be discarded and sterilized in an autoclave prior to disposing of the contents.

2. While the rate and level of conidiation on different substrates will vary, moisture and temperature must be strictly regulated to promote abundant spore formation.
3. If harvesting conidia for long-term storage or formulation, after conidiation is complete and the conidia have matured, the substrate must be dried to facilitate separation of spores from the medium. Conidia may need to be dried further to improve their stability in storage. Control of moisture is vital to prevent conidial germination; temperature must be regulated to promote drying, but must not be too high to maintain viability.

4-5. To ensure that viable and virulent conidia have been produced before and after drying, when conidiation is complete samples should be collected from several bags (representing 5-10% of the total number) selected at random during any production run (approx. 1 g per bag). Samples are carefully mixed to provide a homogenous representative sample of the production batch. The composite sample should then be sub-divided and tested for moisture content, conidial viability, and virulence. After drying, a similar dry composite sample should be prepared, sub-divided and similarly assessed (time 0). Sub-samples should be held at 22-28º C (room temperature), 10-12º C (cool), and 4-6º C; viability should be determined after 15, 30 and 60-d in storage, noting the level and speed of germination following storage at each temperature. Batches with <85% viability at time-0 may be considered of poor quality. Adjustments to the final formulation may be necessary to ensure the correct number of viable conidia are contained in the final product. Virulence may be estimated using a standardized bioassay against an insect that is known to be susceptible and is available year-round. These steps will ensure the quality of the product prior to application for pest control.
CHAPTER 5. CHEMICALS AND RAW MATERIALS SUITABLE FOR CULTIVATION OF FUNGI

5.1 Chemicals

- Peptone
- Dextrose
- Yeast extract
- Agar
- Citric acid
- Sodium hypochlorite, 5.25% (bleach)
- Tween 80 (or similar surfactant, e.g., Triton X-100)
- Mineral oil (high grade paraffin oil)
- Lactophenol cotton blue stain

5.2 Raw Materials

- Sorghum and waste materials from sorghum processing
- Millet
- Rice and rice bran
- Barley
- Wheat and wheat bran
- Soybean mash
- Maize meal
- Oats
CHAPTER 6. EQUIPMENT

6.1 Routine Small Instruments and Equipment for Fungal Production

- Large glass culture tubes
- Paper
- Fabric (cotton)
- Aluminum foil
- Rubber bands
- Wide-mouthed glass jars (300-500 ml capacity)
- Autoclave-resistant plastic bags or unused polythene bags
- Non-absorbant cotton wool or polyurethane bungs for flasks
- Inoculating loops
- Scissors
- Glass test tubes
- Petri dishes
- Microscope slides
- Cover slips
- Pasteur pipettes
- Pipettes (1 ml)
- Plastic, metal or cardboard ‘rings’ for formation of aeration ‘necks’ in culture bags
- Conical flasks
- Haemocytometer
- Thermometers
- Respirator
- Cardboard trays, large brown paper grocery bags, or plastic trays for drying conidia

6.2 Larger Pieces of Essential Equipment

- Autoclave or pressure cooker
- Refrigerator
- Incubator
- Microscope with phase-contrast capability
- Stereomicroscope
- pH meter
- Laminar air flow hood (ideal), sterile cabinet or room
- Balances (0.01-100g; 0.1-10 kg)
- Water bath
- Still, for production of distilled water
6.3 Miscellaneous Items

- Dustpan and broom
- Eyewash station
- Fire blanket
- Fire extinguisher (suitable for electrical and general fires)
- First aid kit
- Desk for writing
- Safety shower
CHAPTER 7. MASS PRODUCTION FACILITY

7.1 Basic Facility

Below are the rooms needed to mass produce fungi and a description of their specifications.

- Room for working with fungal cultures, ideally equipped with an ultraviolet light, sterile bench or laminar air flow hood. Access to this room should be restricted and air movement within the room limited to prevent air borne contamination.
- Room for mass production of fungi, with 'open' metal shelves to allow air flow around production bags. Access to this room should be restricted to production personnel. Surfaces (floor, shelves, walls) should be constructed of materials that are easy to clean.
- Room for drying and processing fungal material. It should be well ventilated and access to must be restricted. Ideally, safety equipment, overalls, etc. should be easily accessible in an enclosed area outside the main drying room so workers can put on protective gear prior to entry, and store it outside the room away from contamination.
- Room for storage of fungal formulations; limited access.

All rooms should be cleaned regularly using bleach or other liquid sterilant (not dry) to keep them clean and free from contaminants, including residual fungi from the production process. Interior surfaces (walls, floors, ceilings, shelves) should be water-resistant (painted, tiled) for easy cleaning. Bench tops must be impervious to water and resistant to acids, alkalis, organic solvents, bleach, and moderate heat. Ideally, rooms should be temperature-controlled, or located where temperature fluctuations are minimal year round, and can be maintained at 22-26° C. Air circulation is needed in the production and drying rooms to prevent localized build-up of heat and moisture.

7.2 Additional Needs

- Room for storage of all mass production materials (grains), secure from rodents and stored product insects.
- Room for preparation and packing of mass-production media.
- Wet lab for washing and drying equipment, preparation of media, and sterilization of materials.
CHAPTER 8. SAFETY AND SANITATION

8.1 General Health and Safety Guidelines

- Do not eat, drink, smoke or store food in mass-production facilities.
- All work places should be regularly cleaned and not used for storage of non-essential equipment or materials.
- Hands should be washed carefully before and after working with fungi.
- Broken glassware should not be picked up by hand.
- Any breakages or spills should be cleaned up immediately and sterilized as appropriate.
- Do not use equipment unless you have been instructed in its operation.

8.2 Specific Safety Issues

8.2.1 Dry fungal powder

- Respirators, gloves and overall should be worn when working with mature and dry fungal cultures (during harvesting and formulation).
- Pregnant women and people with allergic sensitivities should not be allowed to work with dry fungal biomass.

8.2.2 Alcohol

- Keep containers of alcohol away from excess heat and open flames.
- Do not use in the presence of an open flame.
- Allow to evaporate fully before using an open flame.

8.2.3 Electrical equipment

- Electricity and water do not mix! Do not allow water or aqueous solutions/suspensions to come in contact with electrical chords, equipment or outlets.
- Make sure hands are dry before handling electrical equipment.
- If electrical equipment cracks, sparks or gives off smoke, unplug immediately and do not use it; seek assistance from a qualified electrician to repair.
8.2.4 Fire precautions

- If a gas leak is detected or develops, turn off the gas at the burner and the source; do not use again until the problem is fixed.
- If clothing catches fire, extinguish immediately with water or a fire blanket (staff should be trained in ways of responding to fires).
- Do not use water on electrical fires; dry fire extinguishers should be made available and staff instructed in their use.
- In the case of a small fire, try to smother the flames quickly with a towel or book.
- If a larger fire occurs such as in a waste-paper basket or sink, first raise the alarm; if an extinguisher is available, put out the fire.
- In the case of a larger fire in the laboratory itself, evacuate the room and building as quickly as possible, remembering to turn off all gas burners and unplug electrical items if possible and closing doors behind you; alert the fire authorities as soon as possible.
CHAPTER 9. CALCULATION OF PRODUCTION COSTS

Fungal mass production requires allocation of specialized facilities, equipment and materials, and an uninterrupted supply of electricity and gas. A trained and qualified labor force is also critical to successful production of a high quality fungal final product. Based on the fixed rate of these expenses, it is possible to calculate the cost of producing fungi. This is especially critical for the commercialization of fungal-based products. It is also important to define where costs are incurred and where potential savings can be made so that production is as economical as possible. However, cost cuts need to be made carefully as all inputs will affect the quality of the final product. Costs associated with distribution, sales and marketing of products also need to be factored in to the final calculations.
CHAPTER 10. LITERATURE CITED AND KEY REFERENCES


APPENDICES

Appendix A. Calculation of Spore Concentration

Appendix B. Key Fungal Production Procedures In Pictures
APPENDIX A.


Materials and Methods

Prepare a suspension of conidia prepared from a known weight of grains (e.g., 0.5 g in 9.5 ml 0.01% Tween 80 or similar surfactant) or spore powder (e.g., 0.1 g in 9.9 ml 0.01% Tween 80) in glass test tubes. Addition of glass beads to these and the dilution tubes will help break up spore clumps and produce a more homogenous suspension. Take 1 ml of the primary suspension, and add it to a second tube containing 9 ml 0.01% Tween 80 (-1 dilution). Take 1 ml of the -1 dilution and add it to a third tube containing 9 ml 0.01% Tween 80 (-2 dilution). Take 1 ml of the -2 dilution and add it to a fourth tube containing 9 ml 0.01% Tween 80 (-3 dilution) to achieve a $10^{-3}$ dilution from the original suspension. Prior to each transfer and counting, each dilution tube should be vortexed for 30 seconds. Spore concentrations in the -2 and -3 dilution tubes will generally be appropriate for counting using a haemocytometer.

Place the glass coverslip on the haemocytometer and load each side of the counting area with the appropriate suspension using a capillary tube or a 0.1 ml micropipette. Place the haemocytometer on the stage of the phase-contrast microscope and allow spores to settle for approx 2-3 mins. At 40x objective magnification, focus on the counting grid. The grid will be made up of 25 large squares (or cells in a 5 x 5 array), each containing 16 smaller squares (4 x 4 array). Spores will be visible within the grid (Fig. A1). Count all of the spores in five of the larger squares/cells (the entire 16 smaller squares); while counts can be made in any five squares, it is recommended to count each corner square and the center square, or all five across a diagonal axis. Counts within each large square should ideally fall in the 20-100 range for optimal accuracy; if numbers are greater or lower, then the next appropriate dilution tube should be used. For spores on the border line of the square, count only those touching the top and right side of the ruled grid lines; do not count those overlapping the bottom or left grid lines. If using an Improved Neubauer haemocytometer, there are two grids on each slide; repeat counts on each side, then clean, re-load and repeat, obtaining 4 x 5 counts in total. If using a haemocytometer
with a single grid, repeat the counts at least three times. The average number of spores per (large) square or cell can then be calculated.

To estimate the number of spores per ml of the original suspension, this mean value is first multiplied by a volume conversion factor, in this case $2.5 \times 10^5$. This provides the number of spores/ml in the dilution tube. For example, if a mean value of 20 spores per cell was obtained, then the dilution tube contains $20 \times 2.5 \times 10^5$, or $5 \times 10^6$ spores/ml. This value is then multiplied by the dilution factor to obtain the concentration in the original tube. If the counts were made on the -3 tube, for example, the concentration in the original tube would be $5 \times 10^6 \times 10^3$ or $5 \times 10^9$/ml. From this value, it is possible to calculate the yield/g of substrate or the number of spores in 1 g of dried powder.

Figure A1. Counting grid on an Improved Neubauer haemocytometer. Note the 5 x 5 array of larger squares (cells) and the 4 x 4 array of smaller squares within each cell. When counting spores, total counts should be made in each of five cells (e.g., in cells numbered 1 through 5).
APPENDIX B

FUNGAL PRODUCTION PROCEDURES IN PICTURES

Fig. B1a. Autoclave used to sterilize materials and equipment for mass-production of fungi.

Fig. B1b. Specialized autoclavable rack to create agar test tube slant that provides a large surface area of nutrient media on which to grow fungi.

Fig. B1c. Simple method to create agar test tube slants if specialized racks as shown in Fig. 9b are not available.

Important Note. It is very important to prepare fungal suspension with minimal oil residue.

Fig. B2a. Test tubes containing a fungal culture covered with a layer of mineral oil. These can be kept in a refrigerator at 4°C for long-term storage.

Fig. B2b. Carefully remove oil from surface of medium using sterile pipette.

Fig. B2c. Add 5-10 ml of sterile Tween (0.01%) and scrape surface of fungal culture to loosen fungal material for preparing conidial suspension.
Fig. B3a. Test tubes with *M. anisopliae* with concentration:

A. $10^9$ conidia/ml  
B. $10^8$ conidia/ml  
C. $10^7$ conidia/ml  
D. $10^6$ conidia/ml

Fig. B3b. Test tubes with *B. bassiana* with concentration:

A. $10^9$ conidia/ml  
B. $10^8$ conidia/ml  
C. $10^7$ conidia/ml  
D. $10^6$ conidia/ml

---

Fig. B4a. Petri dishes containing agar medium are inoculated with 1 ml of a conidial suspension having $10^7$ spores/ml concentration to produce larger amount of conidium for test purposes.

Care must be taken to prepare agar dishes. Dish on left was prepared incorrectly—condensation accumulated on the top, making it unsuitable for storage. Dish on right was prepared correctly and shows no signs of condensation or contamination.

Fig. B4b. Test tube slant as shown in Fig. B2, containing agar medium and fungal culture for inoculating Petri plates.

---

Fig. B5a. Petri dishes containing fungal cultures growing on agar medium ready for harvesting conidia.

Fig. B5b. Test tube slants containing fungal cultures without mineral oil (*M. anisopliae* on left, *B. bassiana* on right). These slants are suitable for short-term storage (1-6 months) if held at 4°C in refrigerator.
Fig. B6. Change of volume of substrate after cooking, initial volumes: 100 ml seeds or grain, 750 ml tap water (pH 4).

Food-grade millet  Millet seeds  Sorghum seeds  Wheat grains

Fig. B7a. Jars containing cooked grain ready for closing with paper toweling and foil cover prior to autoclaving.

Fig. B7b. Closed jars prepared for autoclaving.

Fig. B8. Preparation of conidial suspension.

Fungal suspension preparation: A and B, fungal slants of B. bassiana and M. anisopliae; C, D and E, harvested spores; F and G, sterile water and Tween solution, to produce a more dilute spore suspension.

Flasks containing fungal suspensions: M. anisopliae (A) and B. bassiana (B), Concentration 5 x 10^7 spores/ml or more.
Fig. B9. Inoculation procedures for inoculating jars with fungal suspension.

- Fill syringe with fungal suspension
- Remove foil cap and inoculate jar.
- Cover jar again with foil cap after inoculation

Syringes for inoculation

Fig. B10. Position inoculated jars on their sides after inoculation.

Wrong position

Correct position

Fig. B11a. Jars containing fungal cultures of good quality, (A) *M. anisopliae*, (B) *B. bassiana*.

- Jar with too much water
- Jar with too much solid substrate

Fig. B11b. Jars with fungal cultures of bad quality.
**Fig. B12a.** Larger-scale fungal mass production in bags.

Different types of pans to sterilize solid substrate, standard pressure cooker (left), simple cooking pot (right).

Filling mass production autoclavable bags with pre-cooked solid substrate (millet).

**Fig. B12b.** Preparation of grains for mass production in Ethiopia. Autoclavable plastic shopping bags are used for production (arrow).
Fig. B13. Preliminary steps for inoculating fungal mass production bags.

Sterile tap water with citric acid (0.4 ml/liter) to use when inoculating bags.

Sterile beaker used to transfer fungal suspension into mass production bags.

Jars with good quality fungal material ready for inoculating mass production bag.

Fig. B14. Preparation of fungal suspension for inoculating production bags.

Pour contents from small jar into larger jar containing sterile water/citric acid to make a solution of $5 \times 10^7$ spores/ml concentration or higher.

Pour material including grain from big jar into beaker.

Add 0.01% Tween solution into jar of fungal cultures and mix thoroughly.
1. Pour liquid fungal inoculum into bags (~5-10 ml) containing cooked solid substrate.

2. Close bag temporarily and mix inoculum into solid substrate until evenly distributed.

Fig. B15. Inoculating plastic mass production bags with fungal suspension.

Fig. B16. After filling, production bags are gathered together with a collar and closed with a specialized stopper.

Custom-made collars can be constructed from cups or PVC pipe.

Fig. B17. Mass production bag is closed and laid on its side, ensuring space is left above solid substrate.

Right position

Wrong position
Fig. B18a. Production bags with inoculating substrate held horizontally on shelves at controlled temperature.

Fig. B18b. Bag with good quality fungal material produced after 4-5 days. It is ready for mixing to distribute the fungus equally.

Fig. B19. Bags containing good quality fungal material.

Beauveria bassiana

Metarhizium anisopliae

Fig. B20. Bags showing poor quality fungal growth.

Condensation has accumulated in the bag. The bag must be opened to reduce humidity level.

Non-target fungi have colonized the substrate in the bag. The bag and its contents must be autoclaved to destroy the material.
Fig. B21. Fungal drying process.

Use any type of flat trays or boxes. Cardboard boxes work well because they help absorb moisture from fungi.

Fig. B22. Appliances to separate conidia from substrate.

Specialized graduated soil sieves work well for separating fungi from the solid substrate. Sieves have progressively smaller grid sizes and fit together into one separation unit.

Fig. B23. Simple method for separating conidia separating from the solid substrate if sieves and/or a shaker are not available.

Fungal substrate is put into clean, loosely woven cloth bag.

Cloth bag is put into large plastic bag, closed tightly and shaken by hand. Harvested conidia collect in the bottom of the plastic bag, shown with arrow.
Pure conidia of *M. anisopliae* in small tube for checking spore viability and productivity. Spore viability is checked on agar medium. After 20 hr, germination is stopped with blue stain.

**Fig. B24.** Post-harvest procedures.

Pure *B. bassiana* conidia

Pure *M. anisopliae* conidia

**Fig. B25.** Protective equipment must be worn for most phases of mass production.

A protective mask should be used whenever working with dry fungal material. Filters must be replaced regularly.

Worker wearing mask, gloves and specialized protective overalls.