Production Processes for Anamorphic Fungi

These notes[1] describe a simple two stage fermentation process for *Metarhizium* spp *Trichoderma* spp. and similar anamorphic fungi, based on experience gained in the International LUBILOSA project[2] and the USDA-Mars collaboration for cocoa pest management. There are a number of practical options for production of beneficial fungi (see Fig. 1), but the technique that is commonly found to be practical on a pilot scale is 2-stage fermentation where the fungus is first grown in liquid media then added to a solid substrate (often grain: specifically rice) for conidiation. We describe here the process where fungal inoculum is first produced in liquid shake flask culture and subsequently transferred to a rice substrate on which conidiation occurs. At the end of the fermentation period, the conidiated substrate is air dried before extraction of the conidia using a MycoHarvesters, before the product is further dried, formulated and packaged.

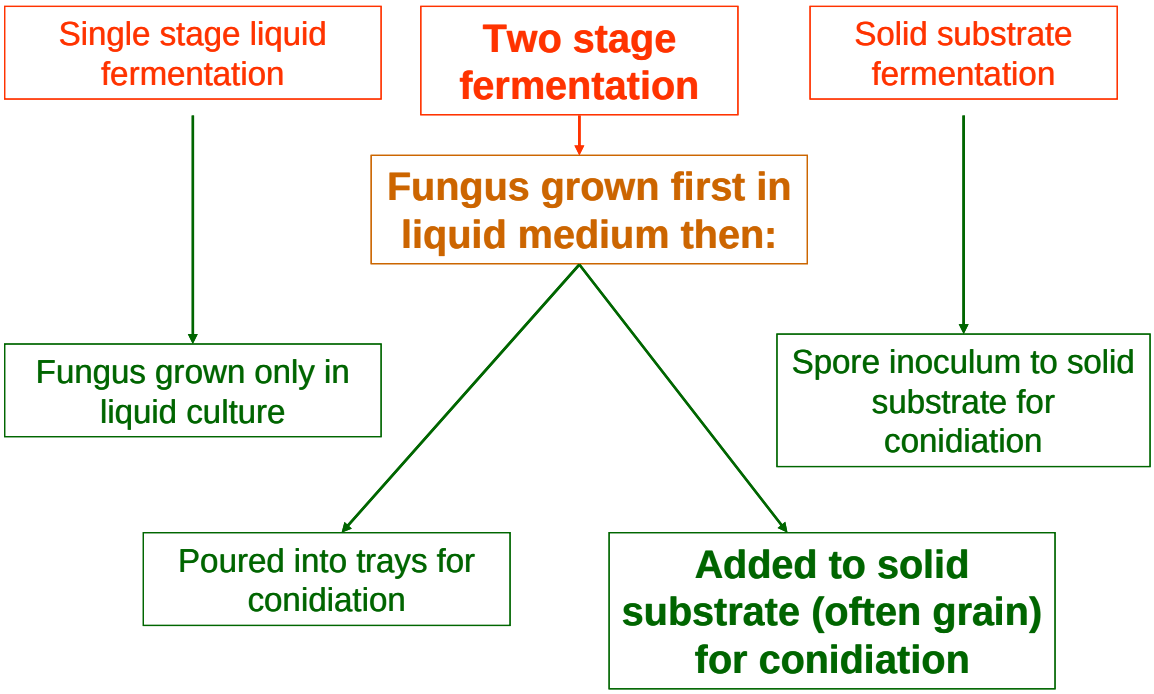


Fig. 1. Options for mass production

Precise needs for individual mycopesticide production units (MPUs) depend on a number of factors, including scale of production required, the properties of the organism to be produced and the economic context (*e.g.* whether it is "lab to field" development, commercial production, *etc.*) and cannot fully be described here. Besides good quality accommodation for the production facility, there is a basic check-list of equipment items that are normally required for successful mycopesticide quality assurance (see Appendix 1).

[1] Part of the biopesticide development series and MycoHarvester instructions (see www.dropdata.org): includes notes prepared by Dr. Nina Jenkins (2002). This version:2 Feb 2007 (RPB).

[2] www.lubilosa.org

Culture Maintenance

A system of maintaining back-up sources of inoculum representing a master culture is vital (see below). In some MPUs, maintenance of the isolate is by regular passaging through the host insect and re-isolation, but we recommend that no more than three sub-cultures on agar should be permitted prior to use in mass production. The practice of re-isolation through the host maintains the virulence/pathogenicity of the isolate, but does introduce the risk of evolution of fungal strains that differ from the field-collected population of microbial control agents (MCAs). Without the back-up of a master culture, this could lead to inadvertent loss of desirable properties found in the original MCA strain. The risk of this happening is fairly low, provided that passaging procedures are supervised by a trained pathology technician.

There are a large number of long-term culture maintenance techniques that can be employed for the preservation of valuable fungal material. These include relatively High-Technology methods such as cryopreservation (liquid nitrogen), lyophilisation (freeze-drying) and storage at –80^{o}C. All these methods involve the use of relatively expensive equipment. For the purposes of storing a single fungal isolate, it would not be cost-effective to purchase such equipment. However, most culture collections provide a culture storage service, which will maintain valuable isolates using at least two of the above techniques. It is recommended that MPUs lodge their isolate with a recognized culture collection under restricted access.

As a back up to the above, a large stock of 'master' material should be stored at MPUs. One simple and reliable method is to dry a large number of infected insect cadavers prior to re-isolation of the fungus. These sporulated cadavers should be dried over silica gel to a constant weight and stored in a freezer in a sealed plastic bag containing silica gel. This material is likely to remain viable for many years and single insects can be removed when a fresh source of inoculum is required.

Liquid culture

Liquid starter culture is used as an inoculum for the solid substrate suitable for the MCA. We describe here a procedure for a *Metarhizium* isolate, as an example; experimentation will be required to optimise methods for different fungal isolates. Liquid medium is composed of 2% yeast extract and 4% glucose (but sucrose is often cheaper) and is inoculated with a small square cut from a 10 day old culture of *Metarhizium* grown on Sabouraud Dextrose Agar (SDA). Flasks are placed on a rotary shaker (approx. 150 rpm) for 2 – 4 days (the number flasks used depends the shaker). The resulting inoculum is diluted 1:40 in sterile water prior to use. Each 600 g bag of rice is inoculated with 25 ml of diluted inoculum.

In the early stages of growth distinct clumps of mycelium may be formed within an otherwise clear broth. This form of growth is less productive than a homogenous colonisation of the culture broth by evenly dispersed mycelium and hyphal fragments. Furthermore, the thick mycelial 'soup' that is produced by the latter growth form provides a uniform inoculum for coating the rice substrate. There are a number of possible causes of uneven colonisation of liquid media, including low temperatures.

To encourage even distribution of mycelium and hyphal fragments in the liquid culture, temperature should be tightly controlled and a spore suspension should be used to inoculate the flasks in preference to agar squares. This may increase the amount of time required to prepare the inoculum, but should be worthwhile given the likely benefit to the production process.

The degree of dilution of the inoculum requires that the preparation of more flasks per production batch. Ideally 20-40 ml of undiluted inoculum should be used for each production bag (typically containing 0.5 – 1 kg). For example, a 190 kg production batch would require approximately 8 l of liquid culture (40 x 200 ml flasks). With a production capacity of 400 kg rice per day, 5 days per week, an appropriate shaker would need to have a capacity for 320 x 500 ml flasks (containing 200 ml liquid culture). Such an industrial sized shaker would be very expensive if purchased from an equipment manufacturer, but it may be possible to design and build simple unit locally.

Alternatively, given that such a large quantity of liquid inoculum is required, a simple fermenter system could be set-up, which could efficiently produce large quantities of inoculum in single batches. An ideal system would consist of a single aeration unit (air pump or compressor) that is capable of running up to 6 x 20 l fermenter vessels (carboys) at any one time. Given the simplicity of the liquid culture stage, it would not be necessary to purchase pH, temperature or oxygen probes, which would reduce the cost of the system considerably.

Solid substrate

Autoclaved rice provides a nutritive solid support on which conidiation occurs. Dry, unpolished rice is placed in autoclavable bags (*e.g.* 200 mm x 500 mm - 600 g rice per bag). 500 ml of water is added to the rice in the bags, which are then heat sealed to ¾ of the way across the top. The water level within the bags comes well above the level of the rice and the bags are loaded upright in to the autoclaves for sterilisation for 1 hour. Autoclaves measure 1 m x 1.5 m and hold up to 160 bags (96 kg rice).

On completion of the autoclave run, the bags are massaged to break up the rice and the open corner of the bag is folded over twice and fixed with a paper clip. The bags are then laid out onto multi-layered trolleys - 4 bags per tier and moved into an air-conditioned room to cool.

Once cool, the bags are inoculated with a fixed amount (*e.g.* 25 ml) of diluted inoculum: an automatic dispenser (*e.g.* Fig. 2) speeds-up the process and reduces contamination. After inoculation clips are usually removed and the open corner of the bags swabbed with alcohol. It is unlikely that swabbing is necessary and may even increase the risk of contamination as alcohol is not sterile and will not kill fungal or bacterial spores. The final moisture content of the rice following inoculation is 50%. The bags are sealed with masking tape and massaged to distribute the inoculum evenly and to break up any lumps. The bags are then laid flat and a slit is cut along half the length of the bag, this is sealed with micro-porous tape

and the bag is placed tape-up on the shelf of a multi-layered trolley. Once loaded, the trolleys are moved to the incubation room, which is air-conditioned and maintained at a fixed temperature (*e.g.* 25-30^{o}C is often good for *Metarhizium*). Bags are inspected for contamination and massaged on two occasions during the incubation period to increase aeration and to break-up any clumps that form during the incubation process. A considerable amount of time is often taken in massaging the rice at various stages of the production process and some MPUs incorporate of a small amount of oil to reduce clumping. The effect of massaging the bags during incubation on final yield should be evaluated in a properly replicated experiment. Good records should be kept on when in the production process bags are massaged, transferred from one room to another and when finally opened for drying.

Fig. 2. Autoclave (left) and semi-automatic substrate bag inoculation at CEPLAC, Brazil.

Conidiation typically takes place after approximately 10 days and bags are incubated for 14 days before being transferred to the drying room. Here, the bags are cut open to expose the conidiated rice for 3-10 days (depending on ambient relative humidity) to surface dry before spore harvesting. The drying room should be air conditioned if possible, but is not dehumidified. An industrial dehumidifier may have to be installed in the drying room/s to permit drying of the conidiated rice to approximately 20% in preparation for extraction. Extraction at higher moisture contents is usually less efficient.

A high quality particle size specification for the spore powder is essential for successful formulation and machines called MycoHarvesters have been developed for such purposes. Notes on the use of the MycoHarvesters are given in the formulation technical manual and on www.mycoharvester.info and a description of the scaled-up version (the MH3) is given in Appendix 2. For pilot-scale production the MycoHarvester 5 (see Fig. 3) has been developed for experimental-scale production

Fig. 3 The MycoHarvester 5

Following extraction, the conidia powder requires further drying to reduce the moisture content to below 5% for long-term storage. In order to maintain moisture content at 5%, conidia powder must be packaged in moisture-proof packaging such as aluminium laminate sachets. When so packaged, conidia powder has a long shelf-life and does not require storage below zero degrees centigrade. A method of drying conidia powder is shown in Figure 4, which shows an low-cost design of spore drier, for reducing the moisture content to 5% prior to packaging. Dry air is emitted from the dehumidifier into the base of the drier box. A layer of conidia powder (up to 10 cm deep) is placed in a specially designed drawer, with a cotton cloth base. The drawer is then placed inside the drier cabinet and the door closed. Dry air passes through the cloth base and up through the conidia powder, finally exiting through small holes drilled in the top of the drier box. The unit can be constructed from plywood, but sheet metalwork would be better for cleaning. The dryer should be operated in a cooled room to prevent over-heating of the conidia during the drying process.

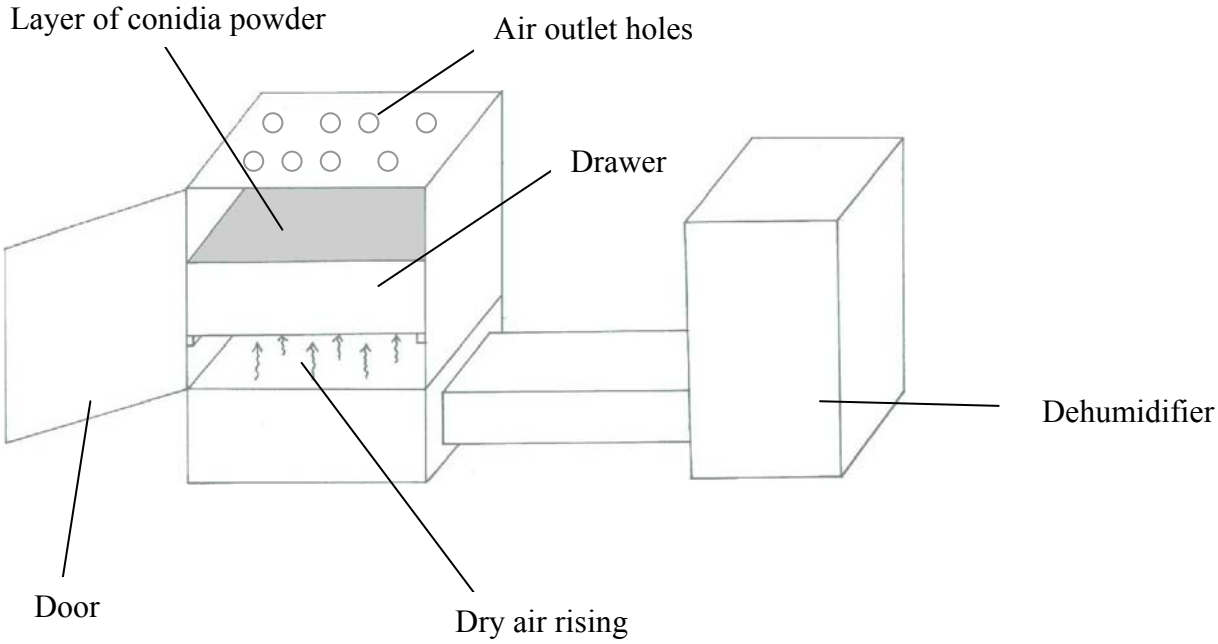


Fig. 4. A low-cost spore drier

Health and safety

The fungal genera *Metarhizium, Beauveria* and others have a good safety record and are not considered as potential human pathogens (provided isolates do not grow at above 37º). However, fungal conidia in general, like all proteinaceous material, have the potential to be allergenic and it is possible for reactions to occur following repeated and prolonged exposure to high concentrations of conidia. Appropriate personal protection gear should always be worn when working in environments with high concentrations of conidia. In particular, this applies to aspects of the production cycle where conidia may be liberated into the environment, including opening of the bags to dry, extraction of conidia, drying of the conidial powder and packaging. In areas where any of these processes are carried out, personnel must wear appropriate personal protection equipment at all times.

MPUs should have safety routines built into standard operating procedures (SOP) followed by a complete safety audit. Some important categories include:

- Electrical safety assessed by qualified personnel.
- The safety of autoclaves, which are used for sterilisation of the rice substrate, should be regularly checked and serviced by qualified pressure vessel engineers.
- Lab-coats or overalls and disposable gloves should be worn at all times when handling conidiated rice and conidia powder. Overall, direct bodily exposure to conidia powder should be avoided and procedures to enforce the use of personal protection equipment should be implemented.
- Full-face respiratory masks fitted with Hepa or P3 filters should always be available (as either powered or un-powered units). For most fungal spores a 1µm filter is sufficient,

but finer particles may be present in the substrate (an important reason to carry out particle sizing checks on the product). Powered units are most suitable, because they provide an air-flow through the mask, which reduces heat build-up during activity, but are more expensive than the un-powered versions. Details of specifications and recommended manufacturers (Appendix 3) should, if possible, be co-ordinated with local suppliers of safety equipment.

Quality control (QC) and record keeping

In-process QC

Standard QC procedures include measures for detecting large-scale problems such as sterilisation failure and the introduction of contaminants due to in-process handling of the solid substrate. It is important to ensure that contamination is both detected early and its source is identified so that subsequent problems can be avoided.

Agar plate cultures are checked visually for the presence of contaminants before use as inoculum for liquid cultures. Liquid cultures are checked under the microscope for visible signs of contamination and the sterility of the rice is verified using un-inoculated bags as controls for each autoclave for each batch. During the incubation process, any contamination present in the bags is identified during the massaging process and contaminated bags are discarded.

A formal set of QC checks must be incorporated into the production process. Checks include monitoring of spore inoculum from, sterility screening of liquid medium and autoclaved rice prior to inoculation and purity checks on liquid and rice substrates post inoculation, all by plating on to generalist media such as SDA. Particle size spectrum analysis is often essential (see appendix 5.4), but may need to be done less frequently than other QC routines.

Record keeping

Records must be kept for each production batch from the date of inoculation of the rice. Batches are split according to the autoclave used for the sterilisation of the rice substrate and separate records are kept for each. Details are kept on date of inoculation, number of bags, incidence of contamination during incubation, type of contamination and final yield of conidia from all bags extracted.

It should be possible to trace each batch produced back to the original stock culture on SDA. Details are normally transferred and stored on a computer following packaging of each batch so that production data are maintained as both hard and electronic copies. Data stored in the form of spreadsheets or data-bases can be analysed at the end of the season to evaluate the effect of minor differences in the production process on final yield of product.

QC on final product

Research groups typically assess for viability and virulence. These tests represent the bare minimum, and are not sufficient for high quality products with predictable, defined properties. Examples of quality specifications for the 'Green Muscle' product are given in Appendix 4.

Notes on packaging are given in the formulation technical manual. A suitable bag sealer will be required to deal with the proposed aluminum foil packaging that is necessary for long shelf life of the product. Moisture content is often the key factor affecting product shelf life and an automated moisture content meter (Fig. 5) is invaluable for optimising the shelf life of mycopesticide products; failing this the oven method (Appendix 5.1) uses more conventional equipment.

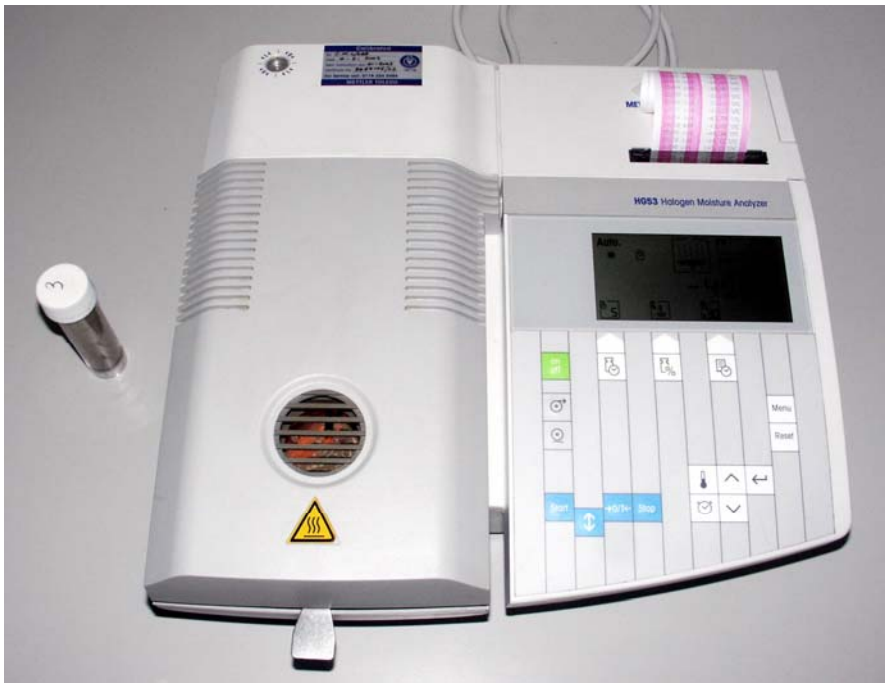


Fig. 5. A Moisture Analyser (Mettler Toledo)

Moisture analyser	US$ 6,000 approx. (as shown)

The purchase of the following equipment is a priority on scale-up from small scale trials:

Industrial dehumidifier	US$ 3,500 upwards
Laminate bag sealer	US$ 2,000 upwards

Appendix 1

Routine Maintenance of Fungal Pathogens and Pilot Production Facility

Essential equipment:
Autoclave
Cooker or gas ring
CT room with shelving
Laminar air flow cabinet (preferably two - class 1 for media prep and class 2 or similar with user protection for manipulation of fungi)
Refrigerators (2 x large)
Access to incubators (10-40 ºC)
Access to balances (2 and 4 decimal places)
Access to microscope (standard microbiology and dissecting)
Oven (for dry weight etc)
Access to sonicator (bath or probe)
Access to pH meter
Access to a Whirlimixer/Vortex
Automatic (Gilson style) pipettes
Microscopy equipment (slides, cover slips, stains etc.)
Haemocytometers (3 if possible)
Bunsen or spirit burner
General laboratory glassware: 500ml beakers, 250ml conical flasks, medical flats, universal bottles, funnels etc.
Rotary shaker (and/or fermenter vessel)
MycoHarvester v.5: sieves (300 and 106 µm mesh) are a poor substitute
Fume hood or powder control cabinet
Respiratory protection for fine dusts
Conical flasks (preferably 250ml capacity - twice as many as there are places on the shaker)

NOTE:
1. Need separate areas for aseptic work and formulation (handling of spore powder etc).
2. Avoid proximity to any insect rearing/entomology areas where possible infection could cause disruption.

Target pest culturing facilities (project dependent)
Constant temperature (CT) rooms
Cages (insect dependent)
Separate areas for infected and non-infected populations
Bioassay equipment (project dependent), *e.g.*: micro-applicator, track sprayer

Consumables:
Petri dishes
Agar
Peptone, yeast extract or supply of brewers' yeast *(is there a brewery in town?)*
Sucrose/Glucose
Surfactants such as Tween 80
Alcohol (for sterilisation *etc*)
Chemicals, general e.g.. K_2HPO_4, $MgSO_4$, NaCl etc.
Non-indicating silica gel
Formulating agents (e.g. Clays; mineral oils such as Shellsol T, Ondina EL for ULV)

Appendix 2
Specification: MycoHarvester version III

MycoHarvesters are normally designed to beneficial micro-organisms (especially fungal spores in the approximate range 3-10 µm) from solid substrates (usually grains). The MH III was designed for larger-scale processing and consists of 2 sections:

a. an agitator for the substrate: usually a rotating drum construction (as required);
b. the spore extractor unit: consisting 4 or more stainless steel "cyclones" linked to a compatible fan which draws air through the equipment and (usually) vents to the outside of the building via standard 100-110 mm pipes.

The **standard construction** consists of a 4 cyclone configuration. The Unit is best installed along an external wall and requires a "working space" depth of approximately 3 m. Substrate agitators used to date have occupied a space 3 m wide, coupled to the cyclones (≈1.5 m) and an air extraction system taking up another 0.5 m. Allowing for working space, approximately 6 M of wall is usually sufficient.

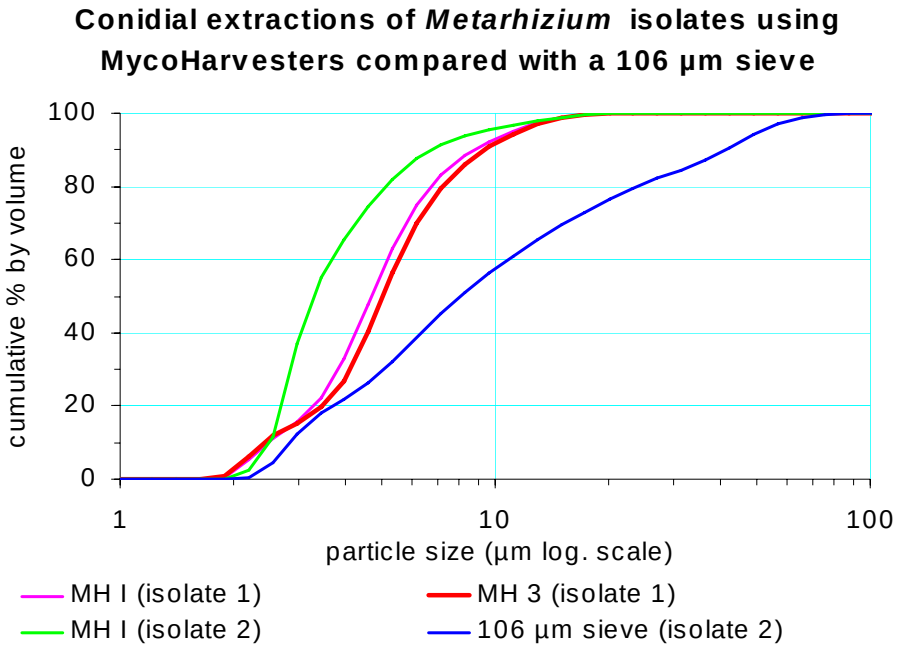
Important: clients must specify both the **pipe configuration** and **electrical supply** required for the fan unit (both voltage and frequency for 3 phase electrical supply).

Packed dimensions: Cyclone and Fan Unit (4 cyclone configuration)

- The agitator drum is usually constructed locally to comply with clients' specific needs.

Particle size spectra

MH III units have been shown to separate conidial preparations of *Metarhizium anisopliae* that are equivalent to the MycoHarvester version I and substantially better than other processes. This is illustrated in the following diagram:



Appendix 3
Safety: Prevention of inhalation of dust
Suppliers of full face respiratory protection (one unit required for each technician)[3]

Type of respiratory protective equipment	Manufactured by
Easi-Air 7800 or 7800S full facepiece fitted with W3200 powered air purifier, W3210 high efficiency filter, W3213 breathing tube and W2954 CA battery pack	3M, St Paul Minnesota 55144-1000 USA
Martindale Mark V Positive Pressure Powered Respirator fitted with three standard efficiency filter and a half mask or a full facemask	Martindale Protection Ltd Neasden Lan London NW10 1RN
RPFF 81 Positive Pressure Powered Respiratorfitted with one RC 351 (formerly RCHEF 81P) high efficiency filter and a RFF 80 full facemask; RC 350 (formerly RCHEF 81) high efficiency filter and a half mask	Protector Safety Limited Great George Street, Wigan Greater Manchester, WN3 4DE, England
Powerflow (LL) 055-00-01P8 Positive Pressure Powered Respirator fitted with one 450-01-02 high efficiency SP3 filter, a 055-00-01 full facemask, a 007-00-06 battery pack and a 024-00-04 turbo unit	Racal Safety Limited Beresford Avenue, Wembley Middlesex, HAO 1QJ, England
Dustmaster DM1 ventilated visor respirator partno. 045-00-01 P5 with a headpiece 045-00-01, a main filter part no. 021-02-06 and a motor part no. 500-02-05	Racal Safety Limited Beresford Avenue, Wembley Middlesex, HAO 1QJ, England
Powerflow Positive Pressure Powered Respirator part no. 055-00-01P6 fitted with one PM3 high efficiency filter canister part no. 009-00-13P, a full facemask part no. 055-00-01P and battery partno. 007-00-03	Racal Safety Limited Beresford Avenue, Wembley Middlesex, HAO 1QJ, England
Racal Breathe Easy 7 Positive Pressure Powered Respirator part no. 055-00-01P1 fitted with two P3 high efficiency filter canisters part no. 009-01-00,a full facemask and a battery part no. 007-00-05	Racal Safety Limited Beresford Avenue, Wembley Middlesex, HAO 1QJ, England
Phantom powered respirator fitted with 071.345.00 full facepiece, 034.029.02 blower, 034.018.00 high efficiency filter and 025.033.03 battery	Sabre Safety Ltd Ash Road, Aldershot Hampshire, GU12 4DE
Willson 6783 full facepiece fitted with R73 filter, RP41 blower assembly and RP40 lead acid battery	Willson Safety Products P O Box 622 Reading PA 19603-0622 USA
3M #6800PF Powerflow™ Face-mounted Powered Air Purifying Respirator (PAPR) fitted with 6800DIN full facepiece, 450-01-01 SP3 High Efficiency Particulate Air (HEPA) filter,024-00-02 Powerflow™ motor/airflow unit and 520-01-17 battery pack	3M St. Paul MN 55144-1000 USA

Approx. costs (US$)

Racal/3M Breath Easy 7 system $800 each; spare filters (P3) $160 (set of 6)

[3] From: http://www.labour.gov.hk/text/eng/faq/oshq9_4d.htm

Appendix 4

Specification for 'Green Muscle' technical material (see: Cherry *et al*, 1999[4])

	Specification	Method of determination	Test routine[i]
1. Appearance:	Uniform dark green powder, easily separable from desiccant or other packaging material	Observation	lot
2. Viability:	> 90% at packing. > 80% acceptable after storage	Suspend conidia in paraffin[ii]; store for 24 hours before plating out on Sabouraud's dextrose agar. Count 3 separate Petri plates (300 spores per plate) after 24 hours at 25°C.	lot
3. Moisture content:	< 5% at packing (NB. may rise to 6 - 7% soon after opening)	Halogen moisture balance	lot
4. Contaminants	<0.002% (<1 : 5 x 10^4 or <10^6 particles/g product); c.f.u. determined by number. No human pathogens	Dilution series from approx. 5 x 10^7 conidia/ml to 50 conidia/ml. Techniques for identification of contaminants are responsibility of manufacturer (site specific).	lot
5. Number of conidia	5 (±1) x 10^{10}/g dry powder[iii]	Routine part of contaminant assessment	lot
6. Particle size: The technical material (TC) is available in 2 qualities:			
i. <u>minimum standard</u> for small batches (some SU formulations for use in small hand-held sprayers)	by weight: • 100% must pass through a 100 mesh (150 µm) sieve • < 2% should be retained by a 200 mesh (74 µm) sieve • < 15% should be retained by a 45 µm (BS 410) sieve	Wet sieve method as described in section 2.4.4. formulation manual	batch
ii. <u>high specification</u> for large scale operations (and OF formulation)	by volume: • <10 µm: 80% • <60 µm: 99.9% • <100 µm: 100%	Malvern 'Master' or 2600 particle size analyser (or equivalent spatial PSA)	batch or >4 times per year
7. Virulence	A dose of 5 x 10^4 conidia per insect kills >95% of test insects in 5 days.	Conidia suspended in oil. Bioassay at 30°C, 35% RH: ≥20 insects/assay (both sexes may be used); ≥20 insects as controls.	batch

i: "Routine" means every lot; virulence and particle size will normally be carried out with every batch (mass production lots originating from each re-isolation through locusts[3]).

ii: Purified light paraffinic oil such as Shellsol T.

iii: This value for *Metarhizium anisopliae* isolate IMI 330189 at 5% moisture content should be similar for other acridid isolates, but may be quite different for other *Metarhizium* isolates (*e.g. M. anisopliae* var *majus).*

iv: Normally *Schistocerca gregaria*, but other species may be selected where appropriate.

[4] Cherry, A., Jenkins, N., Heviefo, G., Bateman, R. and Lomer, C. (1999) A West African pilot scale production plant for aerial conidia of *Metarhizium* sp for use as a mycoinsecticide against locusts and grasshoppers. *Biocontrol Science and Technology*, **9**: 35-51

Appendix 5

Standard Operating Procedures for Product Quality Control

5.1 Moisture Content Determination (Oven Method)

Purpose

To determine the percentage moisture content of solid substrates or pure conidial powder.

Method

1. Label all universal bottles or glass Petri dishes and their associated lids with indelible marker pen before drying.

2. Place bottles and lids (separate) in pre-heated oven and allow to dry for at least one hour at a minimum temperature of 80°C.

3. Remove bottles from oven and quickly replace lids before allowing to cool for 1.5 to 2 hours in a bench top desiccator. Ensure silica gel is fresh by regeneration for at least 3 hours at 80°C.

4. Weigh all bottles, complete with lids and record weights to at least 3 decimal places. Use latex laboratory gloves to avoid the transfer of moisture from hands.

5. Add a representative sample of substrate or conidial powder to each bottle, replace the lid and re-weigh. Three bottles should be prepared for each sample to ensure reproducible data. For solids, a sample quantity of 3g is recommended while a 2g sample is adequate for conidial spore powder.

6. Place bottles containing the samples and lids (separated) in an oven-proof vessel. Colonised substrates require drying for at least 4 hours at 130°C. Spores dried at 103°C for 17 hours.

7. After the drying period, remove bottles from oven, quickly replace lids and allow to cool to room temperature in a bench top dessicator for 1.5 to 2 hours.

8. Final dry weights can then be recorded.

9. The percentage moisture content of the samples can then be recorded as follows :

% Moisture = Wet wt (g) – Dry wt (g)/Sample size (g) *100

Where,

Wet wt = Wt. of bottle, lid and sample before drying
Dry wt = Wt. of bottle, lid and sample after drying
Sample size = Wt. of bottle, lid and sample before drying – Initial wt. of bottle & lid

NB. To ensure the correct drying time has been selected for the sample under investigation, repeating steps 6 to 9 will show any change in moisture content results. If there is any discrepancy, repeat until consistent values are obtained.

5.2 Germination Test

Purpose

To determine the percentage germination of conidia of *Metarhizium spp*. and *Beauveria spp.* Conidia should have a percentage germination of ≥ 85% for mycoinsecticide use.

Method

1. Prepare fresh Sabouraud dextrose agar (Oxoid) as instructed on label in small 50 mm Petri dishes. Three plates of a 5 mm minimum depth are required per test sample. Dry agar after pouring to reduce build up of condensation after refrigeration. Refrigerated agar plates must be less than a week old. Remove any condensation which will cause problems if mixed with oil.

2. Wearing face mask, spectacles and gloves, transfer a small quantity of spores onto the upturned lid or base of a clean, dry, plastic Petri dish and place in a humid chamber for 30 min to allow the spores to re-hydrate. Label each dish if multiple samples are being processed.

3. After 30 min use a clean microspatula to add a small quantity of hydrated spores to a labelled, capped, plastic tube containing 9 ml Shellsol T (Alcohols Limited, Bishops Stortford, Herts). The resultant spore concentration of the suspension should be 10^5 spores /ml. Shake vigorously. **Clean the microspatula between samples using 70% alcohol or IMS and dry.**

4. Dip the clean microspatula into suspension and spread evenly over the surface of the agar - prepare 3 plates per sample.

5. Incubate plates for 24 h at 25°C.

6. After 24 h transfer plates to the fridge, to reduce the growth rate . Remove a maximum of 9 plates from fridge, open lids to allow the agar to dry. Examine plates microscopically under x300 magnification and using a separate tally counter for germinated and non-germinated spores, count a total of ≥ 300 spores. A germinated spore is defined as a spore having a germ tube. Record results on appropriate record sheet and sign and date.

7. Calculate the percentage germination as follows:

   % Germination= [a /(a+b)]*100

   Where,

   a= Number of germinating spores
   b= Number of non germinating spores

8. Calculate the average percentage germination of the three plates.

5.3 Biological Purity & Colony Count

Purpose

To assess the microbial purity of dry, conidial powder or colonised substrate and to determine the number of conidia/g of product. The specification of the LUBILOSA product is ≤ 0.002% ie. a *Metarhizium anisopliae* var *acridum* batch with a percentage contamination level greater than 0.002% will fail the QC assessment.

Method

1. For each sample, 8 universal bottles containing exactly 9 ml of 0.05% Tween 80 and 1 bottle containing 10 ml of the Tween solution should to be prepared and sterilised. Loosely screw on plastic lids and cover each with aluminium foil before autoclaving for 20 min at 121°C, 15 - 20 psi. If an automatic dispenser is used to fill the bottles, ensure an exact setting of 9 ml by weighing.

2. Allow bottles to cool after sterilisation and add approximately 0.1g of conidial powder or 0.5 - 1g of colonised substrate to the bottle containing 10 ml of 0.05% Tween 80. The exact weight, however, must be recorded to at least 3 decimal places. Agitate vigorously to ensure a homogeneous suspension.

3. Under sterile conditions, preferably within the Laminar Airflow Cabinet (LAF), carry out the serial dilution of the stock suspension as follows. Remember to switch on airflow at least 15 min prior to use and to thoroughly swab down both the inside of the cabinet and all objects entering the cabinet with alcohol and then with bleach. Remove aluminium foil and label bottles from 10^{-1} to 10^{-8}.

4. Remove exactly 1 ml from the stock suspension using a sterile pipette tip, and decant into the bottle labelled 10^{-1}. Replace lids and agitate suspension manually. With a clean, sterile tip remove exactly 1 ml of the 10^{-1} suspension and decant into the bottle labelled 10^{-2}. Repeat this procedure until 1 ml of the 10^{-7} suspension has been decanted into the bottle labelled 10^{-8}.

5. Label two, 90 mm Sabouraud Dextrose Agar (Oxoid – prepared as instructed on label) plates for each dilution. Transfer 200 µl of the homogenous suspension aseptically onto each of the two plates.

6. Using absolute alcohol flame a glass spreader, allow to cool, and spread the fluid evenly over the surface of the agar. Flame the spreader between each dilution.

7. Incubate plates at 25 - 27°C for 3 - 4 days. Check plates after 1 - 2 days and count any bacterial or yeast contaminating colonies which may be present. After 3 - 4 days count the number of *Metarhizium* colonies and the number of contaminating fungal colonies, if present. Count the dilutions which have fewer than 300 colonies, take the average

number for both plates of each dilution and record results on the record sheets.

8. The percentage contamination (all types) of the product sample can be calculated using the following equation:

   % Contamination =
   [No. contaminant colonies x dilution / No. product colonies x dilution] x 100

9. A colony count of the sample under investigation can be calculated as follows to give the number of viable colonies per g of initial product:

   Colonies/g = [No. product colonies x 5 x dilution x 10 ml] / weight (g) dry sample

10. A total count of conidia/g of spore powder or colonised substrate and be made from the 10^{-1} or 10^{-2} dilution using a haemocytometer in combination with the calculation below:

    Conidia/g =
    [conidia/ml from haemocytometer count x dilution x 10 ml] / Sample Weight (g)

5.4 Particle Size Spectrum

Particle size assessment and specification depends on the needs of the formulator (which in turn is related to the intended method of application); further notes are given in the formulation technical manual. Measurements normally require the use of expensive laser particle sizing equipment, but particle sizing can be carried out as sub-contracts for approximately $1800 per day, with approximately 12 samples processed in 1 day.

Particle sizing normally must be carried-out when new facilities are commissioned and when changes are made to the production process (*e.g*. modifications to equipment, substrate, *etc*) or fungal isolate. Routine particle sizing again depends on the process and the specification required for formulation, but is often less frequent than other routines, such as moisture content and checking for contaminants.

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