

## Review Article

# Development of mass production technology for aerial conidia for use as mycopesticides

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### Abstract

In any microbial control programme, production of sufficient quantities of good quality inoculum becomes essential to its success. The inoculum produced must be both consistent and compatible with the intended formulation and application technology. Each of the stages in the development and optimization of a mass production technique for the production of aerial conidia is discussed. Full details of the LUBILOSA mass production system for aerial conidia of *Metarhizium flavoviride* are given, along with recommended contamination and quality control procedures. This technique has been used successfully to develop the specifications for a commercial product for the biological control of locusts and grasshoppers.

### Introduction

The mitosporic fungi, previously classified as the Deuteromycotina (Hawksworth *et al.*, 1995), are increasingly being investigated for their potential as microbial control agents for a wide range of weeds (Bowers, 1982; Boyette *et al.*, 1991; Jackson *et al.*, 1995), plant pathogens (Jackson *et al.*, 1991; Lewis *et al.*, 1991) and arthropod pests (Ferron, 1978; Doberski, 1981; Kurtz & Champe, 1982; Milner *et al.*, 1993; Bateman, 1997). A number of successful products based on the conidia of these fungi have already been developed and are produced and sold commercially by companies such as Koppert, Mycotech, Natural Plant Protection, Agricura and EcoScience. However, commercial companies rarely invest large sums of capital into the early stages of development of biopesticide products. More often fundamental research and development up to the stage of small-scale field testing is carried out by public funded research laboratories and university departments (Bowers, 1982).

The initial stages of mycopesticide development begin with collection of fungal isolates and screening for virulence to the target pest. Early in the development of any mycopesticide product there is a requirement for relatively small quantities

of the fungus for both laboratory and field testing. The methods of production vary according to the experimental design and the stage of development within the research project. For bioassays and general isolate screening, maintenance and production on agar slants in bottles is usually sufficient. As the project develops and one or more isolates have been selected for further development, greater amounts of material are required and it becomes necessary to demonstrate the feasibility of large-scale production. This requires the development of a production system which not only produces conidia in sufficient quantities for experimental use, but which also are of consistent quality and compatible with the selected formulation and application equipment. A stringent quality control procedure running alongside the production process, allowing variation and contamination of the product to be carefully monitored and rectified, is essential.

One of the most important considerations in the design of a mass production procedure is compatibility of the product with both formulation and application techniques. For example, the use of oil formulations for application at ultra-low volume (ULV) rates requires the production of lipophilic conidia which suspend easily in oils. Despite the advantages

of submerged liquid fermentation, and the possibility of production of conidia of many mitosporic fungi in submerged culture (Hildebrand & McCain, 1978; van Winkelhoff & McCoy, 1984; Hegedus *et al.*, 1990; Jackson & Bothast, 1990; Jenkins & Prior, 1993; Khurana *et al.*, 1993; Lascaris & Deacon, 1994), submerged conidia are hydrophilic and are not easily formulated in oils. Blastospores are similarly produced in submerged liquid fermentation, but are also hydrophilic and have been found to lose viability relatively quickly during storage (Rombach, 1989; Kleespies & Zimmermann, 1992). Although these problems will no doubt be overcome, they have so far precluded the adoption of single phase liquid production for many mycopesticide products.

Recent developments in solid state culture systems (Bradley *et al.*, 1992a; Roussos *et al.*, 1993) have presented the opportunity for industry to consider production of entomopathogens in specially designed, sealed aerated bioreactors with automated process control along similar principles to those of liquid fermentation. This reduces labour costs and allows close control and standardization of the production process. In developing countries where labour is relatively cheap, different economic constraints exist and the capital cost of these automated processes may preclude their establishment. There are many mycopesticide producers around the world, particularly in Latin America (Aquino *et al.*, 1977; Alves & Pereira, 1989; Antía-Londoño *et al.*, 1992; Mendonça, 1992) and China (Feng *et al.*, 1994), who supply sufficient quantities of product for niche markets in their immediate area. These production systems are basic and often labour intensive; however, due to the low cost of labour in many of these countries and the relatively small size of the market supplied these products can be economically viable.

A recent survey carried out by Swanson (1997) compared two model systems, that of the LUBILOSA Programme<sup>a</sup>, representing a labour-intensive model, which is described in full later in this article, and the proprietary system developed by Mycotech Corporation as representing a capital intensive model. Swanson's findings clearly illustrated the economies of scale offered by the capital intensive system, but found that a production output of at least 80,000 ha p. a. was required before the capital intensive system could attain economic viability. In contrast, the labour intensive production system was more economical at output levels of 20,000 ha p. a. Hence, depending on the likely demand for a given product, a simpler, labour intensive production system may prove appropriate, particularly for niche market products and for application in developing countries. In such cases, a locally produced solution to a pest problem may reduce the requirement for commercial pesticide products and save valuable foreign exchange expenditure.

However, applications for appropriate technology production systems need not be limited to the scenarios above. A feature of the majority of research and development programmes is that large amounts of capital are not available for the design and construction of an industrial scale

<sup>a</sup> An international collaborative research programme for the biological control of locusts and grasshoppers, carried out by the International Institute of Biological Control (IIBC), the International Institute of Tropical Agriculture (IITA), Centre pour Agronomie, Hydrologie, Meteorologie du Comité Inter-État de Lutte contre la Secheresse au Sahel (AGRHYMET-CILSS) and Deutsche Gesellschaft für Technische Zusammenarbeit (GTZ).

production system. This should not be regarded as a major obstacle to the success of a biopesticide programme, as the development of a simple and reliable production system as part of the research process is invaluable for defining parameters of production. The development and optimization of a simple production procedure will not only be invaluable for the supply of reliable material for laboratory and field efficacy trials, but will help to ensure the compatibility of the product with the formulation and application system. A tremendous amount can be learnt about the fungal isolate being developed, permitting its production characteristics, stability and long-term storage to be established well in advance of eventual commercial development and production.

This paper describes the developmental stages involved in designing and optimizing a simple system for the production of aerial conidia. In addition to studies on yield and optimization of a mass production system for a given fungal isolate, the importance of a thorough quality control (QC) procedure is discussed. Finally, the techniques and production methods currently employed in the LUBILOSA programme are presented and the relevance of each stage in the process is described in the context of wider mycopesticide applications. The areas covered include not only the production process but also essential information on fungal culture maintenance for mass production, maintenance of culture virulence, stability, prevention and monitoring of contamination and quality control.

### Current Information on Mass Production of Aerial Conidia

Many scientific papers have been written on the subject of 'mass production' of fungi. The majority describe investigations into optimization of conidia production by a specific isolate on a given substrate, but rarely go further than laboratory scale investigations (Agudelo & Falcon, 1983; Lewis & Papavizas, 1983; Maheva *et al.*, 1984; Silman *et al.*, 1991; Ibrahim & Low, 1993). Comparison between these published production systems is often difficult due to differences in the way in which yield and output parameters are reported. This makes comparison of the efficiency and capacity of similar production systems at best difficult if not impossible. In order to gain the maximum value out of published literature on different production systems, the following parameter values are essential:

**Yield** – described as number of conidia produced per gram or kilogram substrate, taken as an average value over a number of production cycles.

**Concentration of conidia in end product** – described as number of conidia per gram final product, e. g. after extraction/formulation.

**Substrate handling capacity** – number of kilograms of substrate used for each production cycle, taken as an average over a number of cycles.

**Throughput** – described as maximum number of production cycles possible per annum (or other relevant time interval).

**Field application rate** – quoted as number of grams of product applied per hectare (this parameter may require further

clarification if for instance the product is a seed or seedling treatment or for use in a lure or trapping system where the number of hectares protected may be considerably larger than the area actually treated).

Given the above information it would be possible to compare the efficiency and capacity of any number of production systems regardless of whether their end product is sporulated substrate for soil application or a conidial powder separated from its growth substrate for subsequent formulation and spray application.

### **Routine Culture Maintenance in Mycopesticide Development**

Having selected a virulent isolate or isolates for development as a mycopesticide, an essential step in the maintenance procedure is to make single spore (ss) isolates from the original multispore isolation. This is important to maintain virulence and morphologic homogeneity as the mitosporic fungi in particular are likely to contain genetic information from more than one source. The use of multispore isolates in mass production may lead to the accidental selection of material best suited to growth on artificial substrates with a subsequent loss in virulence. Details of how to perform the ss isolation technique can be found in Johnston & Booth (1983). A number of ss isolates should be taken from each multispore isolate and each ss isolate should be tested for virulence to the target insect. One ss isolate should be selected for future work and cultures of both the ss and multispore isolates should be stored safely, using as many long-term storage techniques as appropriate/available. By far the safest mode of storage is to lodge any interesting isolates with a recognised culture collection such as the CABI BIOSCIENCE UK Centre (Egham) [formerly the International Mycological Institute (IMI)], the Agricultural Research Service Collection of Entomopathogenic Fungi (ARSEF, USA) or Centraalbureau voor Schimmelcultures (CBS, Netherlands). Cultures can be lodged at any of these collections under safe deposit which will prevent unauthorized access to the material.

Working cultures and cultures for short-term storage can be maintained on agar. Potato carrot agar (PCA) is suitable for most mitosporic fungi. Cultures maintained on PCA at 10°C can remain viable for up to a year, although this will vary from isolate to isolate. Working cultures can be grown on standard mycological agar such as Sabouraud dextrose agar (SDA) and malt extract agar (MEA). On these richer media, cultures should be kept no longer than six weeks before use in mass production. Sub-culturing should be kept to a minimum and isolates should never be subcultured more than four times before use in mass production. Many mitosporic fungi are known to attenuate during successive sub-culturing, resulting not only in a loss of virulence but also variation in conidiation characteristics. Routine maintenance of isolates for mass production should include regular passage of the pathogen through its original host or closely related species.

### **Experimental Aspects of Production System Development**

Different fungal genera, and even different isolates within the same fungal species, vary greatly in their requirements

for nutrition, pH, water activity ( $a_w$ ) of the substrate (moisture content), temperature optima for mycelial growth and conidiation, light, aeration and incubation period (Johnpulle, 1938; Bartlett & Jaronski, 1988; Latgé & Moletta, 1988; Kleespies & Zimmermann, 1992). All these parameters must be thoroughly investigated and optimized to ensure that the mass production process is not only efficient, but also produces conidia of high quality and reliability. Many papers reporting the optimization of such parameters describe experiments performed on a small scale (Lewis & Papavizas, 1983; Harman *et al.*, 1991; Vimala Devi, 1994). Small-scale experiments such as those carried out in Erlenmeyer flasks generally produce good quantitative data, but while useful in identifying general requirements for conidiation, results are often difficult to repeat at the production scale. One reason for this is that as the volume of the substrate is increased, similar levels of aeration are difficult to maintain. Optimization of any parameter which is affected by aeration must be carried out on the production scale. In addition to the physical problems of scale-up, operational problems can also be encountered. Aseptic technique, for example, is easily performed when handling small bottles and flasks, but maintaining this procedure when dealing with kilogram quantities of solid substrate and large volumes of water is often difficult. Thus, it is not until certain techniques are attempted on a large scale that their feasibility can be assessed. It is therefore important to consider the consequences of scale-up on all parameters that are assessed on a small scale before assuming their validity on the production scale.

Obtaining statistically reliable data during the optimization of fermentation parameters can be a highly intensive and time consuming process and the use of holistic analytical techniques can help to shorten this process. Silman *et al.* (1991), for example, used a fractional factorial experimental design for the comparison of culture methods for *Colletotrichum truncatum* (Schwein.) Andrus & Moore. This design reduced the number of experimental trials necessary to obtain data from multiple variable combinations.

### **Use of a liquid stage for inoculation of solid substrates**

Many appropriate technology systems and the majority of industrial fungal production systems utilize a two stage system in which fungal inoculum of mycelium or hyphal bodies is produced in liquid culture, either in shake flasks or fermenters and transferred to a solid substrate for production of conidia (Samsinakova *et al.*, 1981; Bartlett & Jaronski, 1988; Vimala Devi, 1994; Guillon, 1997). The advantages of a two stage production system which utilizes an actively growing liquid culture as inoculum are: (1) The competitiveness of the fungus is enhanced, reducing the risk of colonization of the substrate by contaminating micro-organisms. (2) Colonization and conidiation are more rapid thus reducing incubation time and economizing on space. (3) The liquid culture stage can act as a screen for contamination which may have been present in the original stock slope culture. (4) It ensures an even coverage of the solid substrate particles resulting in homogenous growth throughout the substrate.

#### *Optimization of liquid growth medium*

In general, mitosporic fungi grow readily as mycelium or hyphal bodies (also referred to as blastospores) in liquid

media. Optimization of a liquid medium to promote rapid growth of a given isolate is one of the simpler steps in the development of a mass production procedure. As with all fungal media, the liquid phase must contain both nitrogen and carbon sources, along with the common trace elements and growth factors. Most mitosporic fungi grow well on very simple media; *Metarhizium flavoviride* Gams & Rozsypal<sup>b</sup>, for example, grows well on a medium composed of brewers' yeast and sucrose in tap water (Jenkins & Prior, 1993). The carbon:nitrogen ratio of this medium was optimized to promote maximum mycelial production, the optimum concentration of total nitrogen was 1.5 g/l (equivalent to 20 g yeast granules per litre) in combination with 20 g sucrose per litre in tap water, and this was found to supply all essential trace elements.

One feature of fungal growth in liquid media is that some isolates tend to grow as discrete mycelial pellets. Unfortunately, these are not suitable for use as inoculum of a solid substrate as they do not permit even coverage. There are a number of methods which have been shown to limit pellet formation and encourage even mycelial growth or formation of hyphal fragments. Inch & Trinci (1987) and Humphreys *et al.* (1989) reported that the addition of polyethylene glycol (PEG) 200 suppressed the formation of pellets in liquid cultures of a number of entomopathogenic fungi. Kleespies & Zimmermann (1992) have also reported increased blastospore production and reduced pellet formation of *Metarhizium anisopliae* (Metschnikoff) Sorokin using PEG 200, Tween 80 and high or low pH according to the isolate tested.

Once a suitable liquid medium has been selected, a simple growth curve should be plotted to determine the period of exponential growth. This should be carried out at the optimal temperature for mycelial growth (see below). The actively growing liquid inoculum should be transferred to the solid substrate during the exponential growth phase.

#### Dilution of mycelial inoculum

It is possible to dilute the mycelial inoculum before addition to the solid substrate. This can be particularly useful if shaker or fermenter space is limited. In studies on *M. flavoviride*, it was demonstrated that dilution of the mycelial inoculum up to 15 times is possible without significant loss in conidia yield on the solid substrate (Jenkins, 1996). However, on a large scale, dilution to this extent was found to be impractical.

<sup>b</sup> Species of the genus *Metarhizium* have been widely evaluated and used for the microbial control of agricultural, plantation and horticultural pests in many parts of the world. While the genus *Metarhizium* is indigenous to many countries, the specific isolate IMI 330189 was isolated in Niger, from the insect *Ornithacris cavroisi* (Finot) (Acrididae). Before 1996, many scientific papers have referred to IMI 330189 (and similar isolates from acridoid hosts) as *Metarhizium flavoviride*, but recent taxonomic studies using molecular methods have indicated that they are genetically more similar to *M. anisopliae*. A monograph which revises the taxonomic status of the genus *Metarhizium* is in preparation (F. Driver, R. J. Milner & J. W. H. Trueman, pers. comm.) and takes account of extensive and independent molecular studies in several laboratories, and formally defines these isolates as *M. anisopliae* var. *acridum*. This reflects wide-ranging and consistent molecular evidence that they have more characteristics in common with *M. anisopliae* than *M. flavoviride*, despite similarity to the latter in conidial morphology, and that they are sufficiently distinct to warrant variety status. To avoid confusion, we will continue to use *M. flavoviride* until the taxonomic revision has been confirmed.

**Table 1.** Substrates used for production of aerial conidia of mitosporic fungi.

Cereal/nutritive	Inert/non-nutritive
Rice, rice bran	Vermiculite
Wheat, wheat bran	Cloth
Buckwheat	Clay/clay granules
Soyabean mash	
Maize meal	
Maize cobs	
Millet	
Oats	
Barley	
Sorghum	
Potato	
Bread	
Groundnut hull meal	

#### Choice of solid substrate for conidiation

There is a wide range of solid substrates available for use in the production of fungi for biological control. A list of those previously reported as suitable for production of conidia is given in Table 1. The choice of substrate will depend on a number of factors, including local availability, cost and isolate preference.

For maximum sporulation, a good surface area to volume ratio is essential. Individual substrate particles should remain separate after hydration and sterilization. Substrate particles which clump together when water is added reduce the surface area to volume ratio, limiting the space on which sporulation can occur. An ideal substrate should not only contain particles of the correct dimensions, but also maintain its structural integrity during preparation for the production process (Maheva *et al.*, 1984; Bradley *et al.*, 1992 a,b).

As illustrated in Table 1, solid substrates may be either nutritive or non-nutritive. One advantage offered by the use of non-nutritive substrates is the opportunity to fine-tune the addition of nutrients to the substrate for optimal sporulation of the isolate(s) to be produced (Jenkins & Lomer, 1994). Used this way, solid substrate fermentation mimics more closely deep tank liquid fermentation in its flexibility for adjustment to different isolates or species of fungi. Additionally, nutrient solutions can be carefully balanced to ensure complete or near complete utilization of the nutrients provided. The biocontrol manufacturer, Natural Plant Protection, Pau, France, uses a clay granule substrate for the production of a number of mitosporic fungi including *Beauveria* spp. and *Trichoderma* spp. A feature of this system is that the clay granules have a naturally low level of microbial contamination, avoiding the need for a sterilization step in the production process (Guillon, 1997).

Nutritive substrates such as cereals are generally more widely available than specialized inert substrates, but they permit far less control over the nutritional environment. Cereal grains vary considerably in their nutrient status not only between species, but between variety and from crop to crop. This variation can affect production, however it is not reasonable within the scope of experimental scale production to run nutrient analysis on different batches of cereals to control for variation. It is far better to select a substrate which gives consistent results from batch to batch.

By far the most commonly selected substrate for production of fungal conidia has been white rice (Aquino *et al.*, 1977; Alves & Pereira, 1989; Mendonça, 1992; Ibrahim & Low, 1993; Milner *et al.*, 1993). This is probably due to a combination of factors including nutritional balance, cost, world-wide availability, physical characteristics such as grain size and shape, hydration properties and structural integrity even after colonization by fungi.

### Isolate selection

The development of a good mycopenicidic relies on the biological properties of the isolate. The following parameters need to be considered in selecting an isolate as a potential microbial control agent:

- laboratory virulence
- field performance
- genetic stability
- productivity
- stability of conidia in storage
- stability in formulation
- field persistence (i. e. tolerance to environmental factors such as UV, temperature extremes and desiccation)
- mammalian safety
- low environmental impact
- capacity to recycle in the environment

The development of a good bioassay procedure will enable the determination of laboratory virulence to the target organism for a large number of isolates. From these laboratory assays, ten or so of the most virulent isolates should be selected for further testing. Genetic stability will be determined during the laboratory manipulations involved in scaling up to produce sufficient conidia for small-scale field tests; any isolates found to lose virulence or conidiation competence should be eliminated. Preliminary mammalian safety testing may also be appropriate at this stage to screen out any dubious isolates.

Field performance should be compared to allow the selection of two or three isolates. Productivity and stability will then be the factors determining which isolate to submit for full mammalian toxicity testing. Stability of the conidial product during storage will be affected by the production process and should thus be assessed only once a suitable production system has been developed.

Any fungal production system will require fine tuning for each isolate to be produced, occasionally major alterations are required from isolate to isolate. Hence a set of particular parameters need to be identified for optimization of the system for each isolate.

### Aeration

All mitosporic fungi are aerobic and require the provision of oxygen for growth and conidiation. However, different fungi vary greatly in their requirements for aeration (Churchill, 1982). In a simple, labour intensive production system, where electronic monitoring and control of the fermentation parameters are not carried out, it is difficult to determine precisely the requirement for oxygen of a given fungus. Observations can be made on the degree of sporulation of a given fungus in small laboratory scale experiments where air supplies can be increased or decreased to varying degrees, in Erlenmeyer flasks for example. This kind of investigation can give some indication of the likely oxygen requirements of the fungus such as low, medium or high, but will not give precise data on oxygen levels or utilization rates as is possible in standard deep tank liquid fermenters and modern proprietary solid substrate fermenters.

The addition of forced aeration to a fungal production system generally improves yield (Bradley *et al.*, 1992a), has been shown to speed up the process of sporulation (Guillon, 1997) and can help to remove excess heat from the system. However, the introduction of forced aeration into an appropriate technology production system is likely to greatly increase the capital cost of the system and, if not designed and implemented carefully, could introduce complications such as contamination and premature desiccation of the substrate. The air to be introduced into such a system must therefore be sterilized and humidified to minimize these risks. Most simple production systems, such as those in Latin America (Aquino *et al.*, 1977; Alves & Pereira, 1989; Antia-Londoño *et al.*, 1992; Mendonça, 1992) and China (Feng *et al.*, 1994) and the LUBILOSА Programme, rely on the passive exchange of air between the growth chamber and the external environment.

### Moisture content

The optimum moisture content for fungal growth and conidiation needs to be identified for each substrate/isolate combination. Moisture content plays a significant role in the final yield of conidia, although the optimization of this parameter can be complex. Most mitosporic fungi prefer humid situations; however, different substrates vary in their moisture sorption curves and will reach maximum adsorption at different moisture content levels. In general, moisture contents within the range 35% to 60% (calculated as percentage water present in the wet substrate on a weight for weight basis) are most commonly used. The determination of optimal moisture content for a given fungus/substrate combination can be carried out initially on a small scale in conical flasks. However, there is a close relationship between moisture content and oxygen availability. Increases in the moisture content of the substrate tend to decrease oxygen availability as the inter-particle spaces become filled with water and air is forced out (Moo-Young *et al.*, 1983). This problem may be compounded during scale-up due to compaction resulting from larger masses of substrate. It is therefore necessary to identify an optimal balance between these two factors at the production scale.

### Incubation temperature

Incubation temperature of the liquid and solid production stages should be matched to the optimal temperature for the

type of growth required. Temperature optima vary greatly not only between fungal species but between isolates of the same species (Thomas & Jenkins, 1997). Additionally, the temperature optima for mycelial growth and conidiation are often different (Alasoadura, 1963). Simple laboratory tests can be used to determine temperature optima for a given isolate, thus allowing the provision of optimal or near optimal incubation conditions. Care should be taken during scale-up as heat build-up during metabolism can cause areas of localized heating way above the temperature optimum.

### Light

Some fungi require light for sporulation to occur (Alasoadura, 1963), other fungi appear to be relatively unaffected by normal intensities of daylight, while in others some degree of inhibition of the sporulation process is observed at certain light intensities (Vouk & Klas, 1931). The effect of light on sporulation of a given fungus can be assessed easily in the laboratory using aluminium foil to prevent light reaching the growing culture. Some early work has suggested that conidia of *Beauveria* produced in the dark were more virulent than those produced in the light (Masera, 1936). Williams (1959) reported that conidia of many species of fungi produced larger conidia when grown in the dark in comparison to those grown under continuous illumination. These factors should be taken into account when selecting a suitable container in which to carry out the solid substrate fermentation.

## The LUBILOS A Mass Production System

This section gives a full description of the procedure developed for the production of aerial conidia of *M. flavoviride* for use in the LUBILOS A Programme. The fermentation parameters given here are those which give optimal production of the LUBILOS A standard isolate (IMI 330189). It is envisaged that the methods described here would be suitable for the production of aerial conidia of a wide range of mitosporic fungi.

The LUBILOS A production facility is housed in a purpose-built wing of the International Institute of Tropical Agriculture (IITA), Plant Health Management Division in Cotonou, Republic of Benin. A feature of the LUBILOS A system is that the purchase of expensive capital equipment has been avoided wherever possible and low-cost, locally available alternatives are used in preference. The production process is a typical two stage system in which liquid inoculum is produced in shake flasks and used to inoculate autoclaved rice, on which conidiation occurs. A flow diagram illustrating the various steps in the production process is given in Figure 1.

The LUBILOS A production system represents one of the more complicated mycopesticide production scenarios as the conidia must be completely separated from the substrate, in order to meet the requirements of formulation for controlled droplet application.

### Routine culture maintenance

The standard isolate of *M. flavoviride* is passaged through the desert locust (*Schistocerca gregaria* (Forskål); Acrididae) at six-monthly intervals. A simple conidial suspension in mineral oil is used to inoculate the insects, which are then incubated

in boxes at 30°C until death occurs, following the methods of Prior *et al.* (1995). The infected cadavers are then further incubated under humid conditions (~100% ERH), to allow the fungus to sporulate externally. A sample of the conidia is then streaked onto PCA plus antibiotics (0.2 g Streptomycin sulphate and 200,000 units Penicillin G. sodium salt per litre).

Conidia from antibiotic plates are used to inoculate at least 30 ordinary PCA slopes in universal bottles. Cultures are grown at the optimum temperature for conidiation (25°C) for 10 days, following which the lids are sealed and the cultures placed at 10°C until use. Every two weeks, a batch of 30 SDA slope cultures are prepared from one of the stored PCA slopes. The cultures are again grown at the optimum temperature for conidiation for a period of 8 - 10 days, cultures are monitored carefully during this incubation period to ensure that mycelial overgrowth does not occur. Isolates which are grown at temperatures above the optimum for production of conidia tend to break into mycelial overgrowth before conidiation is complete and are prone to the same tendency during mass production ultimately resulting in lower yields (Heviefio & Jenkins, unpublished data). Once the incubation period is complete, the lids of the bottles are tightly sealed and the cultures stored at 5°C (for a maximum of six weeks) until use. All production runs originating from the same batch of SDA slope cultures are given the same 'batch number'. Slope cultures for use in mass production are never more than four sub-cultures removed from a re-isolation through *S. gregaria*.

### Preparation and inoculation of liquid medium

A liquid medium is prepared using waste brewers yeast, provided free of charge from the local brewery (Brasserie Beninoise, Cotonou). The yeast is obtained as a liquid slurry and processed by first autoclaving at 121°C for 20 min. The yeast solids are then separated by filtering off the excess water through triple thickness muslin; the yeast forms a solid cake which is then crumbled into granules by hand. The resulting granules are air dried and stored at room temperature until use. Yeast prepared in this manner is good for use up to six months after preparation.

At the start of the mass production procedure, the liquid medium is prepared by re-suspending yeast granules in tap water (20 g/l) with heating. The yeast solution is then homogenized in a commercial Waring blender at high speed to further break up the yeast cells. Locally purchased sugar (sucrose) is then dissolved in the hot broth (20 g/l), 75 ml of the medium is then distributed into 250 ml Erlenmeyer flasks. The flasks are plugged with polyurethane bungs, covered with aluminium foil and autoclaved for 30 min at 121°C.

Once cool, each flask is inoculated with 1 ml of a spore suspension (ca.  $6 \times 10^6$  conidia/ml) in 0.05% Tween 80 using conidia obtained from SDA slopes. Liquid cultures are then incubated on a rotary shaker at 150 rpm for 3 days at  $27 \pm 2^\circ\text{C}$ . This first phase of the production procedure provides an inoculum of suspended mycelial fragments in active growth phase for transfer onto the solid substrate. The use of this actively growing inoculum ensures the rapid establishment of the fungus on the solid substrate. The mycelial inoculum is diluted by 50% with cold sterile water before transfer to the solid substrate.

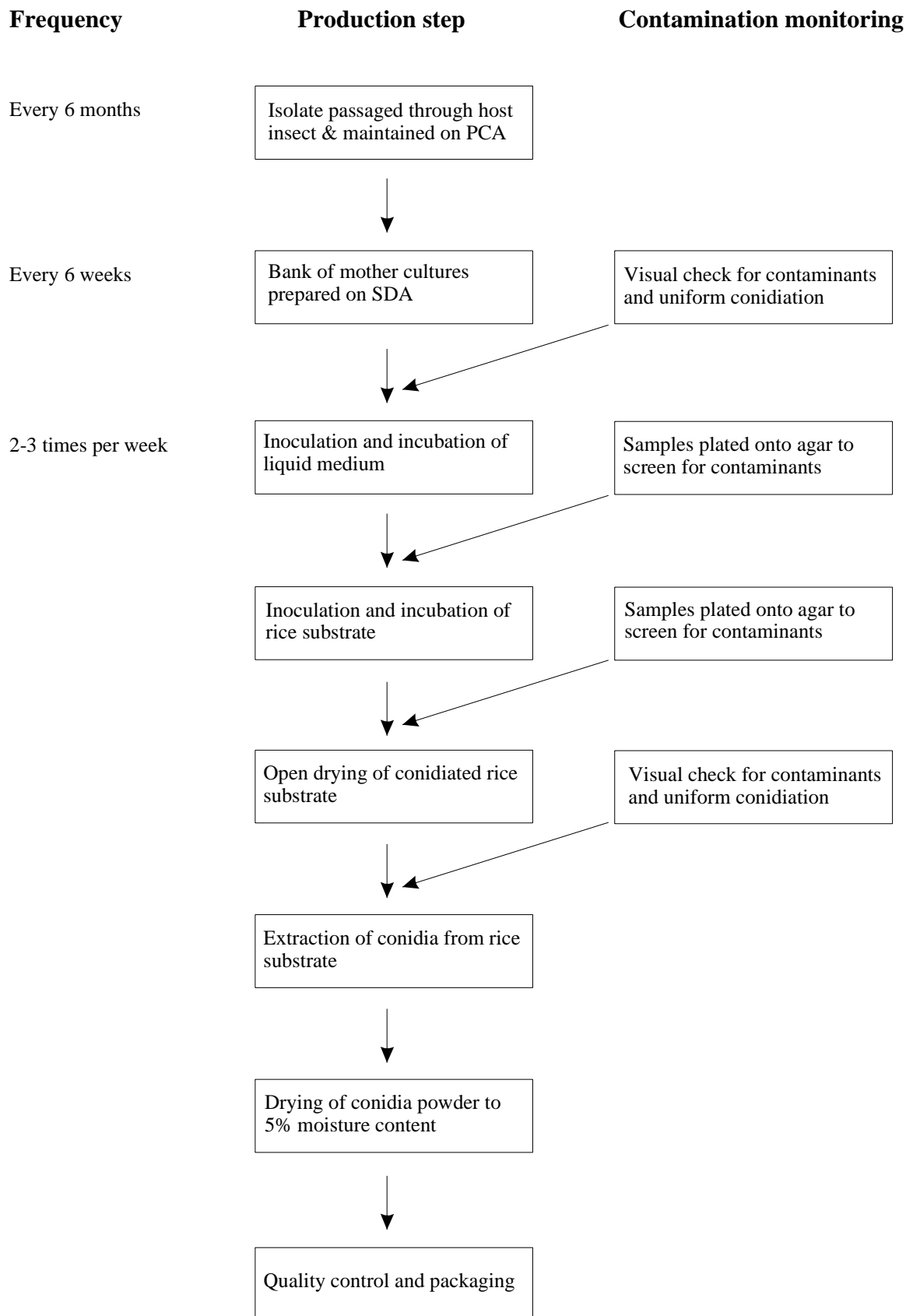


Figure 1. Flow chart showing the various stages in the LUBILOSA production procedure.

### Preparation of rice for the second stage of production

Rice for use in production is carefully selected for consistent appearance and quality. The rice is washed and allowed to drain before the addition of water at a rate of 300 ml/kg and groundnut oil at 20 ml/kg. The rice is then par-boiled to accelerate the absorption of the water before autoclaving. The cooked substrate is then distributed into locally made polypropylene carrier bags (1 kg dry rice equivalent per bag) and autoclaved for 60 min at 121°C. Once cool, the bags are transferred to a laminar air flow cabinet and 150 ml of ready diluted liquid inoculum is added to each kilogram bag of rice and mixed thoroughly from the outside of the bag. The inoculated bags are then placed in disinfected (but not sterile) plastic basins (380 mm diameter × 210 mm deep) with lids to enable stacking. The bags are not sealed within the basins, but are loosely folded over to minimize the entrance of contaminants. The basins are adapted to allow passive aeration during growth and conidiation by drilling four holes (25 mm diameter) around their circumference, these are plugged with sterile polyurethane bungs.

The basins are stacked and incubated at 25±2°C for 10-12 days. On completion of the closed incubation period, the lids are removed and the plastic bags cut away to expose the whole surface of the conidiated rice. The basins are then re-stacked using wooden supports to allow the substrate to dry to approximately 20% moisture content. During the drying period, the room is air conditioned and dehumidified to facilitate the drying process.

### Extraction of the conidial powder

After approximately ten days open drying, the conidia are separated from the substrate using either a two person, hand made sieve or a mechanical agitator fitted to four cyclones.

#### *Extraction by sieving*

A two person sieve, consisting of a 300 µm mesh fixed around a wooden frame is loaded with the conidiated substrate. Plastic sheeting is taped around both the top and bottom edges of the sieve and sealed at the top. A collecting vessel, such as a bucket is fitted to the plastic sheeting at the bottom of the sieve so as to create a funnel into the collecting vessel. The sieve is shaken until all the loose conidial powder has been removed from the rice and has collected in the vessel below. The conidial powder is then further sieved using a 106 µm sieve (Endecotts, UK) to separate the larger rice dust particles from the conidial powder. Even following this second sieving, some rice dust particles remain in the product. Each lot is therefore carefully checked to ensure that it meets the particle size specifications described in the quality control section below.

#### *Mechanical and cyclone extraction*

The conidiated rice is loaded into the drum of a modified domestic tumble dryer. A hopper system is fitted to the door of the drier to permit constant loading of the substrate as the drier operates. The dryer is run without heat to agitate the substrate and remove the conidia from the rice grains. From the tumble drier, a powerful fan at the final outlet provides a negative pressure to draw conidia and other fine particles down a manifold and into a parallel array of four dual-cyclone separators which remove the conidia and rice dust from the

air-flow. Larger particles such as rice dust and mycelium are collected in the outer cyclone chamber, whilst the finely divided pure conidial powder is collected in the inner cyclone chamber and falls into a collecting vessel. After passage through the cyclones clean air is exhausted to the outside of the equipment and is expelled outside. Conidial powder collected from the inner cyclone easily meets the particle size specifications demanded for quality control (R. P. Bateman *et al.*, unpublished data).

### Drying of the conidial powder

The conidial powder resulting from either method of extraction is further dried to a final moisture content of 5% in a locally made spore dryer consisting of a dehumidifier which feeds dry air through a layer of silica gel into a large wooden box containing the conidial powder. Care is taken to ensure that the temperature within the drying box does not exceed 40°C. The drying process takes between three and five days. Once dried, the conidial powder is weighed into plastic-lined foil sachets (100-300 g/sachet) and small packets of non-indicating silica gel are added at a rate of 20% w:w before the bags are sealed. Once packaged, conidia remain viable for >3 years if stored at 10°C or lower, or >1 year if stored at 30°C.

### Quality control and product specifications

The LUBILOSA quality control routine was developed both to monitor the progress of each mass production run by means of a series of contamination checks and to assess the quality of the final product with regard to contamination, moisture content, number of conidia per gram, viability, virulence and particle size distribution. All of these play a significant role in the efficacy of the sprayed product and are a requirement of product registration (Lisansky, 1985; Taborsky, 1992).

Each mass production run is given a unique chronological lot number and a batch number, which refers to the batch of SDA slope subcultures from which the conidial inoculum was taken (see section on routine culture maintenance above). Contamination monitoring checks are carried out on all mass production runs at the following stages:

- (1) Stock slopes are visually inspected before use as inoculum for the liquid medium.
- (2) A sample of the spore suspension and un-inoculated liquid medium is dispensed onto SDA or 2% malt agar plates. The plates are incubated at 25°C for 4 days before checking for the appearance of any contaminants.
- (3) Several samples of solid substrate are placed on SDA or 2% malt agar plates pre- and post-inoculation with the liquid culture. Plates are incubated and checked as above.
- (4) Basins are inspected on opening for presence of contamination; any basins found with contamination are discarded at once.

The final product (conidial powder) is subjected to a number of quality control checks to ensure conformity with the product specifications:

- (1) The moisture content is checked after the drying process to ensure a level of 5% moisture or less.



- (2) Viability is assessed using a standard, 24 h germination test on SDA as described by Hedgecock *et al.* (1995). Viability should be >90% at packaging.
- (3) The presence of micro-organisms other than *Metarhizium* is quantified by preparing a 1 in 10 dilution series ranging from *ca.*  $5 \times 10^7$  conidia/ml to *ca.* 50 conidia/ml; 0.2 ml of each dilution in the series is spread onto two replicate malt agar plates. The plates are incubated for 4 - 6 days at 25°C, following which the number of *Metarhizium* colonies are counted on all plates where single colonies can be distinguished in order to derive the actual concentration of the original conidia suspension. Secondly, contaminants appearing on any of the plates are then counted and the proportion of contaminants to *Metarhizium* conidia is estimated and expressed as a percentage. Contamination levels should be <0.002% by number.
- (4) Virulence of the spore powder is assessed for the first lot of conidial powder produced from a new batch of SDA slopes. Virulence is quantified using a standard laboratory bioassay on *S. gregaria* as described by Prior *et al.* (1995). Virulence is expressed as average survival time (AST) (calculated using Kaplan-Meier survival analysis in SPSS, as described in Jenkins & Thomas (1996)). AST should be within 0.5 days of that obtained using a standard stock culture of the relevant isolate.
- (5) The number of conidia per gram of dry powder is assessed by placing a weighed amount (approx. 0.1 g) into 100 ml Shellsol T or 0.05% Tween 80 in water. This suspension is counted using an improved Neubauer haemocytometer. Conidial powder should contain  $4\text{-}5 \times 10^{10}$  conidia per gram.
- (6) A Malvern® 2600 particle size analyser is used to check the particle size distribution of selected lots. Over 99% of the particles by number should be <6 µm in diameter.

A record of the fermentation parameters and all contamination and quality control checks is kept for each production lot and the information transferred to a database (Microsoft, Excel) at the end of the production process.

### Weekly output and conidial yield from the LUBILOSA production system

Table 2 shows the average yield of conidia obtained from the production unit at IITA Cotonou between January and August 1997. The concentration (density) of conidia in the end product (pure conidial powder) is  $5 \times 10^{10}$ /g and the field application rate is  $5 \times 10^{12}$  conidia/ha. Based on these figures, the current weekly output of the Cotonou plant is  $1.97 \times 10^{14}$  conidia (*ca.* 39 ha at the current application rate), with an estimated annual output of  $1 \times 10^{16}$  conidia (*ca.* 2050 ha).

**Table 2.** Summary of conidial yield and production capacity of the LUBILOSA production unit. (Figures estimated from output of the unit between January and August 1997.)

	Average over production period
Yield	$1.5 \times 10^9$ conidia/g rice
Substrate handling capacity	82 kg rice/production cycle
Throughput	1.6 cycles/week

The variation around the average yield of conidia per gram of rice from this system is huge. Yields of up to  $2.4 \times 10^9$  conidia/g rice have been achieved under certain conditions. The LUBILOSA process only permits loose control over production parameters; as a result, a high degree of variation is inherent in the system. Variation in yield has been reported even in systems with accurate process control over parameters such as temperature, humidity and aeration (Bradley *et al.*, 1992a).

In addition to quantity, quality of conidia is also of absolute importance in the development of a biopesticide product even at the experimental stage (Bowers, 1982; Lisansky, 1985; Lisansky, 1992). A stringent quality control procedure and product specifications helps to ensure that the product of mass production does not vary. This is equally as important at the field testing stage as in the subsequent registration of a product for general use.

The development of the LUBILOSA production system over the past seven years has enabled the Programme to progress from concept to product. In 1998 it is envisaged that IMI 330189 will be registered as a product for use in locust and grasshopper control in Sahelian West Africa and South Africa. The product will no longer be produced in a labour intensive system, but will be produced to the same specifications by licensed commercial companies with industrial fungal production technology. This transfer of technology from labour intensive production to an industrial system has been greatly facilitated by the detailed knowledge gained on the biology of the fungal isolate during development of the LUBILOSA production system.

In the future, the LUBILOSA production unit will continue to be used for research and optimization of production of additional fungal isolates. Further biopesticide development projects for control of pests such as termites, banana weevil, maize stem borers, etc. will benefit from the facilities and expertise gained as part of the LUBILOSA Programme.

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