Chapter 25

USE OF SOLID STATE FERMENTATION FOR THE PRODUCTION OF FUNGAL BIOPESTICIDES SPORES FOR INSECT CONTROL

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Running title: Fungal biopesticides production in SSF

1. Introduction

Numerous viruses, bacteria, nematodes and insects cause significant losses to various crops, including coffee in tropical regions (Table 1). Biological control of coffee pest consists in using natural enemies of coffee pests in order to control their population and proliferation (Dufour *et al.*, 1999; Bustillo, 1999; Muller *et al.*, 1999). There are over 400 species of fungi that can attack and kill specifically nematodes and insect pests (Duponnois *et al.*, 1996; Jenkins, 1995; Schoeller and Rubner, 1994; Segers *et al.*, 1999). The concept of using fungal pathogens to control insects is by no means new (Daoust *et al.*, 1982; Fargues *et al.*, 1979; Ferron, 1967). However, in the last 20 years research stimulated mainly by the resistance of insects to chemical pesticides and by enhanced public awareness of the environment has brought closer the possibility of exploiting such organisms commercially (Hokkanen and Lynch, 1995; Leij, 1992). Recent successes in exploiting nematophagous and entomophagous fungi as biopesticides demonstrated that there could be a high potential in using such micro-organisms to protect coffee plants from known pests (Bustillo, 1999; Dufour *et al.*, 1999; Villain *et al.*, 1999).

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Table 1. Number of diseases affecting some tropical cultures.

Plant	Virus	Bacteria	Fungi	Insects	Nematodes
Pineapple	2	3	3	4	3
Banana	4	2	5	1	1
Cacao	1	-	4	1	-
Coffee	-	-	2	3	2
Sugarcane	2	3	4	3	1
Cotton	2	1	4	3	1
Corn	2	1	3	12	-

A biopesticide is defined as an active biological material with specific action against insects, nematodes or pathogenic micro-organisms (Table 2). Important characteristics of biopesticides include (1) specificity of action, (2) biodegradability, and (3) non-toxicity to pollinic insects (Lopez-Llorca, 1992; Feng et al., 1994).

Table 2. Examples of entomopathogens fungi used in biological control.

Insects		Entomopatogenic fungi
Order	Genera and species	
Ortoptera	*Schistocerca gregaria*	*Metarhizium anisopliae*
Dermaptera	*Forficula spp.*	*Entomophtora formicula*
Eteroptera	*Scotinophora*	*Beauveria bassiana*
Omoptera	*Trialeurodes*	*Aschersonia aleuridis*
Tisanoptera	*Thrips tabaci*	*Verticillium lecanii*
Coleoptera	*Hypothenemus hampei*	*Beauveria bassiana*

In the following sections, we intend to discuss about a few of insects antagonistic fungi used as entomopathogens in biological control. Life cycle of filamentous fungi includes the following five steps: (1) dormancy of the spore with a long time conservation, (2) germination of the spore, (3) apical mycelium growth, (4) conidiogenesis, and (5) conidiospore production (Roussos, 1985). In order to use filamentous fungi as biopesticides it is very important to select strains with high ability to produce conidiospores, commonly named spores (Rivillas Osorio *et al.*, 1999). For large scale spore production on a solid medium, there are different techniques: (1) agar culture media (e.g. potato dextrose agar- PDA) in Erlenmeyer flask, Roux bottle or in a Disc Fermenter, (2) natural substrates such as wheat bran, sugar beet, etc., (3) natural supports (sugarcane bagasse, wheat bran, vermiculite, polyurethane foam, etc) complemented by a nutritive solution containing carbon and nitrogen sources as well as

minerals (Raimbault and Roussos, 1985; Raimbault *et al.*, 1989; Jenkins, 1995; Agosin *et al.*, 1997). Fungal growth and sporulation is commonly carried out using plastic bags containing solid substrates, in column bioreactors (Jenkins and Goettel, 1997) or in a Zymotis which is a bioreactor for pilot and industrial scale spore production (Montero *et al.*, 1989; Roussos *et al.*, 1993).

No worthwhile commercial exploitation of solid state fermentation (SSF) has been specifically elaborated in spite of intensive research and development efforts throughout the world (Lonsane *et al.*, 1985). One of the major reasons for this situation is the lack of efficient techniques to develop active spore inoculum, required in large quantity in SSF system. The form, age and ratio of inoculum are of critical importance in SSF system relying on larger inoculum ratio to control contamination (Lonsane *et al.*, 1992). The spores are usually preferred over vegetative or mycelium in SSF system due to ease in mixing of the inoculum with autoclaved moist solids (Pandey, 1994). The productivity of the system is also influenced by the age of the inoculum. Moreover, viability of the spores is of great significance. The development of a large-scale inoculum has been specified as one of the areas which poses problems in scale-up of submerged fermentation (SmF) processes (Joshi and Pandey, 1999). In fact, this will be more problematic in SSF system due to involvement of lower water activity, complex medium constituents, use of water insoluble polymeric substrates and high heterogeneity (Viniegra-Gonzalez, 1997). Work was, therefore, undertaken to develop efficient strategies for large-scale inoculum development on agar media and also in the SSF system involving the use of bagasse as solid support (Roussos *et al.*, 1991). *Trichoderma harzianum* was selected for the studies due to its significant industrial importance in the production of cellulases (Deschamps *et al.*, 1985), biopesticides (Elad *et al.*1993), antibiotics (Okuda *et al.*, 1982), protein enrichment of cassava flour (Muindi and Hanssen, 1981) and flavour compounds (Sarhy-Bagnon *et al.*, 1997).

2. Conidiospore production on agar medium

Erlenmeyer flasks and disc fermenter have been used for the production of conidiosopres on agar medium (Roussos et al., 1991), which contained (g/l): cassava flour 40; KH_2PO_4 2; $(NH_4)_2SO_4$ 4; urea 1; $CaCl_2$ 1; Agar 15; distilled water 1 l. The pH was adjusted to 5.6 using 2-N HCl. The medium in desired quantity was added to the bioreactors for autoclaving at 110°C for 30 min. Growth and sporulation of the cultures were allowed to take place at ambient temperature (28±1°C) without any pH control. However, the disc fermenter was aerated at the rate of 40 l of humidified and sterile air/h during for seven days (Table 3). Viability of conidiospores was studied using the medium of Douglas *et al.* (1979) as per the methodology of Roussos (1985). The conidiospores were counted using a Malassez's haemocytometer.

Table 3. Sporulation of five strains of filamentous fung igrown on agar enriched with various substrates in Earlenmeyer flasks at 25°C

Filamentous fungi	Spore production* (Number g^{-1} C source)	Carbon source	C/N ratio
Trichoderma harzanum	14.1 x 10^9	Cassava flour	14
Aspergillus niger	5.2 x 10^9	Cassava flour	24
Beauveria bassiana	7.2 x 10^9	Cassava flour	24
Aspergillus terreus	9.9 x 10^9	Molasses	14
Metarhizium flavoviride	6.5 x 10^9	Wheat bran + bagasse	ND
Penicillium roque fortii	7.2 x 10^9	Molasses	2

* Number of co per gram of carbon source

The extent of conidiospore formation per cm^2 of culture surface area in disc fermenter was similar to that on agar media in Erlenmeyer flasks (Table 4). Conidiospores from disc fermenter were sufficient for inoculation of 100 kg moist cassava flour medium for protein enrichment of cassava or 100 kg moist bagasse + wheat bran medium for enzymes production. Calculations are based on rate of inoculation of these media 10^{10} spores/kg moist solid medium with standardized values (Raimbault and Alazard, 1980). Larger inoculum would, however, be required for larger pilot or village level plant of 5-8 tons (wet wt.) per day capacity. Using disc fermenter, the inoculum for such plants can be produced but will require using 20 fermenters working in tandem. However, such a strategy is highly unthinkable to put into practice and it would be cost-intensive with respect to both capital and operating expenses. It is also not practical as the use of agar medium at such large scale is highly laborious and agar itself is expensive. It is, therefore, necessary to search for alternative strategy, simpler and less expensive, for large-scale production of conidiospores.

Table 4: Spore production by *Trichoderma harzianum* in various bioreactors

Bioreactor	Conidiospore production	
	Per gram of cassava flour	Per area (cm^2) or volume unit
Erlenmeyer flask (20 ml)	1.1 x 10^{10}	* 1. 7 x 10^8
Disk fermentor	9.3 x 10^9	* 2.2 x 10^8
FMS Column (18 g)	5.0 x 10^{10}	**8.8 x 10^8
Zymotis (21 kg load)	5.0 x 10^{10}	**7.7 x 10^8

* Results are expressed per area (cm2) or ** per volume unit (cm3) of SSF medium

3. Conidiospore production by solid state fermentation system

The use of inert solid support to absorb liquid medium in solid state fermentation (SSF) system has been pioneered by Raimbault et al. (1989) in order to facilitate selective and homogenous development of mycelia as well as the study of the physiology and growth of fungi. Two different types of bioreactors for SSF systems have been developed and include column fermenter (Raimbault and Alazard, 1980), agitated reactor (Durand *et al.*, 1997), and static reactor such as Zymotis (Roussos *et al.*, 1993). These bioreactors offer excellent potential for conidiospores production (De Araujo *et al.*, 1997).

The use of bagasse as support to absorb starch containing liquid medium in column fermenter gave nearly equal conidiospores production when compared to that on agar medium in flasks as well as disc fermenters (Roussos *et al.*, 1991). The inclusion of feather meal in the medium (10.75 g in 100 g mixture of dry bagasse and starch) resulted in a five fold increase in conidiospores production (Montero *et al.*, 1989). The moist solid medium used in column fermenter and Zymotis contained (g): bagasse 80, cassava flour 20, $(NH_4)_2SO_4$ 0.3, urea 1.3, KH_2PO_4 2.5, $CaCl_2$ 2, birds feathers meal 10.7 and tap water 100 ml. The ingredients were mixed thoroughly and the moist medium was filled in cloth sacks (6 kg, wet weight) for autoclaving at 121°C for 15 min. After cooling to about 30°C, it was mixed thoroughly with the liquid inoculum obtained from disc fermenter so as to provide $3x10^7$ spores/g cassava flour initially present in the autoclaved medium. The final moisture content of the medium was 75%. The inoculated medium was used to fill column fermenters (18g/column). The Zymotis compartments were charged to occupy 5 and 10 cm length with 50-cm height as well as 10-cm length with 30-cm height. In another case, Zymotis was charged with 21-kg moist medium and the medium in this case contained a mixture of sugarcane bagasse and cassava flour at the ratio of 80:20. The medium used was autoclaved at 110°C for 90 min.

The column fermenter assembly and the design of Zymotis have been described elsewhere with operating procedures (Raimbault and Alazard, 1980; Prebois et al., 1985; Roussos *et al.*, 1993). In both cases, fermentation was carried out at 29°C for 6 days with aeration by humidified air at a rate of 4 l/h/column and 300 l/h/kg dry solids in Zymotis. At the end of the fermentation, fermented solids were removed from the bioreactors and the conidiospores were harvested from 10-g material in 0.01% Tween 80 solution as per the methodology described for conidiospores production on agar medium. Productivity of the conidiospores in Zymotis operated in four different substrate load conditions was equal to that from column fermenter (Table 4). In fact, conidiospore production in Zymotis per gram cassava flour was 5 times higher than that on agar medium in flasks. This constituted a tremendous success in development of large-scale inocula. Conidiospores formed in 21-kg moist medium in Zymotis were sufficient to inoculate 5-tons of cassava flour or bagasse and wheat bran media in SSF processes. The maximum working capacity of Zymotis is 42 kg moist medium which

provides sufficient inoculum for 10-tons of wet cassava flour or bagasse and wheat bran media.
Productivity per gram cassava flour has been higher in Zymotis with 21-kg moist medium load as compared to other loads, though productivity in terms of conidiospores/cm^3 of culture surface area is comparatively lower. This is because of the use of 20:80 ratio of cassava flour and sugarcane bagasse in the former as compared to 30:70 ratio in the latter case. Consequently, the effective surface area contained more of the inert solid support. It may be possible to achieve better productivity by using higher cassava flour concentration.

4. Studies on conidiospores preservation and viability

Conidiospore suspensions from the disc fermenter are highly dilute due to the need of large volumes of liquid used to recover conidiospores completely from agar surfaces with an efficiency of recovery of 98%. Unless used immediately, preservation at 4°C becomes essential. Viability of conidiospores after such preservation for 1, 26 and 53 days was 97.3, 84.4 and 83.2%, respectively. Vacuum concentration of the suspension at 40°C to reduce bulk volume, however, resulted in merely 9.4% viability. Reduction in viability was more drastic when temperatures of 50 and 60°C were used along with vacuum concentration. Conidiospores of *T. harzianum* are very sensitive to temperature (Roussos *et al.*, 1989). The strain grows at an optimum temperature of 29°C and does not grow at 35°C. It is interesting to note that the viability was 38.8% when bagasse were added to the suspension before vacuum concentration at 40°C. Bagasse probably absorbed the conidiospores and imparted protection during vacuum concentration. Conidiospores, when used for production of cellulases in column fermenter and Zymotis, performed equally well and produced 19 IU of APF and 200 of ACMC activity per gram substrate dry matter.
It is emphasised that the use of SSF system for the production of the conidiospores could overcome problems associated with the dilute nature of the conidiospores suspensions from disc fermenter. For example, the need for recovery of conidiospores could be avoided and the spores containing fermented mass can be used directly as is done in a number of food fermentation (Lonsane *et al.*, 1992). Alternatively, the fermented bagasse containing *T. harzianum* spores was dried at 20°C to a moisture content of 8-12%, without any appreciable loss of spore viability for use at a later date (Table 5).

In this respect, Zymotis offers advantages as it is possible to dry the fermented solids *in situ* by passing dried hot air through the loop used during fermentation to supply humid air. The inoculum grown on wheat bran and other solid substrates has been stored after drying up to six months without appreciable loss of viability of spores.

Table 5. Kinetics of spore production by *T. harzianum* grown on a mixture of sugar beet and sugarcane bagasse (75/25). Spores viability before and after air drying at 20° C.

Incubation at 29° C (days)	Substrate moisture (%)		Spores produced (x 10^8 g^{-1} DM)		Viable spore (x 10	
	Fresh	Dry	Before drying	After drying	Before drying	After Drying
4	80.1	3.4	38.7	35.0	5.0	13.1
5	86.6	3.9	45.8	48.9	21.5	40.2
6	81.4	3.3	54.0	36.4	41.2	40.2
7	85.9	2.4	47.9	35.5	50.0	45.4

The data indicate high potential and many advantages in producing large-scale conidiospores (inoculum) in Zymotis for pilot and large-scale SSF systems. Conidiospores, thus, produced could also be used as such in SmF processes or if necessary, after recovery in sterile water. The dried fermented solids with its high concentration of conidiospores of *T. harzianum* could also be used directly as biopesticide.

5. Summary

It is estimated that over 400 species of fungi can attack and kill specifically nematodes and insect pests. In the past two decades, much attention has been paid on research and commercial development of biopesticides to control insects. This is stimulated by the increased resistance of insect's pests to chemical insecticides and by a greater public awareness of the environmental impacts of agro-chemicals. Recent success in using nematophagous and entomophagous fungi as biopesticides demonstrated their high potential to control coffee pests. Studies on the fungal sporulation physiology have allowed defining optimal conditions for the mass production of conidiospores of filamentous fungi such as *Beauveria bassiana, Metarhizium flavoviride, Paecilomyces fumosoroseus,* and *Trichoderma harzianum.* The pilot plant scale production of conidiospores can be achieved using solid state fermentation

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