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Mass-production of fungi for bioherbicides

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Summary

Mass-production of fungi to be developed as mycoherbicides requires good knowledge of fermentation technology. Selection of the best performing strain, maintenance and stabilization of the isolate and development of a reliable bioassay procedure are necessary prerequisites to the development of small-scale production systems and scaling-up of the selected fermentation process to industrial level. Solid-state, submerged and diphasic fermentation technologies are the principal methods available for the production of fungal biomass. Selection of the most appropriate fermentation process should take into consideration the advantages and disadvantages associated with each available system and the type of biomass desired as the end-product. Optimization of fermentation systems depends on a multitude of factors such as growth medium, physico-chemical conditions and type of seed inoculum. Fermentation processes should be selected or manipulated to trigger the production of stable, durable and virulent propagules. Spores produced in submerged fermentation are known to be more sensitive, unstable and less virulent than propagules produced in an aerial environment. The production and formulation of mycelial biomass is often considered for fungi unable to sporulate in submerged fermentation. The composition and balance of the growth medium deserves great attention because it can markedly influence yield and the biological characteristics of fungi.

Introduction

Expertise in mycology and fermentation technology are important in the manufacture of mycoherbicides. A major requirement in their commercial production is the development of efficient processes for large-scale production. Several techniques to mass-culture fungi have been developed to produce fungal metabolites such as antibiotics and organic acids (Miall 1975), flavouring agents (Solomons 1975), alternative food protein (Trinci and Wiebe 1990) and infectious propagules for biological control of pests (Soper and Ward 1981, Churchill 1982, Stowell 1991).

Appropriate fermentation technology needs to be selected for the industrial production of viable and highly virulent fungal propagules to be formulated as mycoherbicides. The cost effectiveness of these biological products is closely related to the efficiency of the fermentation methods and the type, quality and quantity of the propagules produced. The stability, germinability and pathogenicity of the fungal propagules are influenced by the nutritional and physical environments of the fermentation process.

This paper presents the important phases and aspects in the mass-production of biological weed control fungal agents, the advantages and disadvantages of the available fermentation technologies, the ideal fungal propagules to produce and market and the influence of growth medium on the quantity and quality of the biological product.

General methodology

Fungal species and strains are biologically different in respect to host spectrum, pathogenicity, environmental tolerance and culture characteristics. The key biological characteristics signifying mycoherbicide potential are pathogenicity and selectivity. The maintenance and stability of the selected strain can be ensured by using single-spore isolates preferably stored in liquid nitrogen or in a cryofreezer. These precautions minimize potential variation of the fungal characteristics and prevent contamination of stock cultures (Churchill 1982). A reliable bioassay procedure is crucial to all the steps involved in the development of any microbial pesticide (McCoy 1990, Stowell 1991). Bioassay methods allow quality control of the biological product since the number of viable and active propagules is far more important than the number of particles per se. A good quality control program should detect unexpected changes in host range and in pathogenicity.

Small-scale laboratory production of the fungal pathogen is required to provide sufficient quantity of inoculum to perform initial glasshouse and field tests and to assist scale-up fermentation studies. Factorial experimental designs are generally employed for the screening of media and the optimization of the physico-chemical culture conditions (pH, temperature, agitation-aeration) (Latgé and Moletta 1988). These studies are commonly performed in 50–2000 mL shake flasks (Churchill 1982) or 20–200 L pilot fermenters (bioreactors) (Kristiansen and Sinclair 1980) for submerged fermentation and in petri dishes, flasks, bags, stationary trays or rotating drums for solid-state fermentation (Aidoo *et al.* 1982, Goettel 1984, Mudgett 1986). Hegedus *et al.* (1990) have established an innovative methodology using 0.5 mL cultures growing in 1.5 mL micro-centrifuge tubes on a rotary shaker. This technique allows the evaluation of several combinations of nutritional parameters under constant culture conditions in a limited space. Biomass yield from pilot scale production systems, however, should not be extrapolated directly to large scale fermenters because their agitation-aeration characteristics compare poorly and affect the optimum nutrient balance required for fungal growth (Rombach *et al.* 1988).

Scaling-up the most appropriate fermentation process for the production of a particular fungus is usually achieved in stages. At each stage, the volumes of the fermenter vessel and medium are increased and the various fermentation parameters are monitored and adjusted to support maximum production of infectious propagules or mycelial biomass. Sophisticated large pilot plant fermenters (500 L or more) monitored with comput-

Table 1. Comparison of submerged and solid-state fermentation systems.

	Advantages	Disadvantages
Submerged fermentation	Equipment readily available in Western countries Well-documented technology Short fermentation cycles (3–7 days) Easy maintenance of sterile environment Low requirement for manpower Ease to monitor and adjust fermentation parameters Efficient substrate utilization	Requires sophisticated equipment High installation and operation costs High requirement for energy Type of propagules produced may not be suitable Aeration/agitation problems with extensive biomass Separation of spores from biomass may be difficult
Solid-state fermentation	Simple, generally inexpensive Aeration easily obtained All types of propagules produced Possibilities to provide illumination if required Conditions of growth are similar to the ones required in nature No problem with high biomass growth	Technologies not easily accessible in Western countries Heat built-up with large quantities of substrate Difficulties in regulating fermentation parameters Risk of contamination Pretreatment of substrate sometime necessary Long fermentation cycles (up to 4 weeks) Large seed inoculum often required Requirement of energy for agitation (if applicable) Possible human allergic responses during harvest of spores Labour intensive Inefficient substrate utilization

ers facilitate the scaling-up of submerged fermentation processes to the typical 200 000 L industrial deep tank fermenters (Churchill 1982). On the other hand, large-scale solid-state fermentation technologies, widely used in the Orient, are not fully characterized scientifically but modern fermenter designs have been developed by the Japanese to manufacture traditional food products, enzymes and organic acids (Aidoo *et al.* 1982, Mudgett 1986).

Fermentation technologies

The type of fermentation process to be selected should take into account the form of biomass desired (spores, resting structures or mycelia), the characteristics of the fungal biomass to be produced, the nutritional requirements of the fungus, the availability and cost of the raw materials composing the culture medium, the accessibility of technology and labour, the capital and operating costs and the market potential. The principal methods available for the commercial production of fungal propagules or mycelia are submerged, solid-state and diphasic fermentations. The commercial mycoherbicides Collego® and DeVine®, registered for sale in the USA. (Boyette *et al.* 1991) and BioMal® registered in Canada (Makowski 1992) are produced in submerged fermentation; the preferred technique in industrialized countries (Churchill 1982). Submerged fermentation is widely used in the pharmaceutical industry and its succession of operations is easy to scale-up and adapt for mycoherbicide purposes. Solid-state fermentation typically implies the impregnation of small particles of the substrate (e.g., inert material, straw, grain) with water or a nutrient solution. In general, the equipment necessary for large-scale solid-state fermentation processes is similar to the small-scale production apparatus, being simple, inexpensive and adaptable. The basic advantages and disadvantages of the submerged and solid-state fermentation processes are listed in Table 1.

Diphasic fermentation systems combine the advantages of both solid-state and submerged fermentation. The fungal biomass is grown in liquid fermentation tanks and then spread in thin layers on trays to allow sporulation. This type of fermentation is widely used to mass-produce insect fungal pathogens; it allows the production of aerial conidia which are often more stable than other propagules (McCoy 1990). Two phase fermentation system have been used to mass-produce the insect pathogen *Beauveria bassiana* (Bals.) Vuill. in the Soviet Union (Soper and Ward 1981) and on a smaller scale to produce the potential mycoherbicide CASST® (*Alternaria cassiae* Jurair & Khan) in the USA (Walker and Riley 1982).

Other techniques have been developed to produce fungi but they are not commonly used. Liquid surface culture, for example, involves the growth of the microorganisms on the surface of a stationary liquid medium contained in flasks (Calam 1969, Stowell 1991) or plastic cushions (Kybal and Vlcek 1976).

In Western countries, submerged fermentation appears to be the most effective method of producing bioherbicides (Stowell 1991). Batch submerged culture where most of the substrates are added at the beginning of the fermentation cycle is a popular process since most fermentation systems have been designed to recover large quantities of secondary microbial metabolites (Solomons 1975). Continuous or fed-batch cultures, however, are feasible, appropriate and economically advantageous for the production of fungal biomass (Trinci and Wiebe 1990).

Table 2. Effect of various liquid media and types of seed inoculum on the production of conidia by *Phomopsis convolvulus* (after Morin *et al.* 1990).

Liquid media	Type of seed inoculum		
	Mycelial agar disk[1]	Pycnidial agar disk[1]	Conidial suspension[2]
Modified Richard's (1:1)[3]	++[4]	+++	+++
Richard solution (5:1)	–	–	+
Modified beef peptone	–	–	–
Tochinai solution	–	–	–
Half-strength PDB	–	++	++
Czapek dox (10:1)	–	+	+

[1] One 6 mm diameter agar disks covered with only mycelium or mature pycnidia was used to seed 100 mL of medium contained in 250 mL flask.

[2] One mL of a suspension containing 10^7 conidia mL^{-1} was used to seed 100 mL of medium contained in 250 mL flask.

[3] Carbon-to-nitrogen ration (C:N)

[4] Microscopic observation of media two weeks after seeding. Index of conidial density (–) no conidia present, (+) = few conidia, (++) = average number of conidia, (+++) = abundant conidia.

In a continuous culture system, fresh medium is continuously added to the fermenter vessel but the total volume of the culture is maintained constant through an overflow line. This allows the fungal culture to reach steady state of exponential growth (Trinci 1991). Continuous culture in comparison to batch culture systems involves less investment in equipment, allows the fungus to grow under constant conditions at a specific growth rate, prevents the degradation of cellular proteins through autolysis and yields more biomass during the same period.

Multiple factors have to be considered to optimize any of the fermentation systems: the selection or design of the 'best' growth medium, the degree of sterility required, the moisture level, the rheological properties of the growing culture, the availability of oxygen and the type of seed inoculum used. The latter does not usually get the attention it deserves even though it has been demonstrated to affect the length of the fermentation cycle and the total yield of fungal propagules. As a general rule, seed inoculum cultures should represent one to five percent of the total medium contained in the fermenter vessel (Calam 1969). Seed inoculum cultures should be vigorous and free of contaminants and have an adequate density of spores or mycelia. Solomons (1975) pointed out that seed inoculum culture containing only spores leads to considerable delay in the fermentation cycle and suggested their replacement by mycelial fragments which do not exhibit a germination lag phase. The type of seed inoculum and the sporal density of the seed inocula composed of spores were demonstrated to have drastic effects on the total yield of conidia by *Phomopsis convolvulus* Ormeno-Nunez in solid-state and submerged fermentations carried out in flasks (Table 2, Figures 1, 2) (Morin et *al.* 1990).

The harvest of the fungal mycelium or spores is performed through centrifugation or filtration of the liquid medium. In solid-state fermentation processes, spores are often recovered using a vacuum collector or the entire biomass is milled with the substrate or carrier. Latgé and Moletta (1988) emphasized that the recovered biomass should be kept wet until formulation.

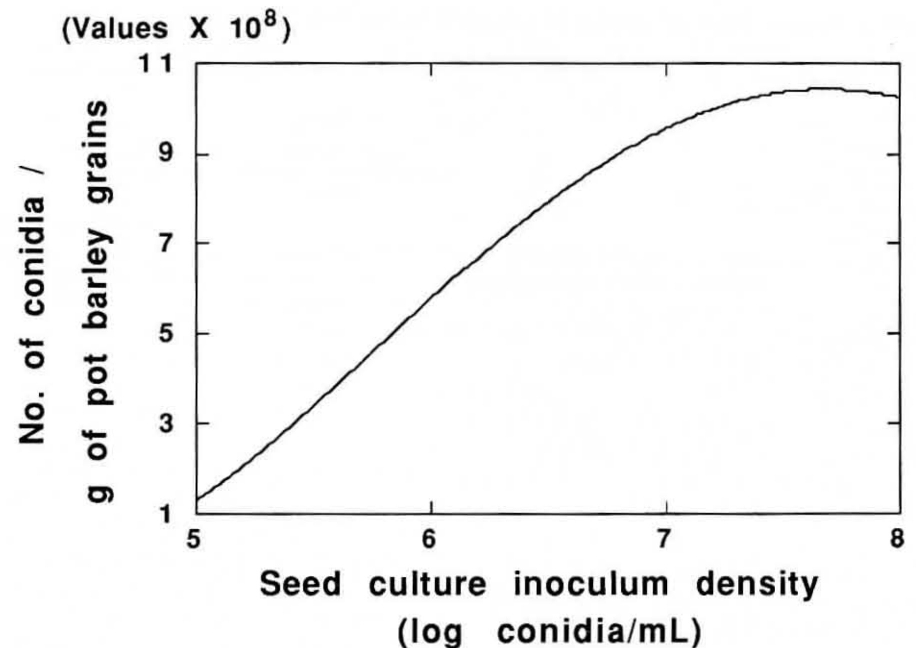


Figure 1. Effect of the density of the spore seed culture inoculum on the production of conidia by *Phomopsis convolvulus* on pot barley grains (After Morin *et al.* 1990).

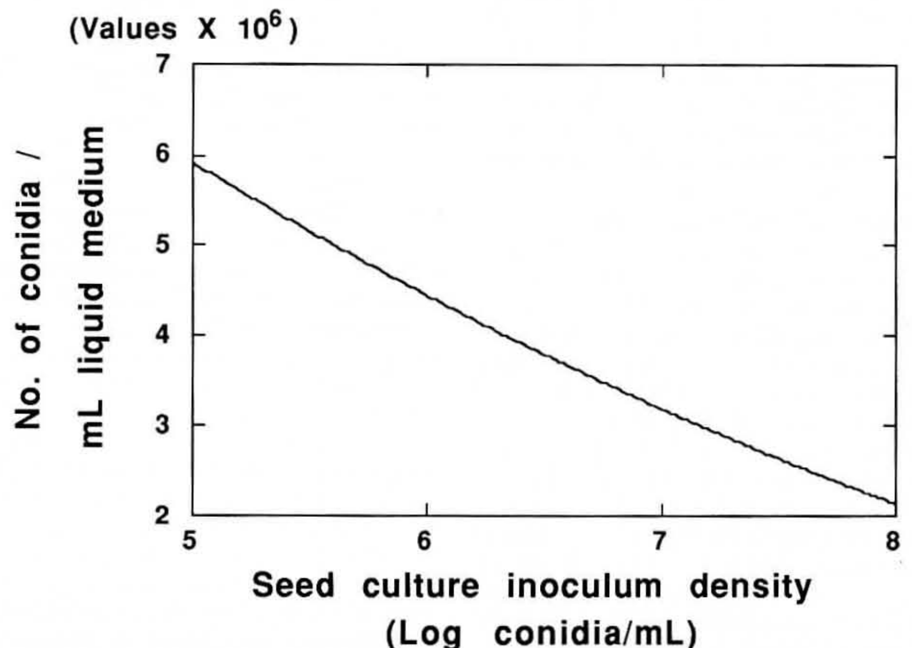


Figure 2. Effect of the density of the spore seed culture inoculum on the production of conidia by *Phomopsis convolvulus* in 100 mL of Modified Richard's (V-8®) liquid medium (After Morin *et al.* 1990).

The 'ideal' propagule

A good understanding of the biology of the potential mycoherbicide candidate and the target weed is a prerequisite to choosing the most appropriate fungal propagule to use in a biological control program. Knowledge of the pathogen's life cycle, the most susceptible growth stages of the weed and the most effective type of damage required to achieve acceptable weed control will assist in defining the favoured propagule. Spores, mycelial fragments, sclerotia and stromatic bodies have the potential to be produced and formulated to become mycoherbicides. The choice of the 'ideal' propagule, however, should be a compromise taking into account the biology of the host-pathogen system, the technical and economic aspects involved in the production, formulation and application of the product, and the durability, longevity and infectivity of the propagules.

The basic attributes of fungal spores as dispersal and infective units generally make them the most suitable propagules to be developed as mycoherbicide (Boyette *et al.* 1991). Stowell *et al.* (1989) classified mycoherbicide candidates in two groups based upon differences in the way they sporulate. Fungi that normally sporulate within the plant tissue (Coelomycetes; e.g., *Colletotrichum* sp.) are generally easily induced to sporulate in submerged fermentation while fungi that sporulate outside plant tissue (Hyphomycetes; e.g., *Alternaria* sp.) do not sporulate in liquid culture and require a solid gas interface to produce spores.

Table 3. Comparison of methods to produce conidia of *Phomopsis convolvulus* (After Morin *et al.* 1990).

Media	Production (conidia/plate or flask) × 10^9	Viability (% germination)[1]	Pathogenicity (Disease rating)[2]
Half-strength PDA plate	0.38	99.2 a[3]	3.8[4]
Pot barley grains	6.91	96.8 b	3.5
Modified Richard's solution	0.25	99.3 a	3.6

[1] Germination of conidia suspended in 0.01 % potato dextrose broth and placed on 1.5% water agar discs in a dark incubator at 24 °C for 24 h.

[2] Ratings: 0 = no visible symptoms; 1 = < 25% necrosis; 2 = 25–50% necrosis; 3 = 51–75 % necrosis; 4 = > 75 % necrosis

[3] Means followed by same letter in the same column are not significantly different (p>0.05) according to the LSD test.

[4] No significant difference among treatments at the 0.15 experiment-wise error rate, according to the Kruskal-Wallis one-way analysis of variance by ranks.

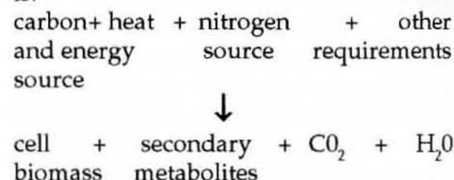
The Coelomycete fungi commonly produce sticky aerial conidia in solid-state fermentation and both blastospores and submerged conidia in submerged fermentation. The blastospores are thin-walled, single-celled hyphal bodies and the submerged conidia are similar to aerial conidia but differ in respect to their mode of production, either arising from mycelial filaments in the liquid culture or through microcycle conidiation (binary fission) instead of being produced in complex conidial fructifications (Thomas *et al.* 1987). Both types of spore produced under submerged liquid conditions are infectious, but are known to be more sensitive to environmental extremes, unstable, difficult to preserve and possibly less virulent (van Winkelhoff and McCoy 1984). Major differences in morphological characteristics and protein analysis have been reported between blastospores, aerial and submerged conidia of *B. bassiana* and possibly explained the fragile nature of spores produced in liquid culture (Hegedus *et al.* 1990). According to scanning electron micrographs of *B. bassiana*, blastospores have a smooth surface, aerial conidia have a rough surface and submerged conidia have an intermediate verrucose surface.

Fungi producing complex fruiting bodies (e.g., pycnidia) are generally believed to have an absolute requirement for a solid attachment to support the development of the sporulating structures and subsequent conidial production. This widespread belief, however, has little scientific basis due to lack of experimental evidence. Interestingly, *P. convolvulus* was observed to produce pycnidia and conidia when grown in complex liquid media (Table 2) (Morin *et al.* 1990). The germinability and pathogenicity of the conidia were similar to conidia produced on agar contained in petri dishes and on barley grains (Table 3). Therefore, the main macroscopic difference between the conidia of *P. convolvulus* produced on solid or liquid cultures was the presence of concentrated extracellular matrix around the conidia arising from solid-state fermentation. However, the extracellular conidial matrix which possibly acts in the protection of spores from desiccation (Nicholson and Moraes 1980) and contains cell wall degrading enzymes assisting in the initial stages of infection (McRae and Stevens 1990) may still be produced under submerged fermentation conditions. This hypothesis suggests the importance of keeping the liquid phase of the culture medium when harvesting the spores.

The production and formulation of mycelial fragments or pellets is usually considered when the mycoherbicide candidate is difficult to induce to sporulate in liquid culture or when it produces very large spores (> 100 μm) making field application difficult with typical spraying equipment (Stowell *et al.* 1989, Stowell 1991). For soilborne pathogens and wood-inhabiting saprophytic fungi, mycelial suspensions or granules often represent the best alternatives to be developed as mycoherbicide since the pathogen's mycelia can easily infect roots or wounded plant tissue. Scheepens and Hoogerbrugge (1989) have observed 90% mortality of *Prunus serotina* Erhr. tree stumps treated with a suspension of fragmented mycelium of *Chondrostereum purpureum* (Pers. ex Fr.) Pouzar. The viability of mycelial propagules, however, is short in comparison to spores (Latgé and Moletta 1988). Mycelia of insect or plant pathogens are commercially produced in submerged fermentation, stabilized with protectants, dryed and milled to the appropriate size (Pereira and Roberts 1990, Stowell 1991) or pelletized with sodium alginate gels and dried (Walker and Connick 1983). The dry mycelial product is applied to the field and sporulate under humid conditions. The newly formed spores can then infect the target pest. Alternatively, the morphology of the mycelium can be manipulated during submerged fermentation to produce small fragments or pellets which do not require milling and therefore may retain more viability (Stowell 1991).

The influence of growth medium

The design of a liquid medium or the selection of a solid substrate traditionally takes into consideration the availability and cost of the material, the ease of preparation and sterilization, the type of fermentation equipment and the total yield of the biological product obtained during fermentation. The medium should provide an energy source and essential nutrients for biomass synthesis. Large-scale fermentation plants normally use cheap sources of nutrients, for example: molasses, cereal grains, starch, lactose, sucrose, glucose as sources of carbon and urea, ammonium salts, nitrates, corn steep liquor or soybean meal as nitrogen sources (Stanbury and Whitaker 1984, Miller and Churchill 1986). Corbett (1980) emphasized that a comprehensive knowledge of the biochemical features of the microorganism is advantageous to design the most appropriate medium but he realized that this is rarely considered a priority. The stoichiometry equation characterizing the fermentation of microorganisms is:



(Stanbury and Whitaker 1984). Experiments using submerged fermentation techniques with chemically defined or partially defined media are necessary to identify the nutritional requirements of the fungus to achieve maximum biomass yield possessing the desired characteristics.

The composition and balance of the growth medium in combination with the physico-chemical conditions of the culture govern mycelial production and sporulation. Yield of conidia of *P. convolvulus* was greatly influenced by the type of substrate or liquid medium supporting growth (Tables 2, 4) (Morin *et al.* 1990). In liquid and solid fermentation experiments performed in flasks, maximum yield was reported with modified Richard's (V-8®) medium and pot barley grains. The differences in availability of nutrients, moisture content, surface area of the solid substrates and the presence of vitamins and oligo-minerals in the complex organic compounds (V-8® juice) added to the liquid medium were possibly responsible for the variable sporulation of *P. convolvulus* (Table 5). The carbon-to-nitrogen (C:N) ratio is also reported to have a marked impact on the yield of spores. Liquid media with a high

Table 4. Effect of various solid substrates on the number and viability of the conidia produced by *Phomopsis convolvulus* (After Morin *et al.* 1990).

Solid substrate	Production (conidia per g of product) × 10^8	Viability (% germination)
Pot barley grains	4.8 a[2]	95 ab[2]
Oat bran	1.5 b	89 a
Lentil seeds	1.5 b	59 de
Flax seeds	1.3 b	82 bc
Wheat bran	1.4 b	53 e
Field bindweed foliage	0.3 c	69 cd

[1] Germination of conidia suspended in water and placed on 1.5 % water agar discs in a dark incubator at 24 °C for 24 h.

[2] Means followed by the same letter in the same column are not significantly different ($p>0.05$) according to the LSD test.

Table 5. Effect of variations in the recipe of the Modified Richard's (V-8®) liquid medium on the production of conidia by *Phomopsis convolvulus* (After Morin *et al.* 1990).

Recipe status	Presence of V-8 juice	Carbon-to-nitrogen ratio (C:N)	Production (conidia/mL of medium) × 10^6
standard	yes	1:1	7.29 a[1]
modified	yes	1:5	0.00 b
modified	no	1:1	0.87 c

[1] Means followed by the same letters in the same column are not significantly different ($p>0.05$) according to the LSD test.

C:N ratio (5:1) stimulated conidiation of *B. bassiana* (Thomas *et al.* 1987) but inhibited conidial production by the potential bioherbicides *P. convolvulus* (Table 2) (Morin *et al.* 1990) and *Colletotrichum truncatum* (Schw.) Andrus and Moore (Jackson and Bothast 1990). The reduction in spore production by *C. truncatum* was associated with an increase in hyphal melanization as the carbon concentration was increased. Very low C:N ratio (1:5), however, completely inhibited sporulation of *P. convolvulus* (Table 5).

The most suitable medium for each fermentation process should trigger the production of the most stable and durable propagules. Inhibition of the production of submerged conidia by *B. bassiana* was reported in liquid media with high phosphate concentrations (Hegedus *et al.* 1990) or media containing peptone, neopeptone, tryptone or yeast extract (Thomas *et al.* 1987). Additionally, the latter media was found to enhance the production of blastospores.

Most studies in fermentation technology have been concerned with improvement of the equipment and the optimization of growth conditions to achieve maximum yield of the biological products at a competitive cost. Baker and Henis (1990), however, have suggested that the most effective way of reducing cost of microbial pesticides is to decrease the field application rate which is basically achieved by increasing the efficacy of the organisms in other words the stability, durability, germinability, pathogenicity and virulence of the propagules.

The nature and quantity of the nutrients in a growth medium can markedly affect the viability and quality of the fungal mycelia or spores. For example, the germinability of conidia of *P. convolvulus* grown on different solid substrates differed considerably (Table 4) (Morin *et al.* 1990). Different protein patterns of aerial conidia of *B. bassiana* produced on different agar media have been observed and may possibly affect their stability and infectivity in insects (Hegedus *et al.* 1990). Further studies have to be undertaken to investigate this hypothesis. McRae and Stevens (1990) reported the influence of growth media on the activity of host penetration enzymes contained in the conidial matrix and on the pathogenicity of *Colletotrichum orbiculare* (Berk. and Mont.) v. Arx. Similarly, variation in viability and efficacy of conidia of *C. truncatum* to cause disease in hemp sesbania (*Sesbania exaltata* L.) has recently been linked to the nutritional environment during submerged fermentation (Schisler *et al.* 1991). The pathogenicity of *Fusarium lateritium* Nees ex. Fr., a pathogen of the weed prickly sida (*Sida spinosa* L.), was profoundly affected by the primary carbon source (starch or glucose) of the submerged fermentation medium (Bannon *et al.* 1990). The presence of glucose in the medium stimulated high production of avirulent spores while the presence of starch resulted in low yield of highly virulent spores. No reference to the type of spores produced, though, was made in the paper. The catabolic repression of enzyme systems essential in pathogenesis is a known phenomenon occurring in other fungi growing in an environment rich in glucose (Stowell 1991).

The physiology of mycelial biomass can also be influenced by the composition of the medium. Mycelium of *B. bassiana* grown in the presence of yeast extract, dried and subsequently rehydrated was observed to produce a higher number of conidia than mycelium grown without yeast extract (Rombach *et al.* 1988). Growth medium can also affect the biochemical activity of fungi. In several submerged fermentation processes, the manipulation of the growth medium is a common method to trigger the production of specific secondary metabolites by fungal mycelia. A good demonstration is the increased production of citric acid in iron and copper deficient culture medium of *Aspergillus niger* Van Tieghem (Corbett 1980).

Conclusion

A multidisciplinary approach is required in mycoherbicide research. Mass-production of the potential fungal candidate is a crucial step in the development of a biological herbicide. Most fermentation technologies have been developed towards harvesting fungal metabolites or transforming raw materials into palatable and desirable products for human consumption. The requirement of infectious fungal propagules, however, is the main concern for biological control programs. Knowledge of the effects of fermentation processes on the physiology of fungi and how they may act upon pathogenesis and survival of the propagules is limited. The economical aspects inherent to the development of a commercial biological product are and will always be of great importance for industry. The enhancement of efficacy of mycoherbicide through manipulation of the fermentation processes represents a profitable avenue of further enquiry that will make this biotechnological innovation more accessible to society.

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