

*Phragmidium violaceum* has all spore forms, but *Puccinia xanthii* has only teliospores and basidiospores.

While some rusts do have a full life-cycle, all stages may not be present in the field. Only the uredinial and telial stages of *Puccinia abrupta* var. *partheniicola* have been found in the field in Mexico. While the teliospores are functional, germination has only been observed in the laboratory after dormancy had been broken by chemical treatment. The rust appears to cycle in the field by the urediniospore stage only. These spores have the ability to remain dormant over winter while retaining their viability.

### Pathogenicity testing

Pathogenicity tests are aimed at selecting the most effective strain of the pathogen for the biotype(s) of the target weed and the environmental conditions under which the weed grows. Specimens of the target weed, grown from seed collected from the target area, are inoculated under the target environmental conditions with the strains of the candidate pathogen that have been collected from different parts of its native range. Pathogenicity is then assessed from symptoms exhibited by the target weed and microscopic examination as for host specificity testing.

### Host specificity

As for insects (Heard this volume), preliminary information about the host specificity of a potential agent pathogen in its country of origin might be obtained by searching the literature, particularly crop protection literature, checking herbarium collections and records, and checking with workers in agriculture to determine whether the pathogen is a known pest of crops.

In the absence of other preliminary information, host range tests against a small selection of plants, usually closely related to the target weed, should be done in the country of origin. This will determine whether

further, more detailed, testing is warranted.

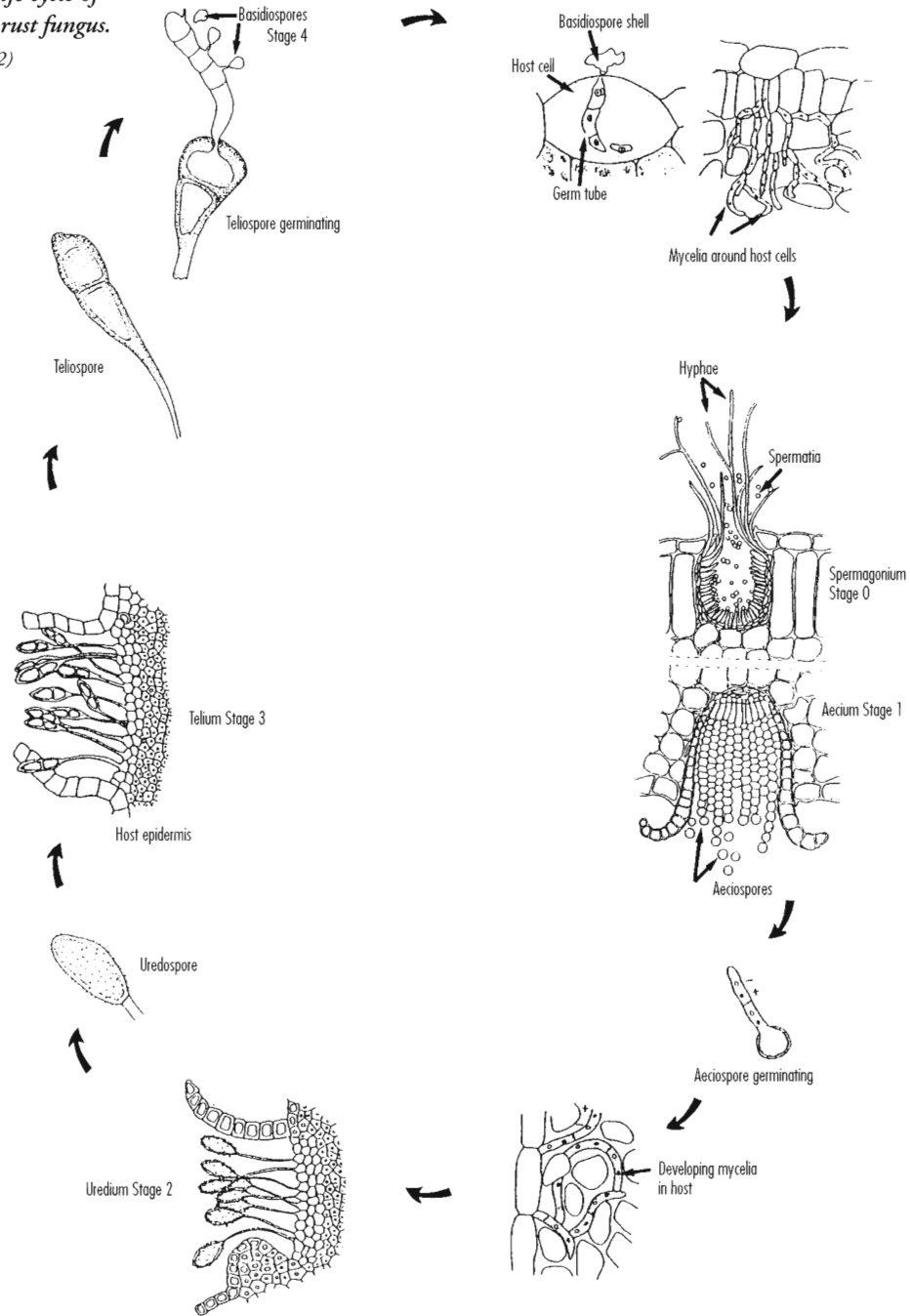
### Supply of test plants

Since testing for Australian weeds is carried out overseas, it is necessary to provide the laboratory carrying out the work with test plants. Where possible test plant seed is sent to the country where the testing is to be carried out. This is the easiest and cheapest method as there are usually fewer problems with quarantine requirements. However, in some cases it is necessary to grow the plants before despatch to the testing agency. In this case, the plants must be able to withstand reasonably rough handling, including being turned upside down, and exposure to extremes of temperature. A packaging method for sending plants overseas which has proven to be reliable involves sealing each plant in its own container which is lined with absorbent paper to prevent sweating and breakdown of the foliage in transit, and tightly packing the individual containers into a polystyrene box which is protected by an outer layer of strawboard.

### Methodology

Host specificity testing involves assessing the response to infection by the test plants at the cellular level. Plants are inoculated in batches of four or five species along with the target weed as a control. Inoculation of each test species should be replicated three or four times. Each replicate is valid only if infection of the control plant is normal. The plants should be inoculated and incubated under ideal conditions for the development of the particular pathogen, and maintained for twice the length of the latent period for the pathogen on its natural host to allow complete development, eg the production of urediniospores. Pathogen development should be observed both macroscopically and microscopically. In the latter case, sample sections of the leaves are examined using techniques such as whole leaf clearing and staining technique (Bruzzese and Hasan 1983), and scanning

Figure 1. Stages in the life cycle of  
*amacro-cyclic rust fungus.*  
 (Alexopoulos 1962)



electromicrographs. Processes examined include: the fate of the spores on the leaf surface, development of infection hyphae, appressoria, penetrant hyphae and haustoria and reaction of the test plant at both organ and cell level, for example, deposition of callous tissue, necrosis of cells to form a barrier, presence of polyphenols, chlorosis and leaf abnormalities (tumefactions).

The methods used for host specificity testing of a rust for rubber vine (H. Evans pers. comm. 1992) are summarised below as an example of appropriate methods for pathogens.

Batches of 4–5 test species were screened at any one time. Three to four plants were included for each species and only vigorously growing (flushing) plants were used. A range of leaf types (young to senescent) were inoculated by brushing their lower leaf surfaces with a dense urediniospore suspension ('saturation' inoculum, ca  $5 \times 10^6$  spores/mL) in sterile distilled water and 0.01% Tween 20, using a fine camel hair brush. In the case of aquatic plants (with stomata only on the upper leaf surface) and leafless, fleshy asclepiads, the sites of inoculation were modified accordingly.

Rubber vine plants (*C. grandiflora*, *C. madagascariensis* var. *madagascariensis* and var. *glaberrima*) inoculated with a spore concentration of  $1.5 \times 10^6$  to avoid defoliation, were used as controls for each test. Plants were maintained for 24 hours at previously determined optimum conditions for rust infection (ca. 23°C, 100% RH). Inoculated plants were then transferred to a greenhouse chamber at 25°C and 50–60% RH during 12-hours light, and 20°C and 60–80% RH during the night.

Plants were observed over a three-week period, i.e. more than double the latent period for sporulation of the rust on control rubber vine plants. Samples of all inoculated leaves were removed at 10 and 21 days for clearing and staining (Bruzzese and Hasan 1983).

Samples from fleshy leaves and stems were cut longitudinally to facilitate subsequent microscopic examination of the inoculated area. In addition to the light microscope examination, all inoculated material was inspected for external symptoms of infection using a stereoscopic microscope.

Each test species was screened on at least two separate occasions, and any plant showing an unusual macro- or micro-reaction to the rust was investigated further. Symptomatology was assessed according to 15 categories (Table 2) and the susceptibility to infection of each test species was rated based on systems devised by Kochman and Goulter (1983) and Bruzzese and Hasan (1983) (Table 3).

Assessment categories within any one test species were, for the most part, consistent. However, variable reactions occurred occasionally between replications, although most frequently within the same treatment. Leaf age was the main factor governing variation in susceptibility ratings and the full range of reactions are shown in Table 3. Weak or non-dominant reactions are represented by parentheses.

Results of the host range tests for plants within the subfamilies Periplocoideae and Secamonideae are shown in Table 4 as examples.

All species and varieties within the genus *Cryptostegia* were highly susceptible to infection by *Maravalia cryptostegiae*. The only other species exhibiting susceptibility to *M. cryptostegiae* were the Madagascan *Gonocrypta grevei* and the Australian *Cryptolepis grayi*. Further testing of *M. cryptostegiae* types against *G. grevei* indicated that there are at least two distinct physiological races or pathotypes of *M. cryptostegiae*, one adapted to *Cryptostegia*, the other to *Gonocrypta*. *Cryptolepis grayi* showed varying levels of susceptibility to the rust, ranging from resistant to moderately susceptible, and the appearance of fertile pustules on one plant of the first test run warranted further

Table 2. Assessment categories for macro-and microsymptoms of pathogen infection.

Category	Symptoms
0	spore lysis, low (<10%) or no germination
1	spore germination (>20%)
2	abnormal germ-tubes
3	abnormal appressorial development, invariably non-stomatal
4	normal appressorial development, invariably over stomata
5	collapsed appressoria, no penetration
6	penetrant hypha with or without evident substomatal vesicle
7	necrosis of penetrant hypha, heavy staining (polyphenol) around and beneath stomata
8	short internal hyphae only, no haustorial mother cells/haustoria
9	collapsed or necrosed internal hyphae, callose or polyphenols present
10	longer internal hyphae, haustorial mother cells and haustoria
11	hyphal collapse, host cell plasmolysis and/or callosed haustoria
12	extensive internal hyphal network, initiation of sorus formation
13	external symptoms; chlorosis or reddening; leaf abnormalities (tumefactions)
14	restricted sporulation (<1 pustule/cm <sup>2</sup> )
15	abundant sporulation (>15 pustules/cm <sup>2</sup> )

investigation. Fertile pustules were observed in 3 of 14 plants in further tests, despite development of host cell plasmolysis and haustorial inhibition which restricted development of sporogenous tissues by the rust. Results of the screening suggest that *Cryptolepis*, like *Gonocrypta*, is a generic host of *M. cryptostegiae*. Decisions based on these tests are discussed by McFadyen and Heard (this volume).

### Importation

Once approval to import the pathogen is given, it is transported in a double metal container. Under

normal circumstances, prior to release in the field, the pathogen is reared through one or more generations in quarantine, in the recipient country, to make sure that it is not contaminated with other fungi, particularly parasites, such as *Darluka* sp. In some circumstances, this step may be waived if the exporting organisation guarantees that the culture is pure.

### Mass Production of Inoculum

Large amounts of spores are produced, using a suitable culture technique, for release in the field. The technique may involve culture on the weed host or

**Table 3. Susceptibility ratings used for assessing the reactions of rubber vine and other test plants to the rubber vine rust**

Score	Rating	Macro/microsymptoms
0	Immune (I)	No visible symptoms; no stomatal penetration
1	Highly resistant (HR)	Visible symptoms: chlorosis, flecking or general discoloration; hypersensitive reaction at the stomatal or substomatal level
2	Highly resistant	Development of internal hyphae but restricted by production of callose or polyphenols
3	Highly resistant	Internal hyphae with more extensive branching producing haustorial mother cells but aborted at cellular level
4	Highly resistant	Development of hyphal network; haustoria abundant but invariably non-functional (collapsed or callose ring), with or without host cell plasmolysis. No visible symptoms
5	Resistant (R)	Hyphal network extensive; initiation of sori, non-eruptive or eruptive and appearing as swellings or blisters on leaf surface, abortive, no sporulation. Host cell plasmolysis and/or haustorial collapse. Macrosymptoms generally present: chlorotic spots
6	Resistant	Eruptive sori, usually small in size; sporulation restricted (few pustules/leaf) and delayed; evidence of mainly collapsed-callosed haustoria. Macrosymptoms generally present: widespread chlorosis, leaf distortion
7	Partially resistant (moderately susceptible) (PR)	As above, but pustules larger and more abundant but still less than 1/cm <sup>2</sup>
8	Highly susceptible (HS)	Numerous pustules (>15/cm <sup>2</sup> ), abundant sporulation; majority of haustoria healthy. Typically chlorotic then necrotic leaves; but premature leaf fall not evident
9	Highly susceptible	As above, but premature leaf fall common; with or without chlorosis or reddening (anthocyanin production)

use of artificial media (Auld this volume). Depending on circumstance these spores might be used in the fresh state or collected and stored for a period prior to release. Drying and refrigeration at 4°C or in liquid nitrogen is a suitable storage method.

### Field Release

For successful establishment in the field, the requirements of the particular pathogen with respect to temperature, humidity/dew period and other environmental factors must be considered. Infection of plants may not be achieved if environmental conditions at the time of release are not suitable.

Table 4. Symptoms and susceptibility ratings of test plants within the subfamilies Periplocoideae and Secamoneoideae

Taxa	Macro/micro symptoms															Ratings	
	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14		15
Subfamily Periplocoideae																	
<i>Cryptostegia grandiflora</i>	-	+	-	-	+	-	+	-	-	-	+	-	+	+	-	+	9 HS
<i>C. madagascariensis</i>																	
var. <i>madagascariensis</i>	-	+	-	-	+	-	+	-	-	-	+	-	+	+	-	+	9 HS
var. <i>glaberrima</i>	-	+	-	-	+	-	+	-	-	-	+	-	+	+	-	+	8 HS
var. <i>septentrionalis</i>	-	+	-	-	+	-	+	-	-	-	+	-	+	+	-	+	8 HS
<i>Gonocrypta grevei</i>	-	+	-	-	+	-	+	-	-	-	+	(+)	+	(+)	+	(+)	5,6,7,8 P, HS,HR
<i>Pentopetia androsaemifolia</i>	-	+	-	-	+	-	+	-	+	+	-	-	-	-	-	-	2 HR
<i>Gymnabthera fruticosa</i>	-	+	-	-	+	-	+	-	+	+	-	-	-	-	-	-	2 HR
<i>G. nitida</i>	-	+	-	-	+	-	+	-	+	+	-	-	-	-	-	-	2 HR
<i>Finlaysonia obovata</i>	-	+	-	(+)	+	-	+	-	-	-	+	+	(+)	(+)	-	-	5 R
<i>Cryptolepis grayi</i>	-	+	-	-	+	-	+	-	-	-	+	(+)	+	(+)	(+)	-	5,6,7 P,PR
<i>C. albicans</i>	-	+	-	-	+	-	+	-	+	+	(+)	(+)	(+)	(+)	-	-	4,4 HR,R
Subfamily Secamoneoideae																	
<i>Secamone elliptica</i>	-	+	-	-	+	-	+	+	-	-	-	-	-	-	-	-	2 HR

Note. Parenthesis indicate variable but replicable symptoms between individual plants in a test.

Spores may be released in the field as water- or oil-based suspensions, sprayed or brushed onto the leaves or stems, or in the dry form, diluted if necessary with a powder such as talc. Alternatively, infected potted plants may be set out in the field amongst the plants to be infected. For short-lived spores this latter method allows a bigger window of opportunity for new infections to occur, as fresh spores are produced constantly so increasing the chance of coinciding with suitable environmental conditions. Where dew is unlikely to form naturally to allow infection, it may be artificially induced by enclosing foliage in plastic bags at night.

### **Integration With Other Control Methods**

It is unlikely that the release of one biological control agent will provide acceptable control at all sites in all seasons. Further studies will be required if the agent becomes widely established to understand its interaction with other biological control agents already present and also how chemical, mechanical or ecological methods of control could best be used to enhance the overall controlling effect (Adkins this volume; Farrell and Lonsdale this volume).

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# Bioherbicides

## Introduction

The notion of using plant diseases to control troublesome plants is an appealing one. Fungi have been used in attempts to control weeds in various parts of the world since the 1950s. Initially researchers concentrated on native or naturalised fungi, spreading them on to target weeds by various means. Results were generally unpredictable, varying with time and location. One fungus to achieve success was the persimmon wilt fungus, *Acremonium diospyri*, which although not commercially available, has been used since 1960 to control persimmon trees in Oklahoma rangelands. The fungus is provided free to local landholders by a benevolent foundation. Suspensions of conidia provided in plastic 'squir' bottles are applied to wounds made in the trees with a hand axe (G.E. Templeton pers. comm. 1988).

A step forward was made in the Peoples Republic of China in 1963. A *forma specialis* of the fungus *Colletotrichum gloeosporioides* was developed by simple fermentation procedures into a product, 'Lu-bao No. 1', used to control the parasitic weed dodder (*Cuscuta* spp.), in soybeans. It was applied in inundative doses of spores to create an artificial and localised epidemic. Although the original strain of this fungus has been replaced, the daughter product 'Lu-bao No. 2', is still

used. It is applied as a liquid suspension of spores like a conventional herbicide (Y.H. Li pers. comm. 1987). This type of product has become known as a 'mycoherbicide' although the term bioherbicide is now generally used to cover the possibility of using other microorganisms.

In the 1970s a number of research labs in the USA concentrated on developing pathogens which were already present in that country. This led to the release of two commercial bioherbicides in the early 1980s. DeVine® was produced in a cooperative venture between the Florida Department of Agriculture and Consumer Services and Abbott Laboratories (Ridings 1986). DeVine® is a formulation of chlamydospores of *Phytophthora palmivora* used to control strangler vine (*Morrenia odorata*) a weed of citrus groves in Florida. Although mass production of chlamydospores by fermentation was possible, long shelf life for the product could not be achieved. DeVine® is handled like fresh milk through its distribution system; it has an expiry date of 6 weeks (Kenney 1986). It is feasible to distribute and market this relatively labile product because of a limited target area.

The second product, Collego™, was developed as collaborative effort between a group at the University of Arkansas led by Professor George Templeton, the US Department of Agriculture and the Upjohn Company. Collego® is a formulation of the pathogen *Colletotrichum gloeosporioides* f.sp. *aeschynomene* which is used to control northern jointvetch (*Aeschynomene virginica*), a weed of rice. Although production of spores by submerged fermentation and drying spores for shelf life was relatively easily achieved on a small

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scale for research purposes, improvements were required for commercial production. Research was required on stock culture and media, fermentation conditions, as well as fermentor impeller designs and baffling (Bowers 1986).

The development of Collego® and DeVine® stimulated widespread research interest in mycoherbicides. The result of this activity led to several potential new products. One of these was registered in Canada in 1992: BIOMAL™ is a bioherbicide for control of round-leaved mallow (*Malva pusilla*) in wheat (*Triticum aestivum*) and lentils (*Lens culinaris*) in Manitoba and Saskatchewan, Canada, and northern wheat-producing areas in the USA. It is a selected strain of *Colletotrichum gloeosporioides* f.sp. *malvae* applied in spore suspensions containing  $2 \times 10^9$  spores/L at the rate of  $3 \times 10^2$  L/ha. Control of round-leaved mallow has been achieved in field tests (Makowski and Mortensen 1989).

A bioherbicide for control of American blackcherry (*Prunus serotina*) in pine forests in The Netherlands has been developed with a strain of the fungal pathogen *Chondrostereum purpureum* (Scheepens 1980). Like the persimmon wilt disease, it requires wound inoculation to initiate the disease development. Weed trees are cut mechanically, and the cut surfaces of the stumps are painted or sprayed with mycelial fragments in agar suspensions in the same manner as the 'cut-stump' herbicidal control method. This idea is currently being investigated in woody weed control in British Columbian forests in Canada.

Another approach combining an insect biocontrol agent and a fungus has been used in an attempt to control the aquatic weed water hyacinth (*Eichhornia crassipes*) in Florida USA, by Professor Charudattan. Spores of the fungus *Cercospora rodmanii* are sprayed in small areas to create 'hot spots' of infection. Two previously imported weevils, which attack the inflated petioles of water hyacinth, spread the fungal spores as they move from plant to plant.

## Outline of a Bioherbicide Research Program

In our research at the Agricultural Research and Veterinary Centre, Orange, the current emphasis is the control of the widespread weed Bathurst burr (*Xanthium spinosum*) by the fungus, *Colletotrichum orbiculare* (Auld et al. 1988). The work has included discovering the most strongly pathogenic isolates, defining optimal conditions for disease development (McRae and Auld 1988; Auld et al. 1990a), fermentation and formulation research and field tests (Auld et al. 1990b). This research project illustrates the work that is typically involved in a bioherbicide project; the steps are summarised in Table 1.

Amongst the problems that may arise in a bioherbicide research program, two areas often provide difficulties. The first is mass production of fungi (Auld this volume). The other is the need to overcome or reduce a requirement for dew which many fungi have. A good deal of research examining formulation has gone into this area recently.

## Formulation of Bioherbicides and Bioinsecticides

Formulation has two main aims: to provide an economical and easily useable form of the active ingredient with long shelf life, and, if possible, to enhance the effectiveness of active ingredient. In many circumstances an aim will also be to minimise the quantity of water required.

An active ingredient may be applied in the dry state as dust or granules or as a liquid or in the presence of liquid. This may be as a wettable powder, in oil, as aqueous concentrate, or emulsifiable concentrate. These concentrates are diluted by the addition of water. Other techniques such as microencapsulation will not be dealt with here, although they may have scope in the application of biological control agents.

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**Table 1. Outline of a commercial bioherbicide research program**

1. Define important target weeds. Factors to consider:
    - Value of infested crop.
    - Availability of other control measures and their costs including external costs (Auld et al. 1987).
    - Special circumstances which favour the use of biological control, e.g., a vineweeder on the crop.
  2. Reduce list (1) to a workable number (three species or less).
  3. Survey the weed(s) for pathogens over its range and growth season.
  4. Carry out Koch's Postulates test with a small number of isolates of each pathogen.
  5. Test a range of media for suitability for spore production.
  6. Examine the effect of increasing inoculum concentration on the hosts.
  7. Identify promising pathogens.
  8. Undertake a literature search for (i) host range, (ii) anything else—e.g., suitable growth media.
  9. Reduce program to one weed and one or two pathogens.
  10. Screen a range of isolates for efficacy.
  11. Carry out a host range test with a small group of selected isolates.
  12. If any reaction in any useful plant species, test a range of cultivars.
  13. Define optimal environmental conditions for infection and disease development.
  14. Examine the possibility of mass production of fungus—e.g., submerged shake flask culture.
  15. Carry out preliminary field tests. Small-scale tests may be done earlier.
  16. Further laboratory experiments may be needed to help interpret field results.
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Given that a biological control fungus can be produced en masse and dried, it can be applied as dry material or formulated as a wettable powder. In both cases clays such as kaolin, silica gel or diatomaceous earths can be used as fillers or carriers. Dusts are prepared so that particle sizes are between 3 and 50  $\mu\text{m}$  in diameter; dusts are, however, vulnerable to wind. Granular formulations are less so, being mostly within the range of 0.3 to 1.0 mm diameter. Granules have been used for mycoherbicide applications using

sodium alginate pellets (Walker and Connick 1983) and a pasta like process (Connick et al. 1991). Wettable powders may contain dispersing or suspending agents as well as inert fillers and wetting agents. Sodium alginate at 0.2–0.5% of final volume, for instance, will help keep some spore/clay powders in suspension. Wettable powders have been the most common form of microbial formulation. They have advantages for storage and transport, as well as minimal interaction between spores and other

components. Moreover, given that most fungi used for plant and insect control require free water or very high humidity for infection, the provision of water at application is a logical tactic.

Because many plant pathogenic fungi have a requirement for free water (or dew period) for infection, recent efforts by many mycoherbicide researchers have been directed towards overcoming this dew requirement via formulation; in particular, formulating fungal spores within the aqueous phase of an invert emulsion in oil (or oil mixtures) (Quimby et al. 1989). Although the technique has been shown to overcome the need for dew in some fungi there are disadvantages with the method: the amount of oil required adds to the cost of the product, and application of the viscous material may be difficult. Air-assist nozzles (McWhorter et al. 1988) have been used to spray inert emulsions, however some recent formulations do not require special equipment (Yang et al. 1993).

The use of non-aqueous carriers such as oil-based suspensions has been investigated by some workers (Agudelo and Falcon 1983) and may show promise for low volume applications of the insect pathogen *B. bassiana* (Prior et al. 1988) and enhance some bioherbicides' performances (Boyette et al. 1991; Boyette 1994).

The sensitivity of spores to any ingredient will override other considerations and viability tests must be made continually as a formulation is developed. For instance, Soper and Ward (1981) report variation in the tolerance of the insect pathogen *Metarhizium anisopliae* to various kaolins.

Another approach to address the dew or free-water requirement of bioherbicides is to use them when rain or dew is expected or in association with irrigation. Unfortunately in many areas this may not be possible and conditions favourable for fungal growth may not

even occur at all when the weeds have to be killed. But in tropical areas where humidity is high, rainfall predictable and irrigation common, bioherbicides may have a very useful role.

## Application

Because of their small size, fungal spores can usually be applied in suspension with conventional equipment. Matthews (1983, 1985) has discussed some particular problems and requirements for spray application in the tropics and developing regions. Obviously equipment will need to be free of residues of fungicides and any other harmful chemicals. Notwithstanding this there may be scope for the addition of low concentrations of conventional herbicides to increase the efficacy of a fungus.

The range of techniques available for application therefore is as broad as those available for conventional pesticides and herbicides. These include high volume (about 1000 L/ha), medium (350 L/ha), low to very low volumes (3–150 L/ha), ultra low volumes (0.5–3 L/ha), controlled droplet application and electrostatic spraying. With medium and lower volumes, high powered fans are usually required; with ultra low volumes centrifugal energy may be employed (e.g. Symmons et al. 1989).

## International Bioherbicide Group

An International Bioherbicide Group (IBG) was formed in 1992. Anyone interested in doing research with bioherbicides can receive our newsletter, 'IBG News'. They should send their name, address and area of interest to me.

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# Mass Production of Fungi for Biopesticides

## Introduction

Mass production methods for fungi as biological control agents are the same whether the target is a weed, an insect pest or another disease. Therefore in this chapter I will discuss techniques that have been used for plant pathogenic fungi as well as for insect pathogens and other plant pathogens.

Production of many fungi for biological pest control may often be relatively easily achieved in quantities suitable for laboratory and glasshouse research and even small-scale field trials. However, if large-scale practical use is ultimately intended, mass production techniques for the fungus must be developed. The purpose of this chapter is to introduce methods of mass production which can be used and indicate where they can be adapted to a pilot scale in a non-specialised laboratory.

## Submerged Culture Fermentation

Many filamentous fungi sporulate in submerged culture (Vézina et al. 1965). In the industrialised world at least, liquid fermentation provides the simplest and most economical way to produce large numbers of fungal spores. This is because existing

equipment can be used without modification. The commercial biological control agents for weed control, Collego™ and Devine® are produced by this method (see Churchill 1982; Stowell 1991). Mycotol® for aphid control and Vertalec® for whitefly, comprising *Verticillium lecanii* as blastospores (Latge et al. 1986), and preparations of some strains of *Beauveria bassiana* for insect control (Thomas et al. 1987) also use this method.

Although a bioreactor (fermentor) would usually be used in industrial submerged culture, it may be possible to produce propagules in flask culture. Oxygen mass transfer is a major problem for aerobic processes as the solubility of oxygen in water is only about 6ppm. Oxygen transfer can be increased by minimising boundary layer resistance and maximising surface area for transfer. This is achieved by agitation.

The type of agitation may be important and the air/liquid ratio as well as the surface contact area may need to be high. Conical flasks of 50–2000 mL may be used. The medium being used is sterilised with the flask, relying on a porous closure, like cotton wool, to act as a depth filter. Flasks with side indentations to act as baffles will encourage greater turbulence and aeration. Reciprocating shakers are inferior to orbital shakers which can give high oxygen transfer rates because of the trailing of the liquid around the flask (Fig. 1) allowing greater area of liquid/air contact. Reciprocating shakers tend to splash the closure increasing the danger of contamination. Sophisticated shakers in which temperature and light are controlled may be necessary.

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Another method is to bubble compressed air into flasks to provide oxygen and (limited) agitation (e.g. see Papavizas et al. 1984). However a pilot fermentor allows greater oxygen input and control as well as control of temperature, pH, agitation and foaming. The speed and diameter of the impellor has a big influence on oxygen transfer. The diameter of the impellor should be about 1/3 the diameter of the reactor. Baffle plates assist turbulence, breaking up the liquid; they should be about 1/10 the diameter of the reactor. However they may be a nuisance with some filamentous fungi. Standard industrial bioreactors are from 1 000 L to 800 000 L. Smaller pilot bioreactors cannot mimic exactly what larger bioreactors will do. For instance there is a marked increase in shear at impellor tips but longer mixing times as volume increases. The minimum size of pilot bioreactors generally available is 1 litre.

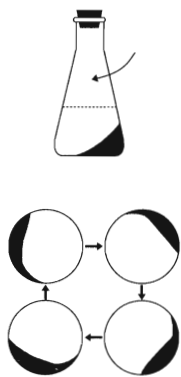


Figure 1. The movement of liquid (shaded) in an orbital shaker as seen from the side (above) and above (below) as the flask is moved by the shaker. This movement maximises the area of liquid/air contact for small volumes of liquid.

Bioreactor operation may be batch culture, semi-batch or continuous culture (e.g. see Trinci and Wiebe 1990). Batch culture is the simplest procedure with less likelihood of contamination. The output of a product such as fungal spores generally follows a sigmoidal curve with lag, exponential, stationary, and perhaps decline, phases (Fig. 2). The growth of product can be affected by substrate limitation and inhibition, product inhibition as well as temperature and pH.

If the production of a given fungus has a long lag phase in batch culture it may be worth investigating semi- (or fed) batch culture to shorten the lag period.

Possibly one of the greatest problems encountered with fermentation will be contamination. Sterilisation is a vital part of the process but the degree of 'sterility' required is a function of the end use.

Media for growth of the biological control organism should be as simple as possible utilising a standard set of inorganic salts and sources of carbon and nitrogen. Production of spores in the fermentor may be

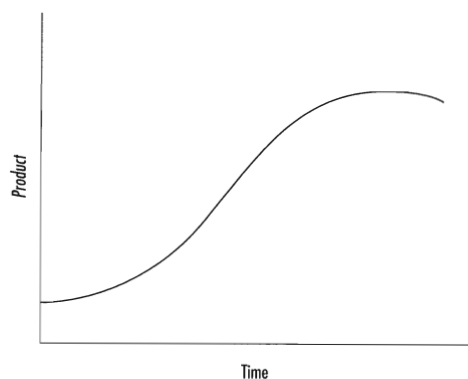


Figure 2. Typical production curve for a fermentation run with time. Note: lag, exponential and 'stationary' phases.

enhanced by changes in media components or simply by diluting the medium (Auld et al. 1990). Production may also be increased by beginning the fermentation process with increasing volume or concentration of starter culture. Latge and Moletta (1988) provide an extended treatment of the production of entomopathogenic fungi in submerged culture and Stowell (1991) gives examples of mycoherbicide production.

Within the bioreactor fungal growth may take the following physical forms: small discrete cells; small compact pellets; larger floccose pellets; or a filamentous form (Solomons 1980).

The method of reproduction in submerged culture may differ morphologically and physiologically from in vivo production. In the production of Collego™ only about 8–10% of the spores produced are normal conidia, most of them are fission spores (Churchill 1982).

Some fungi which do not sporulate in submerged culture may produce mycelium which can be dried (Pereira and Roberts 1990) and applied as fragments or pellets in the field; among them is *Metarhizium anisopliae* produced by the Bayer Company as BIO 1020®. Such fungi may sporulate following dew (Roberts and Wraight 1986; Robmach et al. 1986; Roberts et al. 1987). Walker and Connick (1983) describe the production of sodium alginate pellets of dried mycelium for a mycoherbicide.

It may be necessary to screen isolates for spore productivity under fermentation conditions as well as the virulence of the spores produced. The most virulent isolates may not be the most productive in vitro.

In some cases it may be more appropriate to produce survival propagules rather than infective propagules. Chlamydospores of *Trichoderma* and *Gliocladium* for

instance were more effective in field use than conidia (Lewis and Papavizas 1984; Papavizas et al. 1984).

Jackson et al. (1996) reviewed several years recent work on fermentation of the bioherbicide fungus, *Colletotrichum truncatum*.

## Solid Substrate Fermentation

Solid substrate fermentation has been widely used to produce fermented foods in China, Japan and South-east Asia (Wood and Yong Fook Min 1975). Media may contain some liquid; the 'solid' substrate itself may be relatively inert (eg. paper, wood, vermiculite), allowing for use of defined nutrient levels. The fungus *Sporidesmium sclerotivorum* used against the lettuce pathogen *Sclerotinia minor* is grown on vermiculite moistened with liquid medium (Adams and Ayres 1982). On the other hand some nutritive solid substrates may be available locally at low costs (eg. rice husks, coffee pulp, sorghum grain, straw, groundnut shells). Particle size, moisture content and temperature may all need to be controlled for successful production. Equipment used may be bags, trays or rotating drums.

Industrial submerged culture fermentation production requires considerable capital investment. Production on solid media may be relatively costly in terms of labour and materials in the western industrial environment but not necessarily so where labour is less costly and suitable raw material is freely available in developing countries.

*Beauveria bassiana* has been produced on solid substrates such as heat sterilised grains in Russia and the Peoples Republic of China. In the latter system 500 mL flasks of substrate are used to 'seed' 5 kg lots of steamed grain which is mixed with ten times the amount of wheat bran for fermentation in flat trays or in outdoor pits (Bartlett and Jaronski 1988). The

fungus is produced in liquid surface culture in large inflated plastic bags in Czechoslovakia (Kybal and Vilcek 1976; Samsinakova et al. 1981). Solid substrate fermentation is an alternative for fungi which will not sporulate in submerged culture. Goettel (1984) has also described a technique for producing fungi using cellophane sheets on bran in autoclave bags. Abbott Laboratories in the USA, have made commercial scale up tests for the fungus, using solid substrate to produce either a wettable powder or granular formulation (Bartlett and Jaronski 1988). R.J. Milner (pers. comm. 1991) has developed a solid substrate fermentation system for *M. anisopliae* using rice, inoculating with conidia and harvesting conidia by a washing technique (Australian Patent PK3451/90).

## Two-phase Systems

A two-phase system has been used for *B. bassiana* and *M. anisopliae* production in Russia, where mycelium produced in deep tank fermentation is allowed to sporulate in shallow open trays (Roberts and Yendol 1981; Goral and Lappa 1973). Walker and Riley (1982) described a similar preparation method for *Alternaria cassiae* for control of the weed *Cassia obtusifolia*. In Brazil *M. anisopliae* is produced on autoclaved rice or wheat bran in autoclavable plastic bags following inoculation with blastospores produced in liquid shake culture (Aquino et al. 1975, 1977).

## Recovery of Product and Storage

Recovery of spores from bioreactors may be a problem with filamentous fungi, requiring large centrifuges to spin off spores. Filtration methods often leave a large number of spores behind in the mycelial mass if the latter is formed during fermentation.

Following recovery of spores from a production process it is usually necessary to dry them for long-term storage. It is not always possible to do this and

retain viability of the organism. It is for this reason that the mycoherbicide Devine® is sold in liquid form like fresh milk. Drying should be done as quickly as possible under 'clean' conditions to prevent bacterial contamination. Inert materials such as diatomaceous earth, silica gel or clay may be suitable to hasten drying and to act as carriers.

## Production Capacity

In a non-specialised fermentation laboratory, the demonstration that sporulation of a given fungus occurs in submerged culture may be an adequate goal. Cooperation of fermentation specialists could then be sought. However, ultimately, the maximum production per unit volume of fermentation liquor must be established and this related, as bioreactor capacity (time × volume) to concentrations per unit volume required for field use.

Bartlett and Jaronski (1988) cite typical rates of *Beauveria* conidia per hectare are about  $1 \times 10^{14}$ ; yields for *Beauveria* conidia obtainable from liquid surface culture are  $1 \times 10^{14}$  m<sup>2</sup>, submerged liquid fermentation  $3 \times 10^{11}$  per litre and  $7 \times 10^{12}$  per kg for solid substrate fermentation. Thus if the current submerged liquid production technology were to be used, a fermentation capacity of over 300 litres is required for each hectare treated. Apart from research on maximising production per unit volume or area, improved formulation and application techniques may reduce the density of spores required in the field. Given that there will ultimately be physical limitations to the amount of spores that can be produced in a given area or volume, work on improving application may be required in parallel with production research.

## Developing Countries

As previously stated mass production by fermentation in large submerged culture bioreactors requires considerable capital investment and may not be appropriate in many developing countries. However, many fungi will grow and sporulate on simple solid substrates using basic equipment which could be operated by people with limited training. The need for mass production of fungi should not be seen as a barrier to their use as inundative biological control agents (mycoherbicides or bioherbicides and other biocides). Local mass production could take place with very limited facilities near the end-use field site. This would mean that, at any one location, production could be linked to local needs and would not require vast amounts of fungi to be produced for sending elsewhere.

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# Plant Propagation and Culture

## Introduction

Plants have five basic requirements for healthy growth: light, air, water, heat, and nutrition. Healthy growth will be sustained as long as these requirements approach optimal levels and the plants are free from pest animals and diseases. Most plants also require anchorage in some form of substrate. In most cases, this substrate also serves as the plant's primary source of moisture and nutrition.

A vast literature covers every detail of plant culture from the broadest to the most specific aspects. This paper provides guidelines for routine aspects of plant culture including problems that are likely to be encountered and their rectification. Topics covered are:

- specific requirements
- light
- air
- temperature
- moisture
- potting mixtures
- nutrients

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- containers
- hygiene
- propagation / growing areas
- propagation by seed
- vegetative propagation

For the most part, this paper refers to container-grown plants, although some information may be extrapolated to field culture.

## Specific Requirements

The first consideration in growing any plant species is to determine if the species has specific requirements. This may include questions such as:

- Is the plant a water lover?
- Is it tropical or temperate in its temperature needs?
- Has it evolved on highly fertile heavy soils or those that are very sandy and poor in nutrition?
- Is it a plant that grows in exposed sunny places or does it grow predominantly in the shade of other plants?

The answers to such questions will indicate the conditions that should be provided.

When growing the many plant species on a test list it is not practical to duplicate specific optimal requirements for each species. It is usually only