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Production and evaluation of *Beauveria bassiana* for control of white spruce cone and seed insects

W.H. Fogal, R.K. Mittal, and G.S. Thurston

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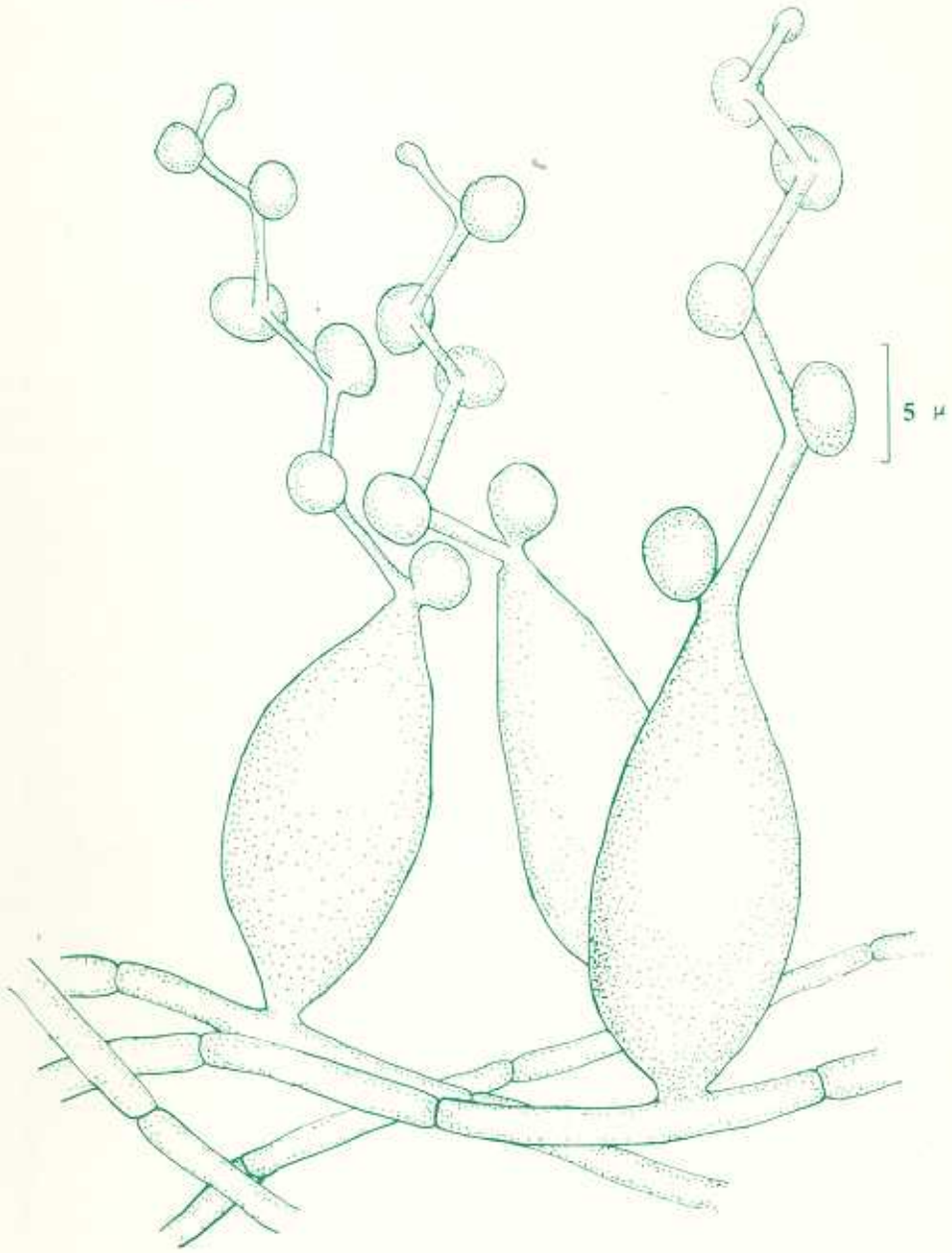
Petawawa National Forestry Institute



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COVER: *Beauveria bassiana*: aerial hyphae, sporogenous cells, and conidiospores. Scale denotes 5 microns.

PRODUCTION AND QUALITY EVALUATION OF *BEAUVERIA BASSIANA* FOR CONTROL OF WHITE SPRUCE CONE AND SEED INSECTS

Information Report PI-X-69

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Caution: Consult with local, provincial and federal health and pest control officials about regulations governing the production and use of biological insecticides.

ABSTRACT

This report provides a detailed description of a method for producing conidiospores of entomopathogenic fungi, exemplified by Beauveria bassiana (Bals.) Vuill., on a bran medium. Procedures are outlined for determining and maintaining viability of spores and ensuring that the product has a high degree of virulence against insect pests. Possible modes of application for use against white spruce Picea glauca (Moench) Voss cone and seed insects are suggested.

RÉSUMÉ

Ce rapport décrit en détail une méthode de production, sur du son, des conidies de champignons pathogènes pour les insectes, en l'occurence Beauvaria bassiana (Bals.) Vuill. Il énonce les modalités pour déterminer et maintenir la viabilité des spores et garantir au produit un degré élevé de virulence contre les insectes ravageurs. On y mentionne des possibilités d'utilisation contre les insectes s'attaquant aux cônes et aux semences de l'épinette blanche (Picea glauca [Moench] Voss).

PRODUCTION AND QUALITY EVALUATION OF BEAUVERIA BASSIANA FOR CONTROL OF WHITE SPRUCE CONE AND SEED INSECTS

INTRODUCTION

Cones and seeds of conifers, including white spruce Picea glauca (Moench) Voss, are attacked and destroyed by many species of insects (Hedlin et al. 1980, Sterner and Davidson 1983, Tripp and Hedlin 1956, Werner 1964). Control of white spruce insects is possible with systemic insecticides applied as foliar sprays (Hedlin 1973, Fogal and Lopushanski 1985) or stem injections and implants (Fogal and Lopushanski 1984). However, results have not been consistent, treatments may cause phytotoxic stress to trees, and chemicals are potentially hazardous to humans and wildlife. Such adverse environmental effects, and the development of resistance to chemical insecticides, are encouraging a search for microbiological agents to supplement chemicals for controlling insect pests on forest trees (Franz 1970, 1971). Insects are susceptible to infectious diseases caused by a variety of microorganisms including bacteria, viruses, fungi, and protozoa. Successful biological control of insects causing defoliation in trees has been achieved with bacteria and viruses, but the use of fungi and protozoans has been pursued less vigorously because they have not provided similar high levels of pest suppression (Coppell and Mertins 1977, Morris et al. 1986). Two species of fungi that show promise in biological control of insect pests are Beauveria bassiana (Bals.) Vuill. and Metarrhizium anisopliae (Metch.) Sor. (Coppell and Mertins 1977, Dunn and Mechalas 1963, Roberts 1973).

Insect-pathogenic fungi were selected as the potential biological control agent for white spruce cone and seed insects because significant control of larch fly Chortophila laricicola Karl and larch coneworm, Dioryctria abietella Schiff by B. bassiana and M. anisopliae had been demonstrated (Tyul'panova et al. 1975), and several insects that feed on cones and seeds of white spruce have also been shown to be susceptible to these two fungi (Timonin et al. 1980). Two of the most damaging insects, white spruce cone maggot Lasiomma anthracina (Czerny) and white spruce seedmoth Cydia youngana (Kearfott), overgrown with B. bassiana are shown in Fig. 1. These fungi have been found effective in reducing both field and laboratory populations of several pest insects (Appendix I). Other fungi might also be considered (Roberts and Humber 1981).

The success of field trials to demonstrate the use of fungi for control depends upon the susceptibility of insects to the fungus, pathogen virulence, environmental factors, and amount of inoculum applied. McCoy and Carver (1941) suggested that success rates might be improved by increasing spore loads and size of areas treated. This requires large and continuous supplies of inoculum with high virulence against the pest to be controlled. Large supplies can be obtained from commercial suppliers[1] but their quality and virulence against white spruce cone and seed insects are unknown. The purpose of this report is to provide a detailed description of a method for

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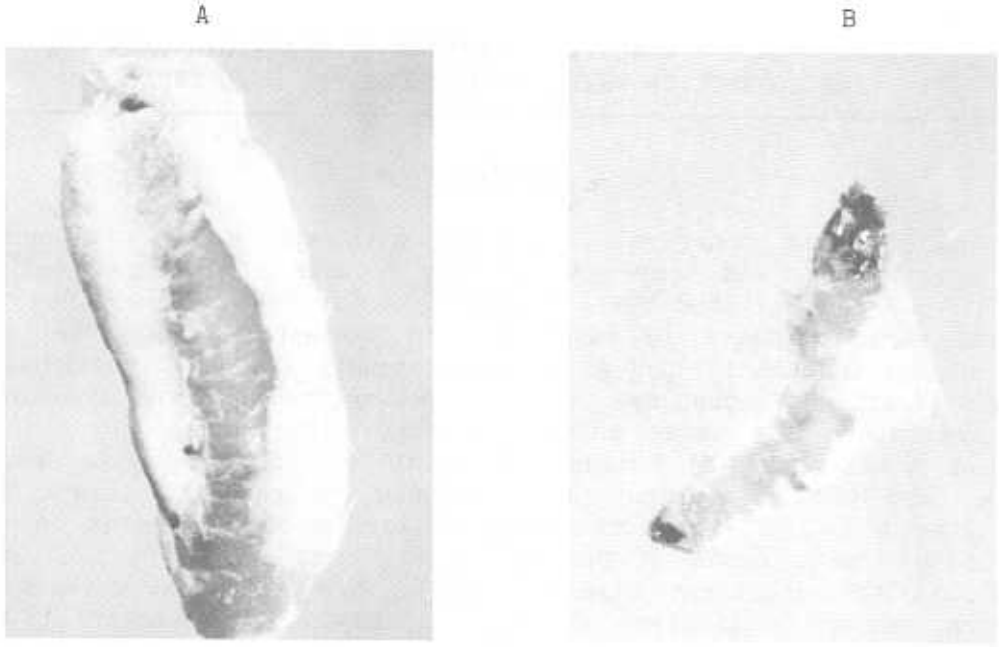

Figure 1. Larvae of A) the spruce cone maggot Lasiomma anthracina (Czerny) and B) the spruce seed moth Cydia youngana (Kearfott) overgrown with Beauveria bassiana (Bals.) Vuill.

producing conidiospores known to be effective against these insects, and to ensure that the product produced or obtained elsewhere has a high degree of virulence against pest insects under investigation. This report is intended to serve as a working guide and source of selected literature for the production of Beauveria bassiana (Moench) Voss.

PRODUCTION OF CONIDIOSPORES

Spores of insect-pathogenic fungi such as B. bassiana can be produced on solid or liquid media under sterile or semi-sterile conditions (Pristavko and Goral 1967, Roberts and Yendol 1971, Roberts and Humber 1981, Samsinakova et al. 1981). In liquid culture the fungus produces blastospores that are shorter-lived than the conidiospores produced on solid media (Pristavko and Goral 1967). Production of conidiospores on a solid medium is achieved by culturing on loose substrates such as bran, whole grains, potatoes, hay, straw, etc. in flasks, bottles, or trays.

The method of conidiospore production described here is adapted from McCoy and Carver (1941) and has been used to produce B. bassiana and M. anisopliae conidiospores for use in laboratory and field tests on white spruce seed and cone insects (Timonin et al. 1980, Fogal 1986, Fogal et al. 1986). A list of materials and equipment is given in Appendix I. For a general discussion of isolation, cultivation, and maintenance of conidial fungi, see Jong (1981) and Müller-Kögler (1967a).

a) Preparing the bran medium

Wash desired number (5-10) of Fernbach flasks with detergent and rinse several times with clear water. Add 150 g wheat bran and 150 mL water to each flask. Mix bran and water with a stiff wire loop or the handle of a large twisted-wire bottle brush, until the medium is damp throughout and contains no lumps. Stopper the flask with a snug-fitting cotton plug and autoclave at 121°C (103.5 KPa) for 30 min. Remove and incubate the flask at 28°C for 24 h, and then autoclave again at 121°C (103.5 KPa) for 30 min. The heat of the first autoclaving may not be sufficient to kill all heat-resistant and dormant fungal and bacterial spores, but the 24 h incubation under ideal conditions (warm, moist) usually activates surviving microbes, leaving them susceptible to the second autoclaving. When the contents of the flask cool to room temperature, the medium is ready for inoculation.

b) Preparing the inoculum

Pure cultures of B. bassiana or M. anisopliae are required for inoculation. Such cultures can be obtained from other researchers but, in the current state of development of biological control with fungi, it is best to isolate strains that are known to be highly pathogenic to the insect pest in question (Fargues and Robert 1978, Timonin et al. 1980). This may require isolation from an insect killed and overgrown with the fungus, or from soil or plant materials habituated by the pest insects. Such sources are contaminated with bacteria and various other fungi so it is necessary to use a selective culture medium that acts against bacterial growth and minimizes the development of other fungi. An effective culture medium for this purpose was developed by Veen and Ferron (1966) and modified to provide better growth by Doberski and Tribe (1980). A recipe for preparing this medium is given in Appendix III.

Samples of mummified insects, soil, or other potential sources of the fungus are crushed and mixed in a slurry or washed with sterile water containing 0.01 per cent Tween 80. Slurries or washings are diluted with an appropriate volume of water, and 1 mL aliquots are spread over plates of selective medium. Plates are incubated at 27°C and colonies identified after 3-5 days growth.

On this selective medium B. bassiana colonies display good growth of white aerial mycelia that are distinct against the violet background. Viewed from the bottom of the plates, colonies are dark violet in color, due perhaps to selective uptake of dye from the medium. Under the high power microscope, conidiospores are one-celled, hyaline, round to ovoid, approximately 2-4μ in diameter, and borne singly on small sterigmata. For taxonomic characters that distinguish B. bassiana from possible contaminants see MacLeod (1954). For other genera of hyphomycetes see Barron (1968), Kendrick (1971), or Kendrick and Carmichael (1973).

Pure cultures are obtained by isolating one colony from a mixed plate. Carefully remove the desired colony under a sterile hood and streak it onto another agar plate. At this stage, standard potato-dextrose agar (PDA) (Johnston and Booth 1983) can be used. After several days growth, when the resultant colonies are identifiable, this process may have to be repeated if

contaminants are present. When no contaminants are present and a petri dish contains numerous sporulating colonies, it is possible to prepare the inoculating liquid. Colonies growing on PDA are shown in Figure 2a. Sporulating colonies in petri dishes can be kept in the refrigerator (4°C) for several months without complete loss of conidiospore viability.

Because the conidiospores of B. bassiana are hydrophobic, an emulsifier must be added to the inoculating liquid. To a 1 L Erlenmeyer flask, add 500 mL tap water and 4-5 drops of Tween 80, shake the flask well, and add 100 mL to each of the required number (5-10) of screw-cap dilution bottles. Leave caps loose and autoclave bottles at 121°C (103.5 KPa) for 20 minutes. Tighten caps immediately after removal from the autoclave. Once the bottles are cooled to room temperature, the conidiospores may be suspended in liquid. Using a large (10 mm), flamed, and cooled inoculating loop in the sterile transfer hood, scrape the sporulating culture off one agar plate and transfer it to one bottle of cool sterile liquid; repeat this for other dilution bottles until most of the sporulating fungus is removed from the plate. Flame the mouth of each bottle, cap it, and shake vigorously for two minutes to suspend conidiospores evenly.

c) Inoculating the bran medium

Using a sterile pipette add 10 mL of the suspension to each flask of bran. When carrying out this procedure do not set the cotton plug down. Hold it in one hand while adding the suspension and make sure the plug is fitted snugly once again. Shake the medium well to disperse the inoculum.

Any suspension that is left after this procedure may be stored in the refrigerator for up to 2 days and used to inoculate more culture plates for making conidiospore suspensions for more flasks.

d) Incubation

The flasks of inoculated bran are incubated at a constant temperature of approximately 28°C. Flasks should be shaken once every day to prevent excess aggregation. Some aggregation is permissible, but if there is too much, conidiospore yields will be reduced because of the smaller surface area on which sporulation can occur.

e) Harvesting

The fungus is ready to be harvested after 6-10 days when the bran is completely overgrown with white mycelium and conidiospores adhere to the walls of the flask when shaken. To enhance conidiospore production and release, the culture must be dried. Drying of cultures to obtain good sporulation should be done quickly after good mycelial growth is observed. This prevents a decrease in the percentage germination of resultant conidiospores (Müller-Kögler 1967a). Drying is achieved by spreading the contents of the flasks on paper towelling in metal or glass trays and breaking large lumps. When the medium is evenly spread, it should be covered with more paper towelling and the trays placed in a well-ventilated drying oven at 35°C until the bran-fungus mixture is dry (generally for 3 days).

When the mixture is dry enough to crumble very easily between the fingers, conidiospores may be separated from bran and fungal mycelia by sieving. Soil sieves with openings of 1.00, 0.50, and 0.044 mm mounted on a reciprocating shaker give a good separation. When sieving and handling the conidiospores, always wear a dust mask, gloves, and a lab coat or coveralls as a precaution against allergic reactions to the dust.

In our experience, 150 g of bran yields 6-12 g of conidiospore preparation, and the preparation contains approximately 7.5-9.5 x 10^7 conidiospores/mg (Timonin et al. 1980).

f) Storage

Conidiospores of B. bassiana should be stored at as low a relative humidity as possible, in the dark and at 4°C (Müller-Kögler 1967a). Under these conditions they remain viable for several years. Clerk and Madelin (1965) found that light and moisture have a detrimental effect on the longevity of conidiospores; 90% of B. bassiana conidiospores remained viable after 635 days when stored at 8°C under conditions of dryness and darkness. Kral and Neubauer (1956) stored conidiospores in flasks with ground glass stoppers at room temperature (18-22°C) and 65-75% relative humidity. Germination capacity was not reduced after 560 days storage but the speed of germination was reduced for the alder spores. To avoid loss in viability it is advisable to use conidiospore preparations within one year of harvest.

g) Safety Precautions

It is generally conceded that insect pathogenic fungi do not cause mycoses of humans or other homeotherms (Roberts and Humber 1981). However, contact with conidiospore dust has been reported to cause allergies (Hall 1954, Heimpel 1971, Müller-Kögler 1967a, York 1958). Symptoms include headaches, aches in the extremities, chills, then fever and heavy perspiration. Containment type air hoods should be used when working with conidiospores of this and other fungi. Dust masks, rubber gloves, and long-sleeved lab coats should be worn to further reduce the possibility of exposure.

QUALITY EVALUATION OF CONIDIOSPORES

The quality of the conidiospore preparation is determined by several factors. These include: number of conidiospores in relation to amount of mycelial material and bran fines in the product, viability of conidiospores, and virulence of the isolate being cultured.

a) Number of conidiospores

The number of conidiospores in a preparation is determined microscopically with a haemacytometer. Conidiospores often form aggregates which must be dispersed for counting. One drop of Tween 80 is shaken well in 50 mL of distilled water. To this, 50 mg of conidiospore preparation is added and shaken or homogenized for 15 minutes to disperse conidiospores. Conidiospores are stained to facilitate counting by mixing equal quantitites of suspension and 0.1% cotton blue in lactophenol (Figure 2a). At least three suspensions

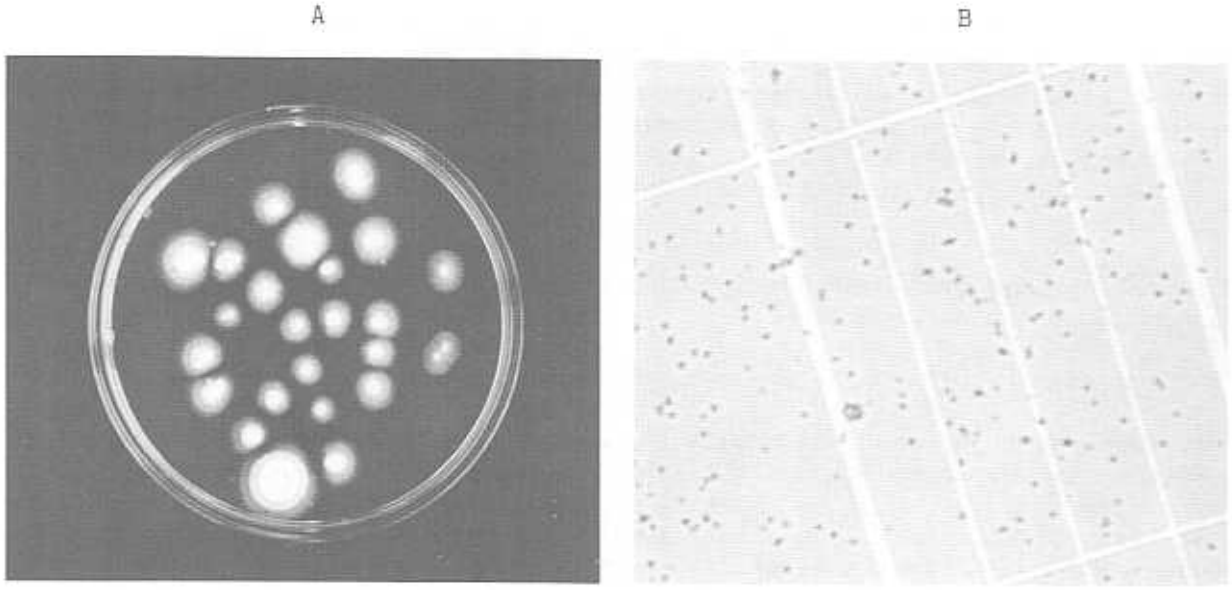

Figure 2. A) Colonies of Beauveria bassiana (Bals.) Vuill. growing on a plate of Potato-Dextrose-Agar (PDA) and B) conidiospores in a counting chamber.

should be made from each preparation with four separate counts of conidiospores in each suspension.

b) Number of viable conidiospores

A count of the number of viable conidiospores in the harvested product is obtained by plating serial dilutions on PDA plates. To break up conidiospore and conidiospore-mycelium aggregates the preparation is thoroughly mixed and repeatedly sieved with sterilized wheat flour. The rate of 100 mg of conidiospore preparation to 50 g of wheat flour gives satisfactory results. Weigh out 100 mg of the conidiospore-flour mixture and add to a dilution flask containing 100 mL sterile water with two drops of Tween 80. Shake the flask well for 5 min to minimize conidiospore aggregations and to distribute conidiospores evenly. Pipette 10 mL of suspension into another dilution flask containing 90 mL water plus one drop of Tween 80 and shake well for 2 min to disperse the conidiospores. Repeat this dilution process several times to determine the desired dilution factor by trial and error. In our laboratory 1/10 to 1/1000 dilution of the suspension yielded satisfactory results. From each dilution accurately pipette 0.1, 0.2, and 0.3 mL onto PDA plates and spread the drop with the edge of a sterile glass microscope slide. After 72 hours, colonies of the fungus should be visible on the plates; contaminants may be present, so, before counting, make sure the colonies are, in fact, those of B. bassiana. It is assumed that each colony represents an individual conidiospore, so by simply counting the number of colonies per plate and calculating back through the series of dilutions, the numbers of viable conidiospores per milligram can be obtained. Plates containing 10-50 separate colonies give the most accurate results. At least five plates for each dilution should be counted (Figure 2b).

c) Pathogenicity

Pathogenicity is determined by the virulence of the isolate used and by the susceptibility of the insect. Different strains of most fungi may display different growth rate, form, and virulence against a particular insect. For example Doberski (1981) found that the time required to cause 50% mortality of Scolytus scolytus F. larvae ranged from 5.6 to 10.3 days for different strains of B. bassiana. Using one strain of M. anisopliae, Zaccharuk and Tinline (1968) reported 2 to 100% mortality on four different species of elaterid wireworms. This difference in virulence among strains of fungi and the variations in susceptibility among insect species could mean the difference between success and failure of applications of the fungus. Therefore, if possible, the strain of fungus with the greatest virulence to a particular target insect should be selected for use. If the strain on hand is the only one available its virulence may be increased by growing it on the insect pest for several generations. Fargues and Robert (1978) found that the simple action of growing the fungus on a pest species increased the virulence of the fungus to that insect species each time the fungus was "passed through" the insect. Since then Timonin et al. (1980) have shown this effect on cone and seed insects. No explanation for this increased virulence has yet been found. Virulence of the strain can diminish with successive subculturing on artificial media and can be increased again by passing it through an appropriate insect host (Roberts and Yendol 1971). This plasticity suggests that a pathogenicity test against the insect or insects to be controlled should be a standard requirement before large scale experiments or control operations are undertaken, so that doses or rates of application can be adjusted accordingly.

If possible, all batches of spores produced should be tested for pathogenicity using the target insect for the assay. However, this is often impractical or impossible, as some insects are very difficult to rear in the laboratory, and others are available only at certain times of the year. A related insect that can be cultured may serve as a potential test species. A simple assessment of pathogenicity can be done by dipping the insect into a spore suspension of known concentration or dusting it with dry spores by either rolling it in a shallow dish of spores or "painting" it with a spore-laden camel-hair brush. The insect should then be placed in a chamber with high humidity such as on a moist filter paper in a petri dish. Mortality is assessed and attributed to the fungus or to other causes at daily intervals. The dose applied to the insects can be varied by diluting the spore suspension with known amounts of water or by mixing dry spores with known volumes of an inert dust such as talc or flour. For insects that are pests of stored food products, spores may be mixed, in different concentrations, with food of the insect (Steinhaus and Bell 1953); tests on soil-inhabiting insects can be done by mixing spores with soil (Zacharuk and Tinline 1968, Fogal 1986). Insects feeding on leaves of potted plants can be sprayed with suspensions of conidiospores in a spray tower (Burgerjon 1956).

These methods can be used to obtain a concentration of fungal spores necessary for mortality of a certain per cent of test insects (LD50 and/or LD90) or time for mortality of a certain per cent at one dose (LT50 and/or LT90). Accurate assessment of LD50s or LT50s requires application and use of appropriate probit analyses (Finney 1971). For large amounts of data, a computer program is available (Robertson et al. 1980).

POSSIBILITIES FOR CONTROL OF CONE AND SEED INSECTS

There are basically two methods for using microbial agents to control pest insects. One involves the introduction of an exotic control agent, with the expectation that it will become established, multiply, and provide adequate control. This has been successfully applied against insects that have been introduced to a location and become pests because no natural enemies existed in the new habitat. A classic example is the control of European spruce sawfly Gilpinia hercyniae Htg. in eastern Canada with a nuclear polyhedrosis virus (Neilson and Morris 1964). The second method involves inundation, and is essentially the application of a microbial preparation like a chemical pesticide. Large quantities of a microbial agent are introduced into an area where it may already occur at levels insufficient to provide control. The latter method is applicable for control of spruce cone and seed insects with B. bassiana.

Based on current knowledge of the life histories of spruce cone and seed insects (Hedlin et al. 1980, Tripp and Hedlin 1956), there are several methods and times for conidia application. These include, for example, spraying or dusting tree crowns with conidiospores before flowering, so as to infect young Eastern spruce budworm Choristoneura fumiferana (Clem.) and spruce coneworm Dioryctria reniculelloides Mutuura and Munro larvae before they start feeding on flowers. Another method is to dust soil and litter around the base of trees to infect adults of insects such as the spruce cone maggot Lasiomma anthracina (Czerny) as they emerge from the soil, and the spruce seed moth Cydia youngana (Kearfott) as they emerge from cones in the litter at flowering time. Spraying or dusting of strobili in tree crowns when adults of several insects are ovipositing on conelets can be effective. Tree crowns can be sprayed and dusted in early summer to infect later stages of the budworm and coneworm. Dusting soil in early summer to infect cone maggot larvae as they emerge from cones and drop to the litter for overwintering is another method.

Two of these possibilities have been tested in the laboratory and in small-scale field tests. Strobili of white spruce trees were dusted with conidiospores just after pollination when insects such as the cone maggot and seedmoth are laying eggs (Figure 3a). A 55% increase in the number of sound seed was obtained, suggesting that insects were killed by the fungus and that such treatments may prevent seed losses (Fogal et al. 1986). However, climatic factors such as rainfall and sunlight may influence the infection process and viability of conidiospores. The second test involved dusting soil with a conidiospore preparation containing 8.5×10^{10} conidiospores/g for control of the spruce cone maggot. Laboratory and simulated field studies (Figure 3b) have indicated that a mixture of these conidiospores and wheat flour or talc, applied and mixed with litter and humus at a rate of 3.5 to 7.5 kg conidiospores/ha, significantly reduced numbers of maggots in the soil (up to 42% mortality) depending on soil moisture content (Fogal 1986). Similar experiments for control of larch cone maggot and coneworm using 20 kg conidiospores/ha of a preparation containing 1.8×10^{6} conidiospores/g provided 48% mortality (Tyul'panova et al. 1975). It may be possible to improve efficacy of the treatments by increasing application rates or increasing virulence and pathogenicity of the spore preparation.

The method described here is capable of producing sufficient conidiospores of desired quality for laboratory and small-scale field testing

A B

Figure 3. A) Dusting strobili of white spruce, Picea glauca (Moench) Voss with conidiospores of Beauveria bassiana (Bals.) Vuill. for protecting seeds, and B) soil bioassay boxes for testing efficacy of B. bassiana against the spruce cone maggot, (Lasiomma anthracina (Czerny).

against cone and seed insects of white and black spruce and other tree species. For larger-scale field trials, larger quantities of conidiospores can be obtained commercially but their effectiveness against cone and seed insects is unknown. The quality of such spore preparations can be determined by following procedures and guidelines presented in this report. Rates of application can then be adjusted by comparison with conidiospore preparations of known quality.

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APPENDIX I

Some insects that have been significantly controlled in laboratory and field experiments with *Beauveria bassiana* (Bals.) Vuill. and/or *Metarrhizium anisopliae* (Metch.) Sor.

Order	Family	Scientific name	Common name	References
Hemiptera	Miridae	*Lygus hesperus* Knight	western plant bug	Dunn and Mechalas 1963
Coleoptera	Chrysomelidae	*Leptinotarsa decemlineata* (Say)	Colorado potato beetle	Clark et al. 1982 Kral and Neubauer 1956 Timonin 1939
	Curculionidae	*Chalcodermus aeneus* Boheman	cowpea curculio	Bell and Hamalle 1970
		Curculio caryae (Horn)	pecan weevil	Champlin et al. 1981
		Hylobius pales (Herbst)	pales weevil	Walstad and Anderson 1971
		Sitona lineatus (Linnaeus)	pea leaf weevil	Müller-Kögler 1967a
	Elateridae	*Ctenicera aeripennis* (Kirby)	wireworms	Zacharuk and Tinline 1968
		C. destructor (Brown)	"	" "
		Hypolithus abbreviatus (Say)	"	" "
		H. bicolor (Eschscholtz)	"	" "
		Limonius californicus (Mannerheim)	"	" "
	Scarabaeidae	*Oryctes nasicornis* Linnaeus	european rhinoceros beetle	Müller-Kögler 1967
		Oryctes rhinoceros Linnaeus	coconut rhinoceros beetle	Latch and Falloon 1967
Lepidoptera	Pyralidae	*Dioryctria abietella* Schiff	larch coneworm	Tyul'panova et al. 1975
		Ostrinia nubilalis (Hubner)	european corn borer	Bartlett and LeFebvre 1934, York 1958
Hymenoptera	Tenthredinidae	*Pristiphora erichsonii* (Hartig)	larch sawfly	MacLeod and Heimpel 1955
Diptera	Anthomyiidae	*Chortophila laricicola* Karl.	larch fly	Tyul'panova et al. 1975
		Lasiomma anthracina (Czerny)	spruce cone maggot	Fogal 1986

APPENDIX II

Materials and equipment required for producing conidiospores of Beauveria bassiana on bran medium under sterile conditions.

1. autoclave
2. incubator
3. sterile hood
4. drying oven
5. refrigerator
6. 2800 ml Fernbach flasks
7. sieves (1.00, 0.500, 0.044 mm openings)
8. receiving pan for sieves
9. reciprocating sieve shaker
10. sterile petri dishes (9 cm dia.)
11. screw cap dilution bottles (150 ml)
12. metal or glass trays (25 x 41 cm is a good size)
13. alcohol or bunsen burner
14. inoculating loop
15. stiff wire loop (long twisted-wire bottle brush)
16. sterile pipettes (1 ml in 1/100 ml; 10 ml in 1/10 ml)
17. non-absorbent cotton
18. potato-dextrose-agar (PDA) culture media
19. wheat bran from untreated wheat
20. Tween 80
21. low power (50 - 100X) dissecting microscope
22. high power (150 - 1500X) microscope
23. microscope slides and covers
24. haemacytometer
25. distilled water

APPENDIX III

Recipe for selective isolation medium for Beauveria bassiana and Metarrhizium anisopliae.

Mix the following ingredients and autoclave at 121°C (103.5 kPa) for 20 minutes.

glucose	40 g
neopeptone (Difco)	10 g
agar	15 g
crystal violet	0.01 g
chloramphenicol	0.5 g
distilled water	up to 975 ml

A solution of cycloheximide (10 mg/ml) is autoclaved separately and 25 ml is added to the rest of the medium when both are cool but before the agar forms a gel.