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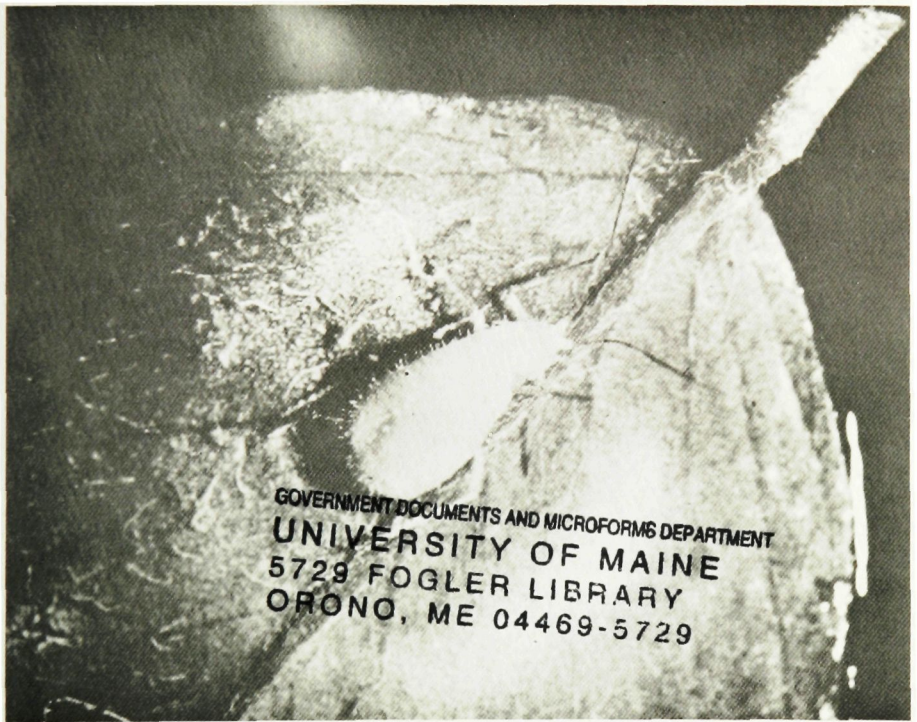
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# **PRODUCTION OF ENTOMOPHTHORA RESTING SPORES FOR BIOLOGICAL CONTROL OF APHIDS**

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Cover Photo: A potato infesting aphid infected with *Entomophthora* nr. *thaxteriana*; fungus has developed conidiophores and produced conidia. These can be seen as a white halo surrounding the diseased aphid.

PRODUCTION OF ENTOMOPHTHORA RESTING SPORES FOR  
BIOLOGICAL CONTROL OF APHIDS<sup>1</sup>

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INTRODUCTION

Many workers have agreed that because *Entomophthora* species are known to cause large scale epizootics and produce resting spores which might be stored for long periods, these fungi are excellent candidates for biological control.

Attempts to control insects have been made in the past by distributing vegetative cultures of *Entomophthora* (e.g. Hall and Dunn, 1958; Remaudiere and Michel, 1971). This method has not been very successful and is impractical for large-scale applications since it does not allow for accumulation or long term storage of material.

The practical utilization of *Entomophthora* species as biological control agents has been hampered by the inability to produce large quantities of resting spores and induce their germination. Resting spore production in artificial media and subsequent germination have been the problem.

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Other workers in the past have reported germination of resting spores, (Nowakowski, 1884; Dustan, 1924; Gilliatt, 1925; Schweizer, 1947; Hall and Halfhill, 1959; and Krejzova, 1968; 1971) but none of these researchers has been able to obtain germination levels greater than 15%. Recently Tzinovskii and Egina (1971) in Latvia have reported using *E. thaxteriana* (Petch) Hall et Bell resting spores for the control of spider mites, *Tetranychus urticae* Koch and *T. cinnabarinus* (Boisduval) and the melon aphid, "*Aphis frangulae* Kaltenbach". Krejzova (1972; 1973) was able to infect insects utilizing *Entomophthora* resting spores although she obtained higher incidence of disease with conidia. During the course of our experimentation with *E. nr. thaxteriana*<sup>4</sup> we have developed techniques for the mass production of resting spores with upward of 98% germination (Majchrowicz and Soper, 1973).

#### MATERIALS AND METHODS

Cultures of *Entomophthora* were obtained from diseased aphids collected from potato plants in northern Maine. The most frequently encountered pathogen was *E. nr. thaxteriana* from the green peach aphid, *Myzus persicae* (Sulzer) and the potato aphid, *Macrosiphon euphorbiae* (Thomas) (Soper and Bryan, 1974; Soper et al., 1974). Initial isolation was made on egg yolk media as described by Müller-Kögler (1959) except that 30 ml of molten Sabouraud maltose agar (SMA) were added to each 100 ml of egg yolk. Insects were collected before eruption of the fungus through the cuticle. These were surface sterilized in streptomycin-penicillin (5,000 mcg-5,000 units/ml) and placed directly on the egg yolk media. The initial isolates were then transferred to slants of either Sabouraud dextrose agar (SDA) or SMA sealed with parafilm and stored at 20°C to serve as stock cultures for future experiments. Production of research quantities of resting spores was possible by using modified egg yolk media. Following incubation at 25°C for four to five weeks the medium had been entirely utilized and the majority of the fungus was in the resting spore state. The contents

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This isolate produces spores similar to *E. virulenta* Hall et Dunn but lacks rhizoids. For this reason, we have chosen to indicate it is near (nr.) to *E. thaxteriana*.

of 50 petri dishes were added to 2 liters of water and blended in a 5 1/2 liter Waring Blendor<sup>5</sup>. This was diluted again with 2 liters of distilled water, blended with a hand mixer, and centrifuged in a batch rotor in a refrigerated centrifuge once at 23,000 g for 20 minutes, twice at 10,000 g for 10 minutes, and once at 2,500 g for 10 minutes. Between each run, the mycelium was easily discernible from the darker colored resting spores (Fig. 1). The mycelium, being less dense than the spores, was at the surface and easily removed. Following initial clean up procedures the spores were re-suspended in distilled water and transferred to 50 ml centrifuge tubes. A similar procedure was followed as with the batch rotor except that mycelium was removed from the surface of the spores with a wash bottle. The purified resting spores were then spread upon 150 mm watch glass dishes and air dried. The drying chamber consisted of a plexiglass box 53 cm x 37 cm x 37 cm. Air was drawn through a desiccant (silica gel) and passed over the spores (Fig. 2). After 18 to 20 hours the spores were removed from the dryer, placed in glass containers, and stored at 4°C. Standardized resting spore germination tests were used throughout the tests. Water agar (2%) was spread evenly over microscope slides and allowed to solidify. These were placed either in desiccators or supported on glass rods within large petri dishes (150 mm x 15 mm) with distilled water to maintain 100% relative humidity. Usually one ml of the streptomycin-penicillin concentration listed was added to each 100 ml of water agar to prevent bacterial contamination. Normal sterile precautions were used throughout.

Resting spore samples to be tested were placed in suspension in sterile distilled water (1 mg/ml). The water agar slides were coated with the suspension (approx. 5 ml) and incubated at 25°C for 48 hours. Germination began in 12 hours and usually was completed in about 72 hours. However, the proliferation of germ tubes and the disintegration of the empty germinated spores made determination of germination levels difficult after 48 hours. By this time, internal changes had taken place within the resting spores which were about to germinate. These spores are characterized by a densely granular endospore rather than a clear oil globule, (Fig. 3). At the end of the test period, the spores were stained with lactophenol aniline blue and a coverslip was added. Random microscope fields were selected until 100 spores had been counted. Each determination was replicated four times. A Leitz Orthoplan<sup>®</sup> widefield microscope was used at 400 x magnification.

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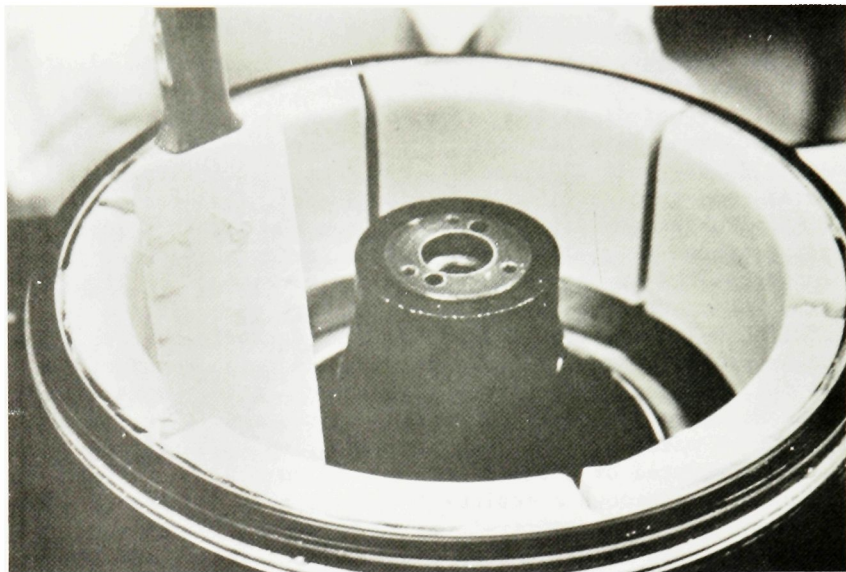


Figure 1. Batch rotor showing yield of resting spores from 50 petri dishes.

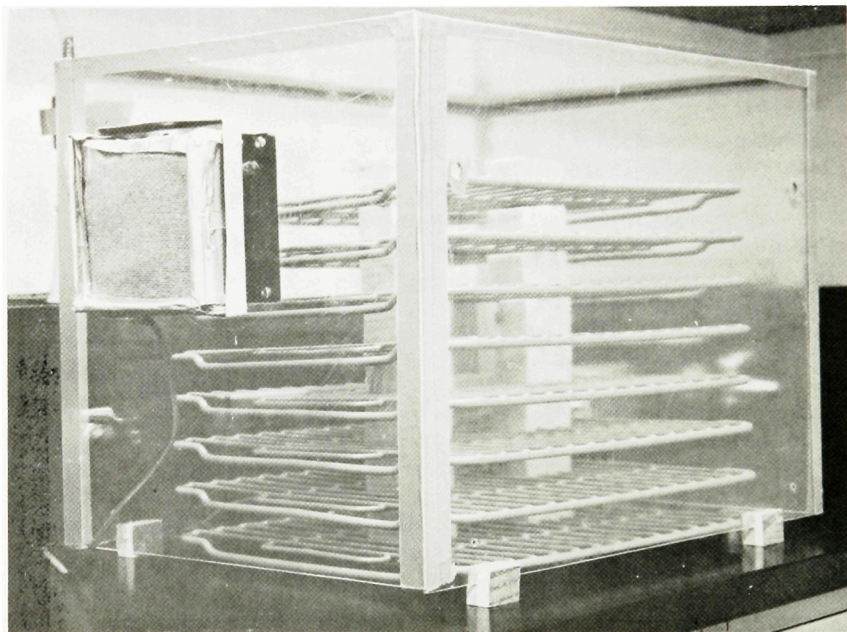


Figure 2. Spore dryer. Air is drawn through silica gel and passed over drying rack.



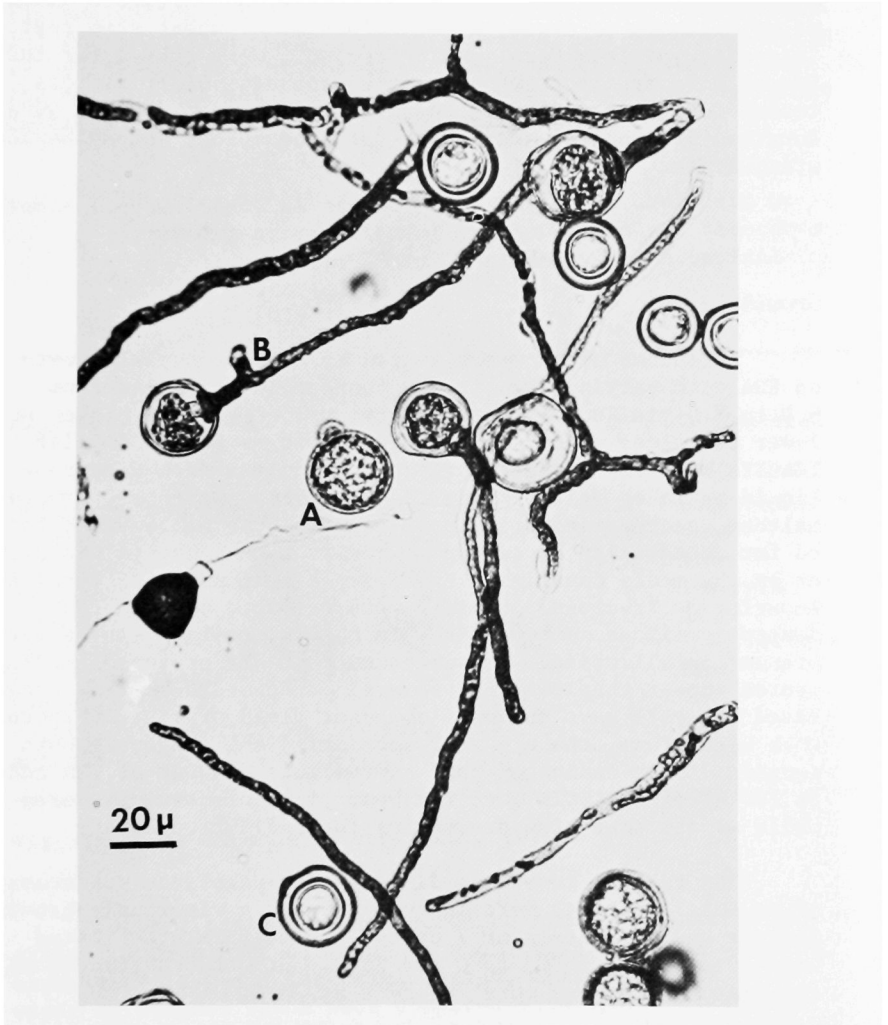


Figure 3. Resting spores of *E. nr. thaxteriana* in various states of germination: (A) germinating spore with developed germ tube; (B) spore with germ tube just forming, note granular contents; and (C) spore still in dormant state.



The method of French and Gallimore (1971) was used to test the influence of various chemicals on germination. The chemical to be tested was diluted using a microliter syringe at the rate of 1  $\mu$ l per 2 ml of water. Some chemicals which were insoluble in water were diluted in 95% ethanol. The alcohol solution was added to water and then serially diluted. The chemicals, thus diluted, were placed in small dishes within the test chamber to ascertain their effect. Representative compounds among those reported by French and Gallimore (1971) as stimulatory to uredospore germination of wheat stem rust, "*Puccinia graminis* Pers. var *triticea*", were chosen for testing with *E. nr. thaxteriana* resting spores. Other factors tested for their influence on resting spore germination were duration of storage, media used for production, and mechanical stimulation.

## RESULTS

### *Growth*

Influence of pH was measured by area of hyphal growth on SDA with varying pH. It was found that pH values from 6.0 to 8.0 yielded similar results, while media at higher or lower pH values would not support growth as well. Similar results were found when tests were conducted with Sabouraud liquid media or malt extract liquid media. Dextrose, fructose, maltose, sucrose and a dextrose-maltose combination were tested for suitability as carbon sources. Hyphal growth was slower on the media containing fructose; all others were equal and superior to fructose. Conidia were produced only on the dextrose-maltose combination. In 6 days, resting spores were present in all media. It was found that SMA produced resting spores sooner than SDA. Of several other media tested, coagulated egg yolk gave the most abundant yield of resting spores. When used alone, the egg yolk soon dried and fungous growth ceased. Tests indicated that approximately 30 ml of SMA added to 100 ml of egg yolk gave the best yield of resting spores while at the same time preventing the media from drying.

The rate of growth at different temperatures was measured on SDA. Cultures were inoculated with a vigorously growing isolate and incubated for 7 days. The temperatures tested were 10°C, 15°C, 20°C, 25°C and 30°C. Growth occurred at all temperatures but was more abundant at 25°C.

### *Germination*

Data presented in Table 1 indicated that the *c*-cinnamaldehyde, *d*-limonene, and *n*-nonanol increased the level of spore

germination significantly. N-octylamine reduced germination. In one of these exceptions (*n*-nonanol), the interaction of ethanol and chemical was one of interference, which tended to mask the expected positive response of ethanol.

Significant increases in germination were obtained by high-speed blending or sonication. Spore samples were treated by: (1) blending for 30 minutes; (2) sonicating for 1 minute; (3) sonicating for 5 minutes; (4) sonicating for 20 minutes; (5) sonicating for 30 minutes; or (6) untreated. Treatments 1-5 resulted in equally good germination; all were significantly better than the untreated control. Bacteria were present in the control and the blended spores, but sonication completely destroyed the bacterial contaminants.

Table 1.--Influence of ethanol atmosphere and chemicals on percent germination of *Entomophthora* nr. *thaxteriana* resting spores. Results of analysis of variance on 2 x 2 factorial experimental design.

Chemical	Atmosphere		Chemical		Interaction
	EtOH	H <sub>2</sub> O	0.5μl/ml	0	
<i>c</i> -cinnamaldehyde	33.4*	19.6	31.9*	20.8	Interference*
citronellal	23.1*	12.5	19.4	15.6	None
<i>d</i> -limonene	32.5*	15.1	34.1*	14.1	None
<i>n</i> -nonanal	24.0*	11.1	17.6	16.3	Synergism*
<i>n</i> -nonane	51.4*	27.0	38.9	37.6	None
<i>n</i> -nonanol	25.9	17.8	27.4*	16.6	Interference*
<i>n</i> -nonyl-mercaptan	22.2*	9.1	15.3	14.6	None
<i>n</i> -octylamine	27.8	32.7	21.0	41.1*	None
<i>n</i> -octyl-thiacyorcte	27.0*	16.9	22.2	21.3	None
safrole	38.8*	15.4	27.6	24.0	None

\*Significance exceeds 5% confidence level.

Further tests were conducted to study the interaction of ethanol with either blending or sonication. In the blending experiment, a 2 x 3 factorial design, atmospheres of water vapor or ethanol vapor were compared with spores receiving (1) no blending; (2) 15-minute blending; and (3) 60-minute blending. Significance was accepted at the 5% or greater level of confidence. Again, ethanol gave greater germination

rates. No difference was found between blending times; both gave significantly greater germination rates than the untreated control. A significant interaction resulted (depression of germination) when spores were blended for 60 minutes and exposed to ethanol.

In the sonication experiment, a 2 x 6 factorial design, the combination of ethanol and sonication were evaluated for influence on resting spore germination. Treatments were sonicated for: (1) 1 minute; (2) 5 minutes; (3) 10 minutes; (4) 15 minutes; (5) 20 minutes; and (6) untreated control. The atmosphere of ethanol again gave higher germination rates. Using Duncan's multiple range test, (5% level of significance) sonication at 5 and 20 minutes was found to be equally good with both better than other treatments. There was no significant difference between 1 minute and control. The 10- and 15-minute treatment results were intermediate in effect between the 0- and 1-minute and the 5- and 20-minute sonication.

A number of storage temperatures were tested. Resting spores were placed dry into vials and stored at  $-12^{\circ}\text{C}$ ,  $4^{\circ}\text{C}$ , and  $25^{\circ}\text{C}$ , or alternation weekly between  $-12^{\circ}\text{C}$  and  $25^{\circ}\text{C}$ . In all cases a sharp rise in germination occurred the fourth week of storage. Germination ceased after 16 weeks at both  $-12^{\circ}\text{C}$  and the alternated temperatures and after 12 weeks at  $25^{\circ}\text{C}$ . No change in germination occurred during storage at  $4^{\circ}\text{C}$  for one year.

#### *Mode of Germination*

The method of resting spore germination is not unlike that of other Zygomycetes such as belong to the Mucorales. In this order the germinating zygosporangium produces a germ sporangium containing many sporangiospores.

The resting spores of *E. nr. thaxteriana* produce another spore stage immediately upon germination. It is believed that the resting spore does not infect directly but that it is this previously unreported stage herein termed the germ conidium which actually causes infection. Although this spore has not yet been described in the life cycle of *Entomophthora*, Speare and Colley (1912) and Dustan (1924) seem to have it illustrated in their drawings. Two or more germ conidia are normally produced per resting spore.

In *E. nr. thaxteriana* the resting spore may produce many hyphal branches (Fig. 4) which eventually give rise to germ conidia, or single germ tube may give rise to a single germ conidium (Fig. 5). The germ conidia produced from the rest-

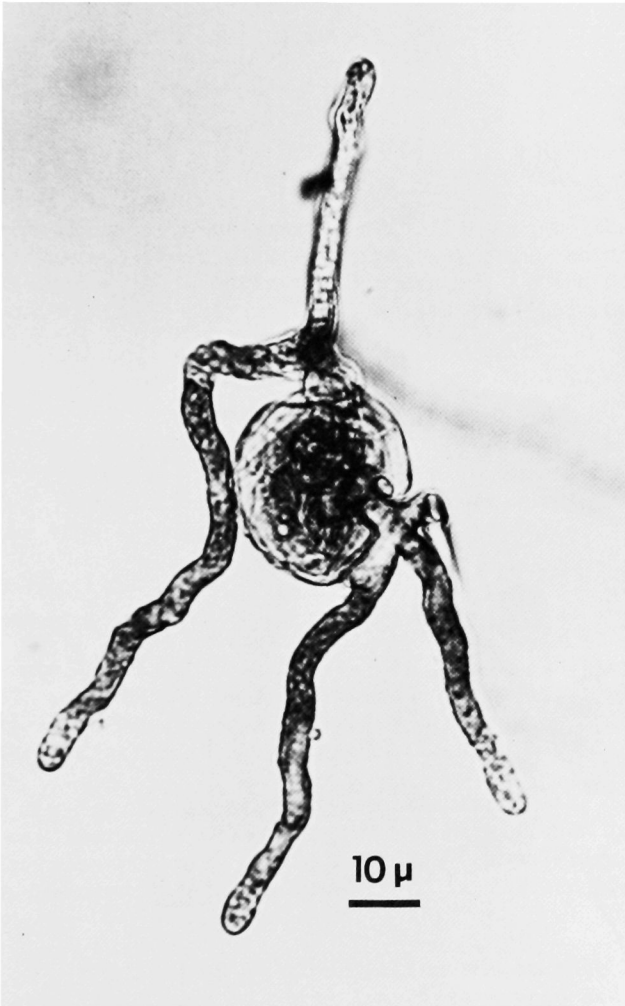


Figure 4. Germinating resting spore of *E. nr. thaxteriana* with many germ tubes.

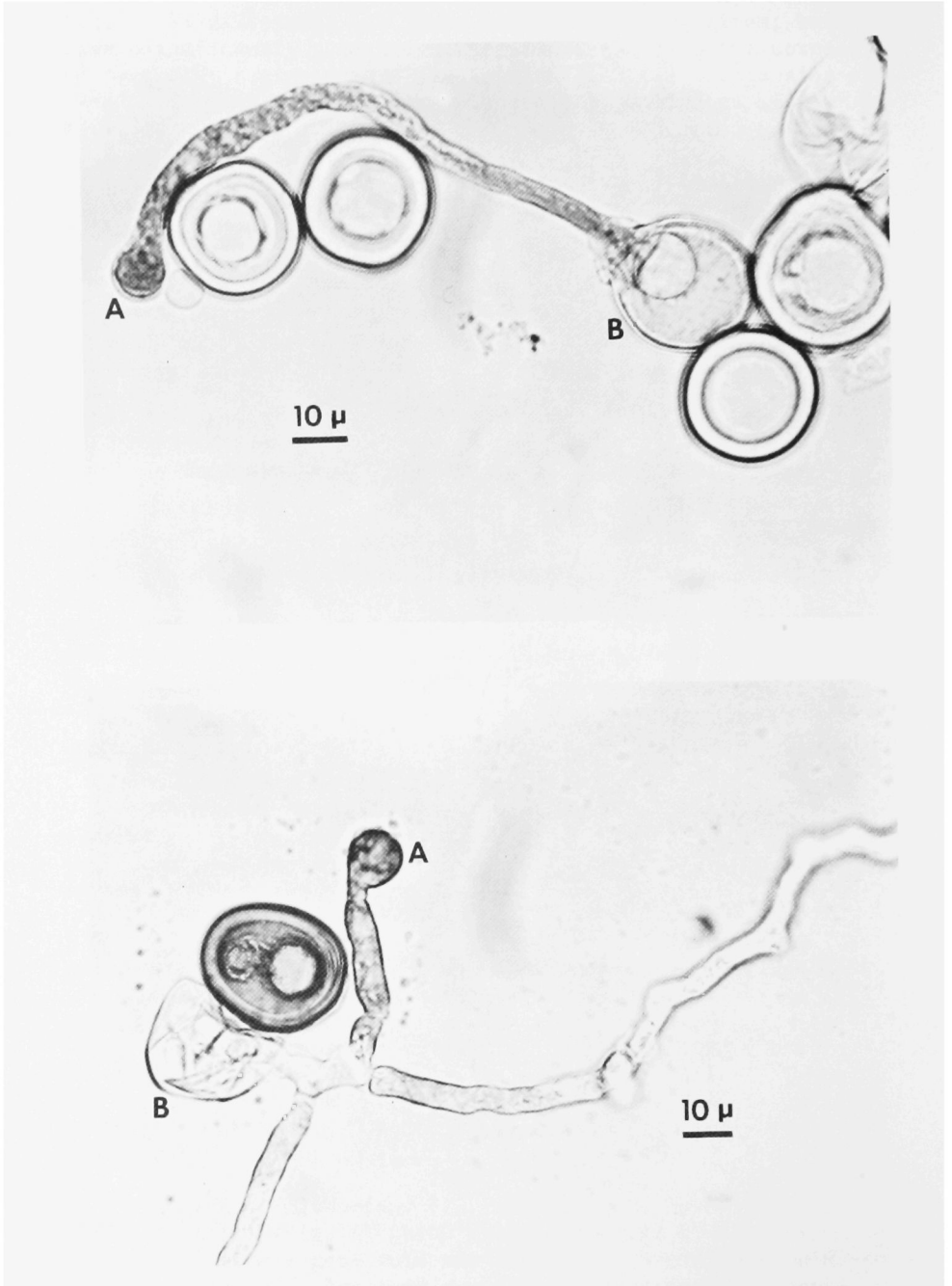


Figure 5. Germination of *E. nr. thaxteriana* resting spore: (A) primary germ conidium; (B) germinating spore.

ing spores in this species are smaller than the conidia produced vegetatively on an infected insect. Similar germ conidia are produced by *E. exitalis* Hall et Dunn, *E. grylli* Fresenius, *E. virulenta* Hall et Dunn, and *Entomophthora* sp. from spruce budworm *Choristoneura fumiferana* (Clemens); in all cases the germ conidia are smaller than the vegetatively produced conidia. It is very likely that a similar stage occurs in all *Entomophthora* species.

Germ conidia of *E. nr. thaxteriana* are produced in greater abundance in a 16-hour light cycle than in complete darkness. They are forcibly ejected vertically up to 3.5 mm. Although they are produced at relative humidities ranging from 80% RH to 100% RH, germination occurs only above 98% RH. They are also able to produce secondary germ conidia (Fig. 6). *E. nr. thaxteriana* spore preparations treated with extracts from other fungi showed a 300 fold increase in the production of germ conidia. The exact chemical nature of these extracts is now under investigation. Since the germ conidia are probably the units of infection, it is crucial in the formulation of *Entomophthora* resting spore sprays that germ conidia be produced.

#### SUMMARY AND CONCLUSIONS

With all of the foregoing experiments in mind, the following technique for *Entomophthora* resting spore production has been developed.

##### *Media Preparation*

1. Surface sterilize hens' eggs in 2.5% sodium-hypochlorite for 30 minutes. If the egg white is to be saved, 50% ethanol may be substituted. It is important to use fresh, unwashed eggs. Eggs that have been washed prior to surface sterilization may be contaminated with bacteria.

2. Separate egg yolks aseptically into sterile containers. Fleakers<sup>®5</sup> (500 ml) have proved to be excellent for this purpose. A small teflon-coated stirring magnet is autoclaved within the Fleaker to facilitate breaking the egg yolks.

3. Add 30 ml of molten SMA to each 100 ml of egg yolk. This must be mixed immediately upon addition to prevent lumping of the agar.

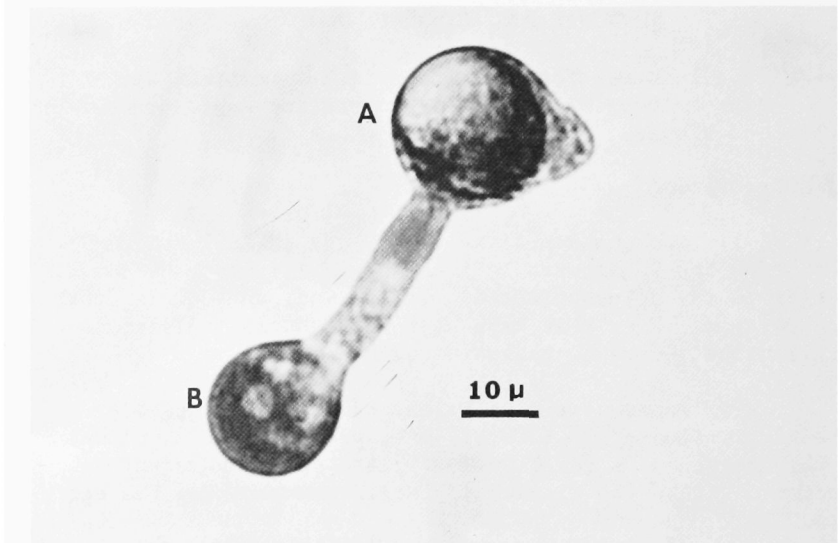
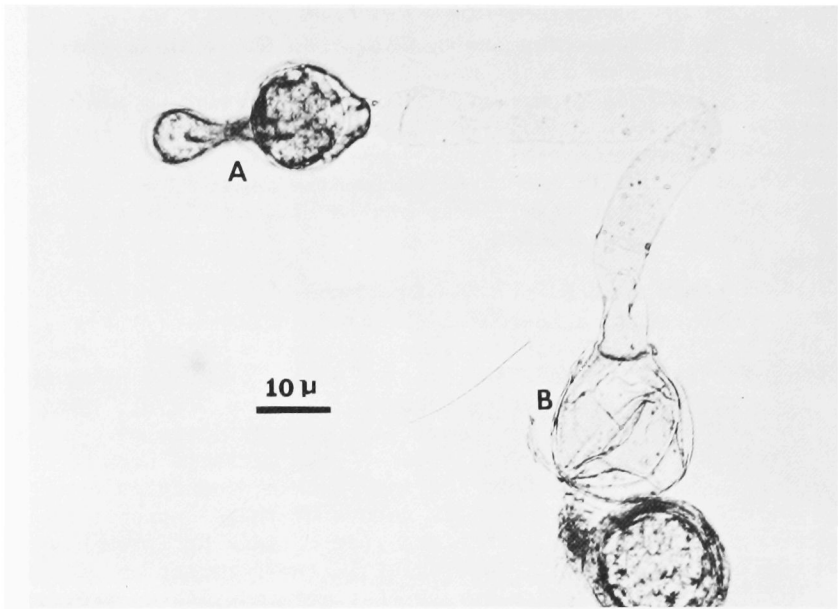


Figure 6. Top photomicrograph of *E. grylli*: (A) production of secondary germ conidium; (B) primary germ conidium has not been released from resting spore. Bottom photomicrograph shows: (A) *E. nr. thaxteriana* primary germ conidium; (B) producing secondary germ conidium.



4. Pour media into 10 cm plastic petri dishes at the rate of approximately 25 ml per dish.

5. Coagulate the media in an isothermal autoclave at 80°C for 7 minutes. Alternatively, coagulation can be accomplished in an oven at 80°C for 20 minutes. If plastic petri dishes are utilized, it is important to note that only certain brands will withstand this temperature.

If the media is overcooked it becomes leathery and the fungi will not grow well. This is more likely to occur if dry heat is used for coagulation. If the media is not coagulated, resting spore formation will be very poor.

#### *Inoculation of Media*

6. Wrap the petri dishes containing the coagulated media in plastic film to prevent desiccation and store for 3 days to check for contamination.

7. Inoculate the plates by streaking with a sterile needle. The source of inoculum should be a vigorously growing culture between 4 and 7 days old.

8. Wrap the cultures in plastic film, incubate at 25°C for 7 days, and unwrap. This allows the fungus to cover the entire medium and prevents excessive desiccation during the incubation period (4 to 5 weeks).

#### *Extraction and Purification*

9. Add the contents of 50 petri dishes to 2 liters of water.

10. Blend for 30 minutes in a 5.5 liter Waring Blendor to which is added 5 ml of 95% ethanol.

11. Store the blended cultures for 24 hours at 4°C.

12. Dilute with 2 liters of sterile distilled water, blend with a hand mixer, and centrifuge in a batch rotor. Centrifuge under refrigeration, once at 23,000 g for 20 minutes, twice at 10,000 g for 10 minutes, and once at 2,500 g for 10 minutes. The resting spores are packed tightly against the rotor wall, and the lighter mycelia are easily scraped from the surface.

13. Place in 50 ml tubes and centrifuge using angular head at 5,000 rpm for 10 minutes for final purification. The mycelia are easily washed from the surface of the spore pellet with a stream of distilled water from a plastic wash bottle.

14. Spread the pure spores on 150 mm watch glasses and place on racks within a plexiglass drying chamber. Air is drawn by fan through silica gel and passed over the spores.

15. Remove the spores after 20 hours and place in sterile containers with a sachet of silica gel. Store at 4°C until required.

Using this procedure, we could obtain approximately 1 kilogram of resting spores from 60 dozen eggs. Resting spores of *E. nr. thaxteriana* thus produced have been found highly infectious both under greenhouse and field conditions. The levels of germination vary among batches; in some cases virtually 100% was obtained. Generally germination is between 20% and 80%.

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