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Evaluation of entomopathogenic fungi for their potential to control the eastern hemlock looper

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Introduction

The eastern hemlock looper, *Lambdina fiscellaria fiscellaria* (Guen.), has had a severe impact in eastern Canada, especially Newfoundland. This looper is particularly destructive in balsam fir (*Abies balsamea*) stands and is capable of causing extensive damage and even tree mortality in a single season. This looper is a defoliator of primary importance because its activities can have detrimental impacts on the economy and environment. In Newfoundland, hemlock looper epidemics tend to last 3 to 6 years and occur in intervals of 10 to 15 years. In recent years, localized high populations of looper, possibly indicating an upcoming outbreak, have been detected in several locations around the island.

In Newfoundland, the most common product used to control the looper is *Bacillus thuringiensis* var. *kurstaki* (*Btk*), a biological based insecticide. *Btk* is a naturally occurring bacterium that targets the larval stage of some lepidopterans. Mimic (active ingredient tebufenozide), an insect growth regulator, was also applied on 30,000 hectares of forest in 2003. This chemical pesticide initiates a premature molt causing the insect to cease eating and die. To be effective, *Btk* and Mimic must be ingested by the larvae. Therefore, application timing is extremely important and should coincide with active feeding. A major concern however is the possibility of the pest insect developing a resistance to insecticides when they are heavily used or not integrated with other methods of control. *Bt* resistance has been detected in some agricultural pests so this is a very legitimate concern.

Increased emphasis has been focused on using entomopathogenic fungi as control agents in Integrated Pest Management programmes to combat these pest insects. In

Newfoundland, two fungus species, *Entomophthora sphaerosperma* Fres. and *E. egressa* MacL. & Tyr. have been identified from looper populations and are suspects in causing the collapse of several looper outbreaks (Otvos et al. 1973). However, scientific data to support this suspicion has never been proven.

This project started in 2001 and continued through the 2002-2003 season. For the 2003-2004 season the goal was to continue the evaluation of the entomopathogenic fungi for its potential in controlling the eastern hemlock looper. Ultimately the goal of this project was to develop alternative methods of biological control for the hemlock looper. The specific objectives for the fiscal year of 2003-2004 were to 1) identify the fungal species collected during the summer of 2002, 2) investigate and determine the superior media for culturing fungi in the laboratory and 3) to test the toxicity of the cultured fungi on the eastern hemlock looper.

Materials and Methods

Fungi Identification

During the summer of 2002, eastern hemlock looper were collected from eastern, central, western and northern regions of the province (Li 2003). Field-collected larvae were individually reared in the laboratory until pupation or death. Fungal infection was the major mortality factor in these field-collected larvae. In order to identify the fungal species responsible for the mortality, larvae killed by a fungus were examined on a daily basis. Once fungal spores were present, photos were taken of the infected larvae (Figure 1), three different culture media were inoculated with spores, and slides were made of the fungi from the deceased larvae. In the fall of 2002, slides were reviewed and divided into

suspect groups. These groups were labelled HLF 1 through 9 (no HLF 7). In 2003, representative slides and pictures were chosen from each group for identification.

Culture Technique Investigation

In 2002, three of the most successful culturing mediums were chosen for mass rearing entomopathogenic fungi. The goal was to find a medium which would culture the prevalent fungal groups. The three mediums tested in this experiment were, Grace's insect medium (Appendix A), sabouraud dextrose agar with yeast extract (Appendix B) and sabouraud dextrose agar supplemented with yeast extract, egg yolk and milk (Appendix C). Plates with each medium were inoculated with fungi from cadavers in the eight groups and then placed in an incubator at room temperature.

In preparation for the winter of 2003, with the risk of possibly losing a percentage of plates while stored in the refrigerator for an extended period, additional plates were inoculated from cadavers or from plates already successfully supporting culture. The plates were incubated until growth had taken place, parafilmed to prevent evaporation and contamination, and then refrigerated. After fungal grabs were taken from the cadavers, they were frozen and kept as a backup in case plates were contaminated or died during winter storage.

In order to compare fungal growth on the three media types, visual comparisons of growth rate and mat character were made. Periodic slide mounts analysed spore and hyphae production.

Toxicity to Hemlock Looper

An important step when experimenting with fungi-host relationships is to test the toxicity of the fungi on the host after it has been cultured on artificial media. A fungus that does not re-infect its host successfully after being reared on artificial media is not very useful in terms of developing a fungus-based insecticide.

Representative samples of the eight groups (HLF 1-9) identified from the 2002 collection were inoculated on plates with their hyphae and spores. The three media types investigated in the culture techniques were tested to monitor their culturing success. The plates were observed throughout the experiment's duration in 2002 and the winter survival in the spring of 2003. Based on the results of these observations, one culturing medium (sabouraud dextrose agar supplemented with yeast extract, egg yolk and milk) was chosen to focus on for the toxicity testing. Overwintered samples were used to inoculate additional Petri dishes and subsequently plates were inoculated from the frozen cadavers in case samples had attenuated.

Harvesting fungi from the cultured plates to prepare for the toxicity testing was only done when spores were present. Using a narrow spatula, a thin layer of spores and hyphae were scraped from the plates removing as little media as possible. The scrapings were placed in a 1.5 ml centrifuge tube in 0.5 ml of distilled water (dH_2O). Manual agitation with a sterile micropestle was used to break apart any large clumps and to separate spores from hyphae. An additional 0.5 ml of dH_2O was added to the centrifuge tube and then vortexed to create a stock suspension. The stock was quantified to determine the number of spores per milliliter using a haemocytometer, and then 0.1 ml was pipetted out and mixed with 0.9 ml dH_2O to make a 10x dilution. The stock

suspension for the direct deposit had a concentration of 3.92×10^5 spores/ml and the spray tower stock had 5.65×10^4 spores/ml.

Eastern hemlock looper eggs were obtained from Canadian Forest Service in Sault St. Marie, Ontario for this experiment. The eggs hatched in the laboratory at room temperature and were reared to third instar larvae for the toxicity tests. The larvae were fed only fresh and untreated balsam fir branches before testing.

Two methods of inoculation were used. Two spore suspensions of 3.92×10^5 spores/ml and 3.92×10^4 spores/ml were used in direct deposit testing. A surfactant (Tween 80) was added to the suspensions to improve spore attachment to the host surface. The direct deposit method involved applying 0.05 μl of fungal suspension directly onto the back of third instar larvae using a micropipette. Ninety insects were inoculated with each concentration, and incubated in three different conditions: room temperature (approximately 24^0C with 50-60% RH), 60% relative humidity (20^0C) and 100% relative humidity (20^0C), with 30 replicates in each treatment. Environmental conditions, especially humidity, can greatly influence the virulence and ecological fitness of the fungi. Control insects were inoculated with a dH_2O and 0.1% Tween 80 dilution. A total of 90 larvae were inoculated as controls, 30 replicates for each treatment. All larvae were fed healthy balsam fir foliage during incubation.

For the second inoculation method, the Potter Spray Tower was used to spray cups containing balsam fir foliage. Each cup contained enough foliage to divide between five individual rearing cups. Cups were sprayed with 3 ml of fungal suspension and were allowed to dry at room temperature. Once dry, the foliage was divided into the five smaller cups where the larvae were reared individually. A total of 60 larvae were reared

at room temperature and 24 were reared in the 60% relative humidity (20^0C) incubator. The larvae were reared through to pupation or death and data was recorded on pupae sex or mortality respectively.

Results and Discussion

Fungi Identification

Two Entomopthorales from the eight tentative groups were positively identified. Our HLF 1 and HLF 4 were identified as *Entomophthora sphaerosperma* Fres. and HLF 2 and HLF 3 as *Entomophaga aulicae* (Reichardt) Humber (=*Entomophthora egressa* MacL. & Tyr.). HLF 5 could not be identified, HLF 6 and HLF 9 appeared to be saprophytic species like Penicillium and Cladosporium, and HLF 8 contained resting spores of *E. sphaerosperma* or *E. aulicae.*

Entomophaga aulicae (Figure 2) has been extensively studied. This fungus has great potential. Mass production of *E. aulicae* was explored before, and a successful report on mass production has been documented. However, commercialisation of this fungus has not been realized.

Entomophthora sphaerosperma (Figure 3) was the major mortality factor in the looper collected in 2002 (Table 1). Its occurrence was far more abundant than *E. aulicae*, which posed the question why more attention was not directed on this species. This fungus has been identified by Otvos et al. (1973) in a provincial report, especially in relation to its role in the decline of looper infestations. However, surprisingly little research has been done on this species despite its prevalence or easy culturing as we experienced with the trial mediums.

Culture Technique Investigation

Although all fungal types grew fairly well on Grace's medium and sabouraud dextrose agar with yeast extract, fungal growth was best on sabouraud dextrose agar supplemented with yeast extract, egg yolk and milk (Figure 4). After plates were inoculated, growth was more prolific and generally performed much better on the supplemented sabouraud dextrose agar than on the others. Plates incubated in the refrigerator during the winter of 2003 were still active and alive when reassessed in the spring. Based on this observation, the sabouraud dextrose agar with yeast extract, egg yolks and milk was chosen to cultivate fungi for the summer of 2003 because of its superior performance in culturing and maintaining the fungus.

One particular fungi species, the *E. sphaerosperma*, did perform better than the others on the medium, hence, this fungus was chosen to test its toxicity to the looper.

During the month of July 2003, the laboratory experienced high temperatures and relative humidity due to a malfunctioning air conditioning unit in the building. Temperatures within the incubator in which our fungal plates were growing also soared. As a result many of the fungal cultures flattened and became slimy, making spore collecting impossible. To combat the effects of high temperatures, a number of fungal plates were placed in the lab's refrigerator with hopes that cooler temperatures would trigger normal growth habits. The plates did appear to recuperate somewhat, but significant damage certainly occurred.

Toxicity to Hemlock Looper

Both inoculation methods were unsuccessful in re-infecting the host. Both spore suspensions should have been sufficient to infect larvae according to published literature, and susceptibility of most insects is dependant on spore dosage. However, no fungus caused mortality was observed from either testing methods (Table 2). The survival percentage was extremely high and the most significant mortality factor was a malfunction in the growth chamber where the 100% relative humidity was held. Temperatures rose well beyond specified settings, and deaths that occurred directly following this malfunction were blamed on this occurrence as noted in Table 2. The other two incubations, which did not have temperature problems, had low mortality.

After scrutinizing the results, several possible explanations may account for the unexpected results from toxicity tests. The first main complication was the malfunction in the building, which resulted in the cultures being 'cooked'. Although the cultures did appear to regain some of their life, it is possible that they did not gain enough strength to recover. Another possibility may be the inoculation method. The rearing conditions may not have allowed rapid germination and infection, which are hallmarks of virulent isolates. However, the direct deposit and internal infection methods are common techniques used for inoculation in previous studies of insect-fungal interactions, so this was not likely a major obstacle. A more likely possibility is that the fungi lost its virulence after being subcultured multiple times on artificial media. Successive subculturing on artificial media can result in attenuation of virulence. The attenuation rate depends on the isolate and the pathogen species. Some can lose their virulence slowly, but others rapidly lose virulence after only a few subcultures on artificial media. Our

cultures were subcultured approximately seven times, and overwintered, before they were diluted and applied to the host.

For further studies an important step would be experimentation with additional fungal storage techniques. A definite goal should be to keep the number of subcultures to a minimum. Other fungal storage methods which have been successful and could be tried with this species are, submerging fresh fungi in mineral oil until required, freeze drying, storing under liquid nitrogen and binding to silica gel. A fungus that has been stored or subcultured multiple times can have virulence regained prior to experimentation with passage through the appropriate host. This may be a possibility in order to bring all fungi up to the same level before testing.

In preparation for the summer of 2004, fresh fungal spore and hyphal samples were submerged in sterile mineral oil and refrigerated to retain their viability during winter storage. Fungi that attenuates readily on culturing medium would be submitted to a fraction of the culture time. When the timing is right for the culture to be started, so it coincides with insect development in the field, plates could be inoculated and run through the artificial medium a minimum numbers of cycles. In this way, the fungus is still as viable as possible.

The potential of the Entomophthorales in pest regulation lies in the fact that they are common and effective naturally occurring pathogens in many insects and can decimate those populations at some times of the year, surviving as dormant resting spores. Their host range is relatively narrow and application over large tracts of land would not put other species at risk and certainly worth considering incorporating into an effective pest management strategy.

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References

Otvos, I.S., MacLeod, D.M. and Tyrrell, D. 1973. Two species of *Entomophthora* pathogenic to the eastern hemlock looper (Lepidoptera: Geometridae) in Newfoundland. Can. Ent. 105: 1435-1441.

Li, S.Y. 2003. Annual Research Report to Western Newfoundland Model Forest. Evaluation of entomopathogenic fungi and viruses for their potential to control the eastern hemlock looper. pp8.

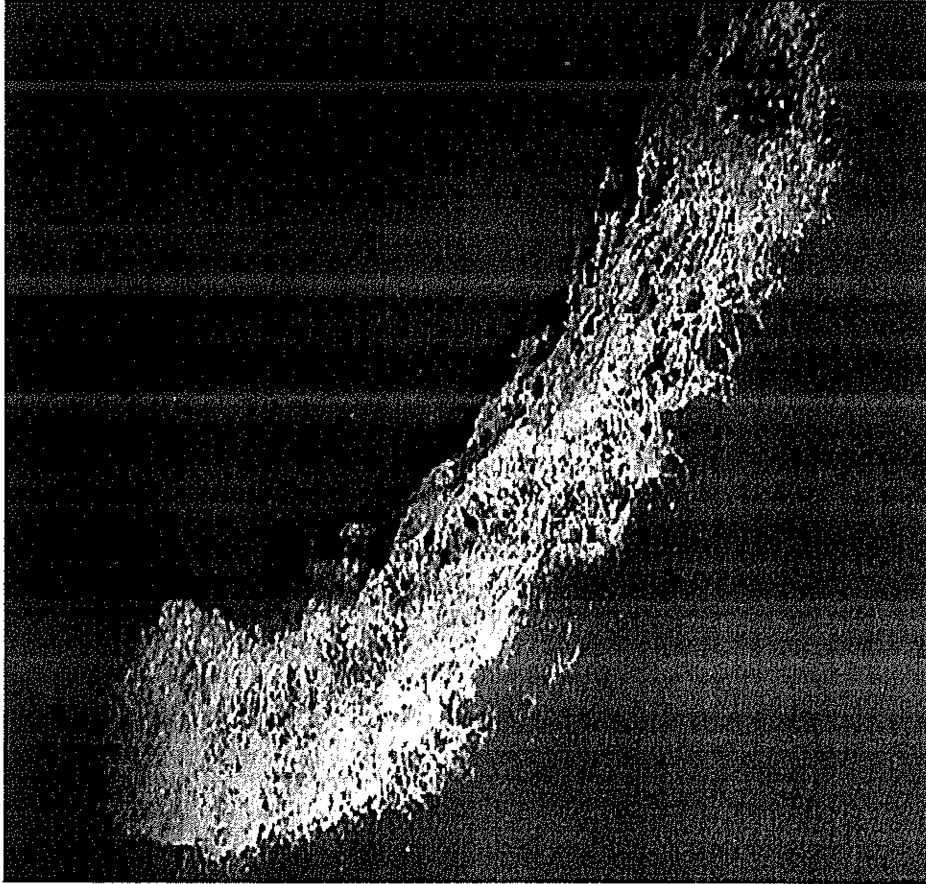


Figure 1. Dead larvae of eastern hemlock looper larvae, *Lambdina fiscellaria fiscellaria* (Guen.) killed by fungi. Cadaver covered by creamy white spores and hyphae.

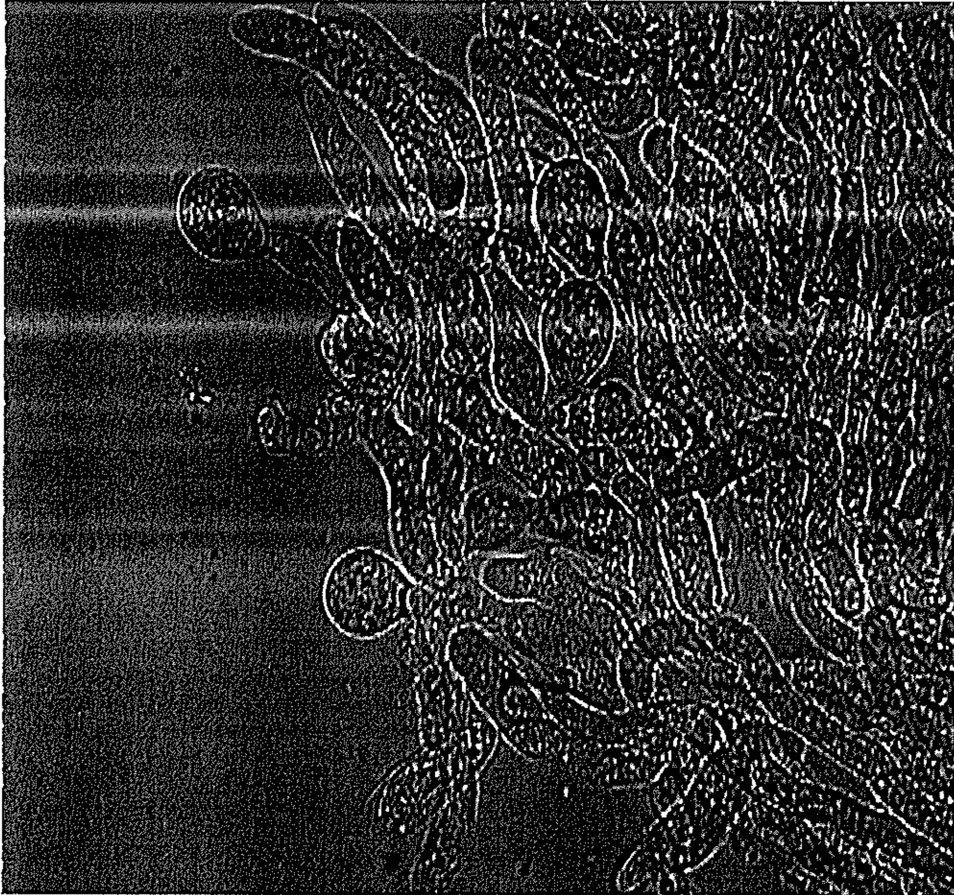


Figure 2. Spores of *Entomophaga aulicae* isolated from dead eastern hemlock looper larvae.

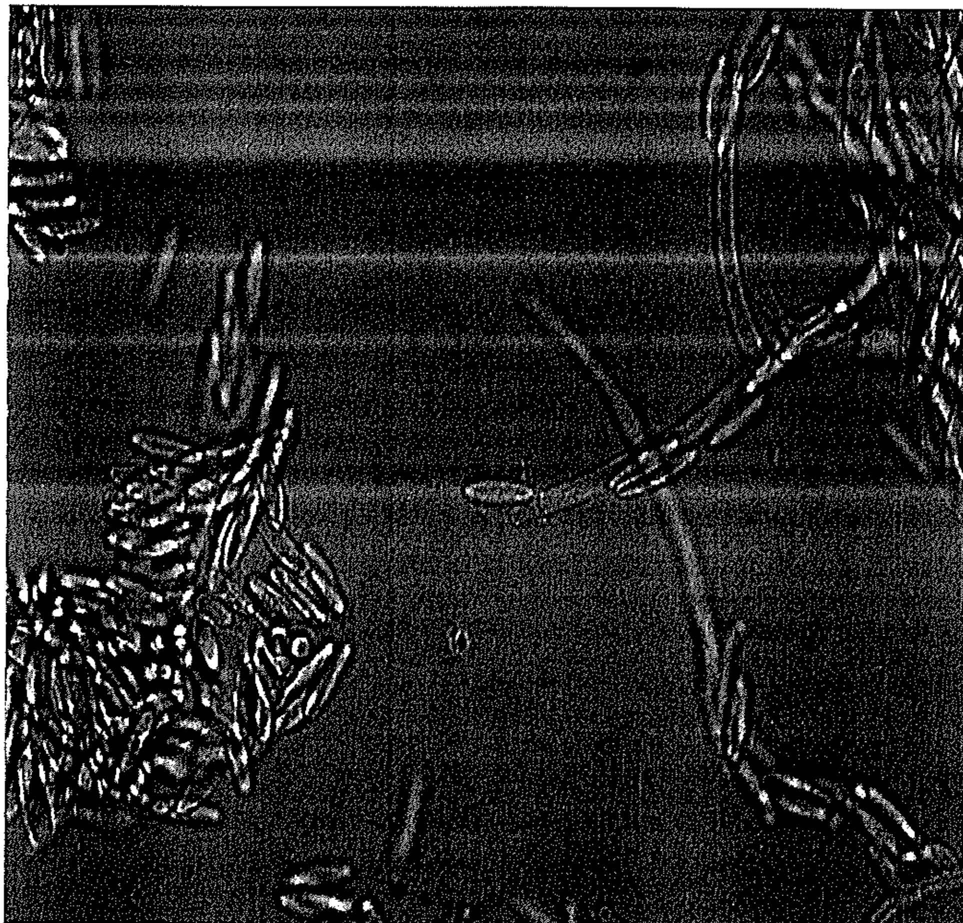
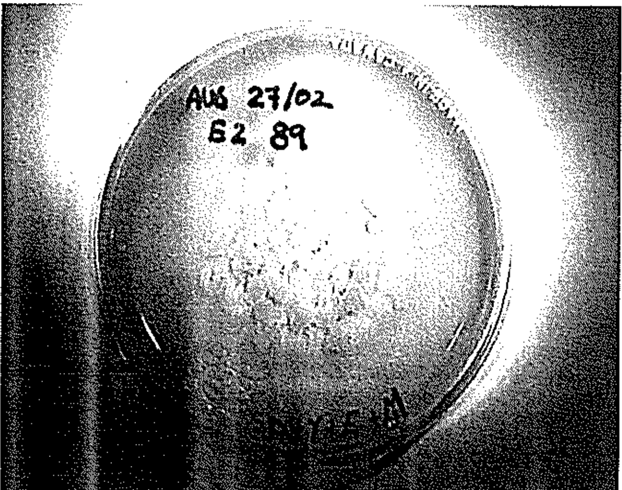


Figure 3. Spores of *Entomophthora sphaerosperma* isolated from dead eastern hemlock looper larvae.

(A)

(B)



(C)

Figure 4. Three media showing fungi growth over equivalent time period

(A) Grace's Insect Medium
(B) Sabouraud dextrose agar supplemented with yeast extract
(C) Sabouraud dextrose agar supplemented with yeast extract, egg yolks and milk

APPENDIX A

Grace's Insect Medium - Preparation Instructions

Step 1: Add 20 ml of dH_2O to a 100 ml bottle of Grace's Insect Medium, 2x. Dispense 100 ml of this complete Grace's medium into 50 ml of culture grade water. Loosely cap (to allow for gas escape) in a sterile container and place in a 37°C water bath until ready for use.

Step 2: Add 2 g of granulated agar to 50 ml of dH_2O to make a 4% agar solution. Autoclave the agar at 15 lbs and 121°C for 30 minutes. After the autoclave cycle has finished, let the agar cool to approximately 70°C.

Step 3: When the agar has cooled to 70°C or slightly lower, add to the 37°C Grace's medium. Pour into sterile Petri dishes. Allow to cool overnight at room temperature before using.

If the plates are not going to be used immediately, Parafilm or Saran Wrap the edges to prevent evaporation. Store them in the fridge until needed.

Reference

Luckow, V.A. in *Recombinant DNA Technology and Applications* ed. Ales Prokop, Rakesh K, Bajpai, Chester S. Ho; New York, McGraw Hill, 4:97-153, "Cloning and Experssion of Heterologous Genes in Insect Cells with Baculovirus Vectors." ISBN :0-07-029075-X. (1991).

APPENDIX B

Sabouraud Dextrose Agar with Yeast Extract - Preparation Instructions

In a screw top bottle combine:

- 500 ml of dH_2O
- 32.5 g sabouraud dextrose agar
- 0.5 g yeast extract

Using a hot plate stirrer, heat and agitate until dissolved. Set this on a metal tray for autoclaving. While the agar is autoclaving it will do a lot of bubbling so make sure the bottle is large enough to allow for this, and that the lid is left loose to allow the pressure to escape.

Autoclave the agar solution at 15 lbs and 121°C for 30 minutes. When it has run its cycle pour immediately into sterile Petri dishes. Allow to cool overnight at room temperature before using.

If the plates are not going to be used immediately, Parafilm or Saran Wrap the edges to prevent evaporation. Store them in the fridge until needed.

Reference

Hicks, B. 2001. History, ecology and potential control of the pine beauty moth, *Panolis flammea,* in Scotland. University of Edinburgh. PhD thesis.

APPENDIX C

Sabouraud Dextrose Agar Supplemented with Yeast Extract, Egg Yolk and Milk
Preparation Instructions

Start about 2 ½ hours before sabouraud dextrose agar:

Surface sterilize three eggs in a beaker containing about 400 ml of a 2% $NaHCl_2$ solution for 3 hours.

In a screw top bottle combine:

- 500 ml of dH_2O
- 32.5 g sabouraud dextrose agar
- 0.5 g yeast extract

Using a hot plate stirrer, heat and agitate until dissolved. Set this on a metal tray for autoclaving (15 lbs and 121°C for 30 minutes). While the agar is autoclaving it will do a lot of bubbling so make sure the bottle is large enough to allow for this, and that the lid is left loose to allow the pressure to escape. Prepare the milk and additional equipment before autoclaving.

In a 250 ml flask or beaker:

75 ml of whole milk

Cover with aluminium foil and autoclave at the same time as the agar.

Additional equipment to autoclave:

2 or 3 stirring rods (wrap in aluminium foil)
1 pair of forceps (wrap in aluminium foil)
500 ml flask (cover opening with aluminium foil)
250 ml beaker (cover opening with aluminium foil)

When the autoclave has run its cycle. Break the eggshells with forceps near the flame of a Bunsen burner. Gently remove the egg yolk and pour it into a sterile 500 ml flask. Add the milk to the egg yolks and stir the contents with a sterile glass rod. Pour the egg yolk-milk mixture into the sabouraud dextrose-yeast agar, and shake. Immediately pour into sterile Petri dishes. Allow the medium to cool overnight at room temperature.

If the plates are not going to be used immediately, Parafilm or Saran Wrap the edges to prevent evaporation. Store them in the fridge until needed.

Reference

Hicks, B. 2001. History, ecology and potential control of the pine beauty moth, *Panolis flammea,* in Scotland. University of Edinburgh. PhD thesis.

Table 1. Field-collected larvae mortality of eastern hemlock looper, 2002 data.

Region	Site	Tot. # larvae	% dead of fungi	Fungi Type Prevalence by %						
				Unknown	HLF 1 and 4	HLF 2 and 3	HLF5	HLF6	HLF8	HLF 9
East	1	149	47.6 %	31.0	46.4	21.1	1.4	0	0	0
East	2	150	68.0	40.2	43.1	10.8	2.9	1.0	1.0	1.0
East	3	149	8.0	66.7	16.7	16.7	0	0	0	0
West	1	149	8.7	46.1	15.4	30.8	0	0	0	7.7
West	2	150	11.3	41.2	17.7	23.9	0	5.9	0	5.9
West	3	150	78.7	30.5	53.4	14.3	0.8	0	0.8	0
Central	1	149	0.7	0	100	0	0	0	0	0
Central	2	150	0.7	0	0	0	0	100	0	0
Central	3	149	1.3	0	100	0	0	0	0	0
Northern	1	150	0	0	0	0	0	0	0	0
Northern	2	150	6.7	90.0	10.0	0	0	0	0	0
Northern	3	150	7.3	81.9	0	0	18.2	0	0	0

HLF 1 and 4: *Entomophthora sphaerosperma*
HLF 2 and 3: *Entomophaga aulicae*
HLF 5: not identified
HLF 6: Saprophytic species (Penicillium and Cladosporium)
HLF 8: Resting spores of *E. sphaerosperma* or *E. aulicae*
HLF 9: Saprophytic species (Penicillium and Cladosporium)

Table 2. Mortality test of eastern hemlock looper, 2003.

Spore Concentration	Incubation Conditions	Total # of Larvae	Dead %	Mortality (%)				Pupae			Adults (%)	
				fungi	high temps.	unknown	handling	Total	Unemerged (%)	Emerged (%)	Male	Female
3.92×10^5 spores/ml	room temp.	30	0	0	0	0	0	30	0	100	47	53
	60% RH, 20^0C	30	10	0	0	10	0	27	0	100	59	41
	100% RH, 20^0C	30	30	0	20	10	0	18	0	100	50	50
10x dilution of stock	room temp.	30	6	0	0	6	0	27	0	100	67	33
(3.92×10^4 spores/ml)	60% RH, 20^0C	30	0	0	0	0	0	30	6	93	43	50
	100% RH, 20^0C	30	27	0	17	10	0	23	0	100	43	57
Control (H_2O + Tween)	room temp.	30	3	0	0	3	0	29	7	93	45	48
	60% RH, 20^0C	30	6	0	0	6	0	28	4	96	57	39
	100% RH, 20^0C	30	6	0	0	3	3	28	11	89	54	36
Spray Tower:												
5.65×10^4 spores/ml	room temp.	30	3	0	0	3	0	28	11	89	25	64
	60% RH, 20^0C	24	4	0	0	4	0	23	9	91	52	39
	100% RH, 20^0C	30	27	0	0	27	0	22	14	91	50	41