Biological Control of Chinch Bug
Research Project, 2004-2005

Year 1 (July 2004- April, 2005)
Up-dated in April 2006 to include related data from Year 2.

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Year 1. Biological Control of Chinch Bug Research. 2004-2005
(Up-dated in April 2006 to include related data from Year 2).

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Executive Summary For Year 1 of Project
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Chinch bugs are ruining a lot of lawns in New Brunswick and elsewhere and the need for alternatives to chemical insecticide controls is increasing. This project stemmed from on-going turf research being done in New Brunswick (see e.g. Wetmore, 2003 and Wellwood, Nickerson and Wetmore, 2003), the goal of which is to identify chinch control measures that minimize the use of pesticides. A very useful review of the literature and description of some potential control options has been prepared by Dr. Patriquin, Dalhousie University (see http://www.versicolor.ca/lawns/secF.html.

Year 1 of this project had three main components: I (Endophytes), II (Entomopathogens) and III (Rearing chinch bugs). Background information, key results and conclusions related to each of these components is presented separately below.

Part I. Endophytes. Many scientific, commercial and popular press reports show that certain fungi (termed "endophytes") that grow inside the leaves and stems of grasses, improve plant vigour and resistance to chinch (http://www.ntep.org/endophyte.htm). Propagules of the fungi are carried from generation to generation through the seeds. In part I, some allegedly endophyte-enhanced grasses were assessed for endophytic fungi using both a commercially-available immunoassay test kit, and microscopic examination. Emphasis was on fescues, since preliminary field results indicate that some of these grow well in New Brunswick and look appropriate for lawns. Also, the literature suggests some fescues are relatively resistant to chinch bugs.

Despite their "endophyte enhanced" status, both assays showed that very little endophyte was present in the field plants. Since the literature suggested that the endophyte can sometimes die in the seed prior to planting, we assessed this in lab tests. It was confirmed the fungus disappeared rapidly even when the seed was stored under the cool and dry conditions that are best for seed storage. This suggests that the fungus-plant association in some "endophyte enhanced" seeds may be so unstable as to be impractical.

Two chewing fescues (Treazure and Victory II) that showed good potential for lawn use were investigated more closely. Tests made with both field and laboratory-grown plants showed that Treazure initially had a relatively high endophyte content, while Victory II had essentially none. The performance of these two grasses in the presence of chinch bugs were compared under lab conditions in "rhizotrons". These were made with thin, glass walls so the roots and shoots of the grasses can be easily observed. The chambers were made so chinch bugs could not escape. Chinch bugs were observed feeding on stems of both Victory and Treazure that were grown in these chambers. When the *specific* Treasure stems that the chinch had fed on were assessed, it was found they contained endophyte . This proves that the presence of endophyte is not a guarantee that chinch will not feed on the plant. It was noted that the amount of hyphae (*intensity* of colonization) in many alledged endophyte enhanced plants was much lower than in some endophyte-containing tall fescues, where it is better established that the endophyte inhibits the chinch. It seems possible that the *intensity* of infection in a plant, as well as the fraction of plants that have any endophyte (*incidence* of infection) may be an important factor governing chinch

feeding. Another factor is whether the endophytic fungus makes compounds (or other factors) that inhibit the chinch. The literature makes it clear that not all strains do.

In short, it is far from certain that using "endophyte enhanced" seed will lessen chinch damage. Vendors of endophyte-enhanced seeds should provide both directions for storage and efficacy data with their product, particularly if they are charging a premium for it. Until they do, it is "Buyer-beware"!

II. Entomopathogens. The literature suggests that certain insect pathogenic fungi (entomopathogens) play important roles in controlling chinch bug populations. Some of the work done during this first year substantiated this possibility. Dead chinch bugs recovered from a lawn in St. Johns Newfoundland were heavily infected by the entomopathogen *Beauveria bassiana*. Preliminary tests using spores from this and a few other isolates of *Beauveria* showed that treated insects at least sometimes died more rapidly than untreated ones. After death, the fungus could be re-isolated from the dead treated insect. i.e. Koch's postulate was confirmed. These results prompted us to undertake the much more extensive testing, results from which are presented in the year 2 report.

With a view to gaining insight into whether entomopathogens might already be a determining factor governing the prevalence of chinch, a variety of soils including some from sites with and without chinch problems, were assessed for entomopathogenic fungi. Two assays were used. The first was a dilution plate count based on use of a selective agar medium. The second was a bioassay using *Galleria* (wax moth larvae). With both assays, the focus was on *Beauveria*, but the assays also detected *Metarhizium*, this being another well known entomopathogenic fungus. The results showed that some soils contained mainly *Beauveria*, others mainly *Metarhizium* and others both fungi. In some, no entomopathogens were detected. Relatively few ($< 10^3$ per gram) spores of either fungus were present in any of the soils. Interestingly, of all the soils tested, those from Angelview (a park in Fredericton which has had essentially <u>no</u> chinch problem) had the most consistently high *Beauveria* counts. Also, the strain of *Beauveria* in this soil differed morphologically from that in other soils, since it made a very white, Isaria-like synnemata. Interestingly, the incidence of entomopathogens in various composts and manufactured topsoils was extremely low. It seems reasonable to propose that grasses growing on substrates without entomopathogens would be more susceptible to chinch.

As a prelude to inoculation trials, tests were made to determine if *Beauveria* spores remained viable after addition to soils. The effects of a variety of factors including soil type, temperature (ca 20C^o, 4C^o, -12C^o), presence of grasses, soil drying, and use of synthetic chemicals (fertilizer, insecticides, herbicides) was assessed. The tests only extended for two months, but no pronounced (e.g. order of magnitude) effects were noted. These results suggest that if spores of are added to soils by e.g. inoculation, the spore levels should remain high in the soil for at least a few months, even if the temperature fluctuates, chemicals are used, etc. This suggests that chinch control by inoculation should be feasible, <u>if</u> the spores have high enough virulence. The inoculation trials to be done in year 2 will address this question.

III. Rearing Chinch Bugs. At the beginning of the project, it became evident that having a dependable supply of chinch bugs at various developmental stages would be useful for testing *Beauveria* isolates, or for assessing endophyte-enhanced grasses. With these goals in mind, we compared a number of approaches. In initial trials, we tried rearing the insect on sod maintained in a screen-covered container in the lab, but this approach failed due to the proliferation of insects and diseases when the sod was enclosed. The literature suggested that the insect could be reared on corn or sorghum, so we grew these plants in closed pots of soil, with 6 inch diameter clear plastic tubes over the upper part of the plants to retain the chinch bugs. These tubes had holes cut in them for ventilation, the holes being covered by a fine screen mesh to retain the insects. Field-collected chinch bugs were added to the tubes.

We succeeded in rearing *some* chinch bugs in these systems, with eggs and the various instars appearing over the course of a few months. However, we could not rear *large numbers* of the insects. One key issue was maintenance of healthy host plants. On two occasions, as the chinch began to multiply, so did thrips. Plant stress, possibly caused by the chinch, may have attracted these. We could not eliminate the thrips without also killing the chinch, so we had to start over. A second problem stemmed from the small size (1 L) of the containers in which the sorghum plants were grown. This small volume made it difficult to maintain the low water conditions believed to encourage development of the insects, without drought-stressing the plants. Including a layer of perlite in the bottom of the pots alleviated this problems, the perlite serving as a water reservoir. A third problem related to the chinch bug's un-surpassed ability as an escape artist. Until we replaced the mesh on the vents of the containers with very fine mesh screen, the nymphs would often be found outside of the pots. However, even when these three problems were resolved, the chinch bug numbers in the systems would not increase. The insects would go through their life cycle, but the numbers dying were greater than the numbers born, so the population decreased. After conferring with a variety of people who have raised chinch bugs, we now believe that our growing facility may be inadequate. Most people have used greenhouses where space limitations are less and light intensity is much higher. If we can locate greenhouse space, we will try this.

A very interesting observation made during year 2, was that adult chinch bugs could be collected from lawns even in the winter. Many insects (hundreds per square meter) were collected from the surface (leaves, crowns, thatch) of a lawn in January. When the sample was thawed in the lab, the insects became active, and appeared to be healthy. These insects have been successfully used in some lab experiments.

Introduction and General Background For the Project

The hairy chinch bug (*Blissus leucopterus*) is ruining lawns in New Brunswick and elsewhere. This insect feeds on the stems and leaves of various grasses, and in so doing causes unsightly, spreading, discoloured or dead areas. Although certain chemical insecticides can kill the insects, use of these is falling out of favour due to their adverse effects on human and environmental health. Also, their efficacy is decreasing as the insects acquire resistance to the chemicals.

The overriding goal for this project is to gain insight into certain factors that may be contributing to the chinch bug problem. This information will be useful for designing an IPM-compatible control strategy. The project stemmed from an on-going turf research program in New Brunswick, some results of which have been presented by Wetmore (2003), and by Wellwood, Nickerson and Wetmore (2003). This program, in conjunction with the literature suggested two microbiological factors that might be of significance. These are "endophyte-enhanced" grasses and "entomopathogenic fungi".

Endophyte -enhanced grasses contain fungi, that are *in* the *plant* (as "*endo-phyte*" indicates). Some species of these fungi (e.g. *Neotyphodium* sp.) can make the plant more resistant to damage by grazing of chinch bugs (Richmond and Shetlar, 2000) or other animals. The fungus is carried between generations in the seed, and when this germinates, the fungus grows up through the stem, leaves, stalk, flower stem, and into the flowers and developing seeds. The hyphae (fungal filaments) in the plant tissue do not damage the tissue, but in contrast, may make compounds that deter insect feeding, or otherwise give the plant an adaptive advantage. Depending on species and cultivar of grass and the specific conditions, any of many fungal strains may be involved in the association. These can differ markedly in their characteristics, but at least some of them have been shown to make the host grass relatively resistant to chinch bugs. Consequentially, some endophte-enhanced seeds are being marketed for chinch control.

There is however some question as to whether all of the fungal-grass combinations that are being promoted are actually effective. For example, it has been suggested that the fungus may not persist well in seeds. Furthermore, the fungal strain in the grass may or may not make compounds that are active against the chinch bugs. These and other issues are being investigated in Section I of the project.

In the second part of the project (Section II) we explored the idea that entomopathogenic fungi may be related to the chinch bug prolem. Again, as the name suggests these are fungi that cause insect disease. There are many species of these, but two that are well known, forming the basis for some biocontrol products that are marketed in certain parts of the world are *Beauveria bassiana* and *Metarhizium anisopliae*.

Both the literature and field observations suggested that *Beauveria* may be an important factor related to the chinch bug problem. For example, in agricultural crops, it has been documented that a sudden decline in chinch bugs can be associated with the appearance of *Beauveria*-covered dead bugs. Turf grass researchers in New Brunswick and elsewhere have also sometimes see dead chinch bugs covered by white fungal filaments ("mycelium") that look like *B. bassiana*. Some scientific papers have confirmed that exposing chinch bugs to *Beauveria* sp spores (or those of Metarhizium sp.) can kill them (Krueger et al. 1990, Samuels et al. 2002, Samuels and Coracina 2004).

It could therefore be hypothesized that factors in chinch-infested lawns in some manner inhibit growth or persistence of the *Beauveria*, or interfere with its ability to kill the chinch. For example under the dry conditions (which seem to favour development of chinch) the *Beauveria* may not be able to infect the insects. Or alternatively (and there is support in the literature for this idea) the *Beauveria* may not sporulate on the dead insects, so high concentrations of fungal propagules do not develop in the soil.

In the previous New Brunswick field work, it was noted that chinch populations were higher in plots where peat moss had been mixed into the soil. It was hypothesized that this peat may have somehow inhibited *Beauveria* thereby allowing the chinch to thrive. The New Brunswick field work also showed that certain anomalous plots never have chinch. One such area is Angelview Park, in Fredercton. One suggestion has been that the soil in this park may contain either high amounts of *Beauveria* sp, or possibly a *Beauveria* strain that is particularly active against chinch. Some of these ideas are being explored in Section II (Entomopathogens) of the project.

During the first field season, it was (ironically) difficult to find large chinch populations in Fredericton. (This may have related to the season being wet). Although we did obtain many chinch bugs from Newfoundland (thanks to Dr. Peggy Dixon and Nancy Hudson), it is debatable whether these were in good health after shipping and storage under refrigerated conditions. It was decided that progress would be improved if we could grow chinch bugs. This became the goal for Section III of the project.

Another goal of the project was to review the literature. The literature concerning endophytes, entomopathogens and chinch bugs is very large. Since the start of the project a useful overview of much literature concerning these subjects by Dr.Patriquin, Dalhousie University (http://www.versicolor.ca/lawns/secF.html) has become available. This gives a good overview of the context for this project. Additional papers with relevance to the subject are available on request from Maritime MicroBiologicals Inc.

Section I. Endophyte-enhanced grasses.

A) Assessing grasses grown in plots or in the lab for endophtes.

A wide variety of grasses that are being tested for performance in field plots at NBDAFA (Garth Nickerson,NBDAFA contact person) were assessed for endophyte content. The same seed lots used for the field plots were planted in pots of pasteurized soil in a controlled growth box (16 hr day at 31 μmol m^{-2} sec^{-1}, 25°C day, 20° night) and tested. A picture of some of these grasses, taken from the field plot on September 1, about 3 months after planting, is below. (Attention is drawn to Victory II and Treazure since these chewings fescues grew well, and had a form that would be acceptable for lawn use. They are discussed in more detail below).

Fescues that are being tested in Fredericton field plots about 4 months after seeding.

For both the field and lab-grown plants, endophyte assays were made in two ways. The first used a commercially-available immunoassay kit from Agrinostics, Ltd. Co. Watkinsville, GA. This test was specific for *Neotyphodium* sp. (Nick Hill, Agrinostics, Pers. Comm.). The other was a staining/microscopy assay. Both of these assays had been suggested by Sylvie Rochefort, PhD candidate, Laval University. Results obtained by the two methods have been shown to correlate well (Hiatt et al. 1999).

The directions that came with the immunoassay test kit were followed, except they were modified to accommodate the smaller stem size of some of the fescues. (The test kit is designed for tall fescues rather than for chewing or sheeps fescues). To this end, in stead of placing a cross section of the stem on the test membrane, a short (ca 1mm) longitudinal section of the stem was used. A picture of the assay membrane, before (left) and after (right) development is below. Red coloration indicates there is Neotyphodium in the tissue. Positive (Neopyphodium sp. abundant) and negative (no Neotyphodium sp) controls are in the right upper corner. It is evident that, at least sometimes, the assay is somewhat subjective since there may be some red formed even when the fungus is not present.

The microscopy assay was made on leaf sheath tissue obtained from plants about 3-6 weeks after germination, depending on the species and growing conditions. The aim was to obtain samples soon after the leaf sheath formed, since when it becomes too mature it becomes opaque, and the fungus in it can not be seen. The tissues were stained using Rose Bengal following the procedures described by Saha et al. (1988). Some pictures of endophyte positive leaf sheathes viewed under 400X magnification are below. The hyphae wend a convoluted path between the cortical cells, this being characteristic of *Neoptyphodium* sp. In a few cases hyphae were seen that were clearly not Neotyphodium. For example, hyphae connected to what looked like *Alternaria* spores were seen. These may have been decay fungi, and were not included in the results.

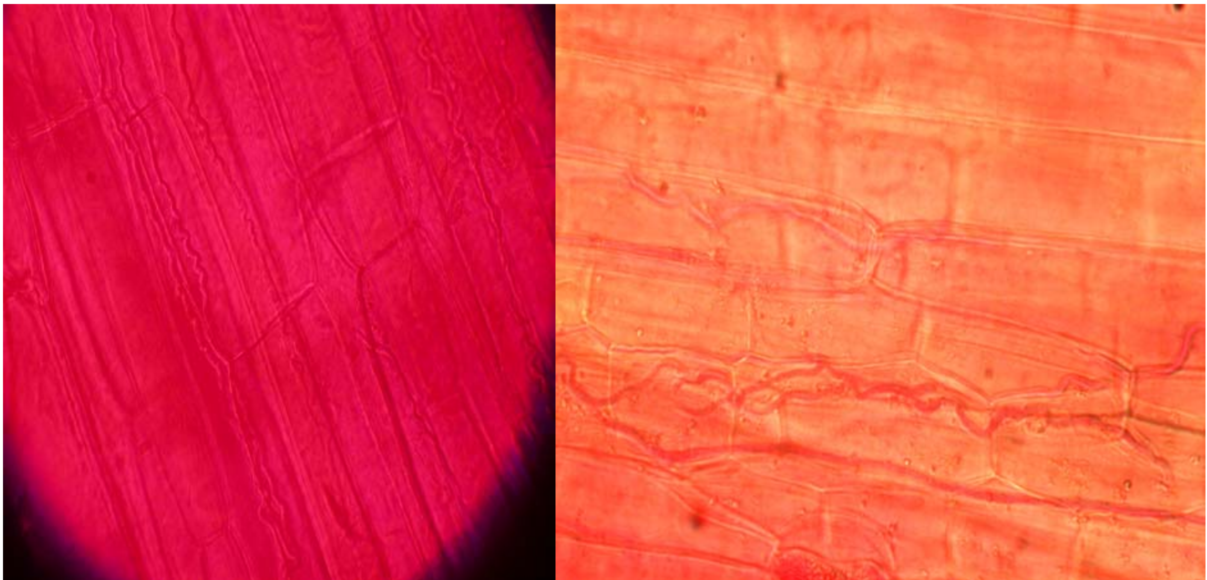


Photo showing endophyte hyphae in cortical cells of leaf sheath after staining. Both at 400X. Both would be rated as endophyte positive.

The results from these two assays (Table 1) did not correlate perfectly, although the general trends were similar. As noted at the base of the table, the number in parenthesis is the number of plants sampled, while the number in bold is the average number of these samples with *some* endophyte (i.e. it is the *incidence* of colonization). It should be noted that neither result gives any

indication about the *amount* of fungus present (i.e. *intensity* of infection) in the sample. It was clear however from the microscopy that in many cases there was not a lot of fungal biomass present.

The positive control (a tall Fescue supplied by Agrinostics with their test kit) gave 60% positive using the test kit, while by microscopy 92% of the plants contained endophyte. Interestingly, when observed by microscopy, the fungal hyphae looked similar to those observed in e.g. Treazure, but they were much more prevalent. (i.e. the intensity of infection was higher).

The Superblue KBG mix had quite high endophyte incidence using the test kit, but it was not clear whether this was from the KBG or from associated grasses that might have dominated the sward. The latter seems probable since KBG does not normally support endophytes. Staining was not done with this mix.

The perennial rye had quite high endophyte incidence measured by either method.

Table 1. Percentage of plants with endophytes present as measured using Agrinostic's immunoassay test kit, or as measured by microscopy*.

Grass cultivar	Test Kit Pooled (#) %	Microscopy Pooled (#) %
Aruba Red	(14) 21	(21) 0
Badger	(14) 14	(12) 0
Florentine	(14) 7	nd
Ecostar	(18) 22	(40) 7.5
Chewing	(14) 14	nd
Victory II	*(14) 7*	*(36) 0*
Treazure	*(22) 64*	*(36) 70*
Berkshire	(14) 0	(12) 8
Brigade	(14) 21	(12) 0
Hard	(14) 7	nd
Blacksheep	(14) 7	(12) 0
Azure	(14) 7	(12) 0
Superblue KBG mix	(14) 57	nd
Scotts Bluegrass	(18) 17	(44) 36
Scotts Perenial Rye	(18) 55	(44) 36
Pos ConTall Fes	(9) 60	(12) 92

*The number of plants assayed is in parenthesis. Numbers to the right of the parenthesis are the percentage of the assayed plants where at least some endophyte was present. Nd=not determined.

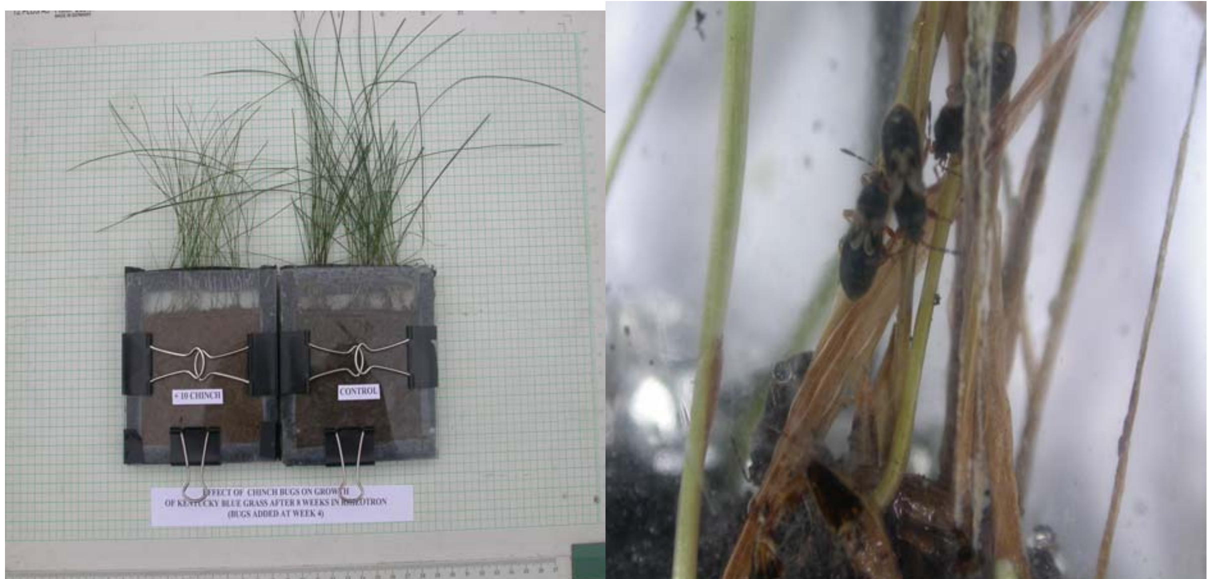
Of note is that both assays showed that Treazure was relatively highly colonized (64% by test kit, 70% by microscopy),while the morphologically-similar Victory was almost endophyte-free (7% by test kit, 0 % by microscopy). Endophyte levels in the other fescues were low despite many of them allegedly being "endophyte enhanced". It is possible the endophyte in the seed died prior to planting. This possibility is explored below.

As mentioned above, we compared Victory II and Treazure in subsequent lab tests since these two are otherwise similar, both showing reasonably good performance (reasonable looking turf) in the field plots. Tests in the lab also showed the germination rate for each was over 80%, and growth rates were similar (data not presented). These cultivars were tested in rhizotrons (see below) to determine if they differed in their ability to support chinch bugs.

B) Testing interactions of grasses and chinch bugs in rhizotrons..

We grew a number of grasses in the lab in various containers, with or without chinch bugs added. In one set of tests we used "rhizotrons", these plant-growing systems facilitating observation of the plants, and retention of the chinch bugs around their leaves. The rhizotrons were made using 10 cm x 10cm x 1 mm glass plates with a strip of 6.4 mm x 9.5 mm x 3.7 mm sticky-backed foam insulation tape stuck around their margins. Two plates were then "sandwiched" around a 1.5 cm-thick layer of pasteurized soil. A strip of filter paper extended out from between the foam strips at the bottom, and up into the soil. As such, placing the chambers into a dish of water wicked the water into the soil. About 20 seeds were planted into the soil along the top edge of the chamber. After about 2 or 3 weeks, the chambers were partially opened at the top, and the leaves were manipulated between the two pieces of foam. Chinch bugs (collected from lawns in Newfoundland and stored in the refrigerator until use) were placed into the chambers at this time. The chambers were re-closed, the clips serving to compress the foam strip together around the grass leaves so the chinch bugs could not escape.

In a first trial, we planted Kentucky blue grass in the chambers. When the plants were four weeks old, 10 chinch bugs were added to some chambers, while others remained as controls. After an additional four weeks, it was evident that the chinch bugs had inhibited the grass' growth. Chinch bugs could be seen climbing on, and feeding on the grass stems. The system was useful for observing the insects' behaviour. (See photo's below. A short movie was also made, this being available from *Maritime MicroBiologicals Inc*.)



***Left photo*. Rhizotrons with 8 wk old Kentucky blue grass with (left) or without (right) chinch, four weeks after the insects were added. *Right photo*. Chinch bugs on stems of grass in rhizotron.**

In another trial, we made three rhizotrons like those above, but 25 cm wide, instead of 10. In each, one side was planted with twenty Victory II seeds, and the other with twenty Treazure seeds. When the plants were four weeks old, twenty chinch bugs were added. Every few days, the bugs in the chambers were observed to see if there were any pronounced differences in distribution pattern, feeding, behaviour, etc. between the two cultivars. No differences were observed. (Data not presented). Unfortunately, many of the insects escaped from, or died in the systems, so by the end of the two weeks, only a few insects were left. Also, it was noted that the insects sometimes clustered together, frequently in a corner of the chamber, so they were not actually on either grass, making data collection difficult. This clustering behaviour also occurs in nature (Dr. Baxendale, pers. Comm.).

On quite a few occasions, insects were observed feeding on grass stems, no particular preference being shown for the Victory II over the Treazure, or visa-versa. (See photos below). On one of these occasions, note was made as to which specific Treazure plant the insect had fed on. At the end of the two week period when the chamber was opened, this particular plant was removed and its leaf sheath assessed microscopically for endophyte. The fungus was present.

In short, no striking differences in chinch bug behaviour was noted between the chinch's behaviour towards the Victory II (no endophyte) and the **Treazure** (endophyte present) grasses. The chinch would feed on Treazure, even when this contained endophyte. It seems clear that the endophyte in Treazure does not confer a high level of protection against chinch bugs.

Left photo. Chinch bug on Treazure stem in rhizotron.
Right photo. Chinch feeding on Treazure stem. Subsequent microscopy showed endophyte was present.

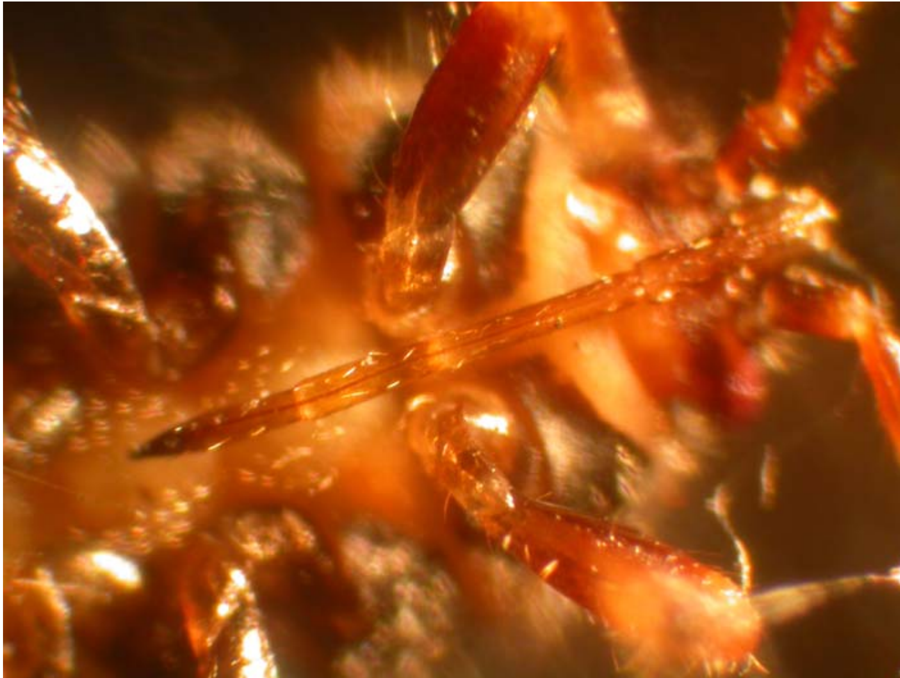


Photo. Chinch from rhizotron. Mouthparts.

C. Effect of storage on persistence of endophytes in seeds. As mentioned above, endophyte was only present in a few of the grasses that were tested, despite their alleged endophyte-positive status. It was hypothesized that this may have been due to the endophyte having died during transit from the supplier, or in the few weeks when they were subsequently stored under office conditions prior to planting. To assess this possibility, we stored samples of both Scotts Perenial Rye and Treazure (both endophyte positive) under a variety of conditions, and then re-assessed them for endophyte after various times.

To establish defined storage conditions, samples of the seeds were placed in 8 ml perforated plastic vials, which were in turn held in 500 ml mason jars. The perforations in the vials allowed passage of air, but not seeds. For dry conditions (ca 0% relative humidity), 20 g of oven-dried silica gel was included in the mason jar with the vials. For 75% relative humidity, a shallow dish containing glass wool and a saturated NaCl solution was included with the vials. The RH above this solution is 75% as described at: http://www.padfield.org/tim/cfys/satslt/satsalt.php. For variable humidity, the mason jar was left open. Replicate jars were kept in either a 25°C incubator, or in a 4°C refrigerator. For variable temperature and humidity conditions, the mason jar was stored open on a window sill from September until when the assay was done. The temperature here ranged from a high (~30 ^{o}C?), when the sun shone in the window, to a low (~5 ^{o}C?), when there was frost on the window. Humidity also would have ranged widely.

After the indicated storage time, 40 seeds were planted into pasteurized Scotts soil in 2 ½ ” diameter square pots in the growth box. After 7 days, the number of seeds that had germinated was counted so percent germination could be calculated. After 5 weeks, the leaf sheath of plants was microscopically assayed for endophyte using the methods described above.

The results (Table 2) showed that the amount of endophyte in Treazure dropped from an initial value of 60% before storage, to considerably less than this (0-25% depending on conditions) after 5 months. Interestingly, endophyte survival was best (25%) when the storage conditions

were most variable, or when the temperature and humidity were relatively high (17%). Under the normally-recommended storage conditions (cool, dry) no endophyte was detected. With the Scotts Perenial Rye, the endophyte was more stable, colonization being significant in seeds stored at all conditions. In contrast to endophyte survival, seed germination remained high with both grasses under all conditions, except with Treazure at 25°C and 75% RH where it decreased to 57%.

Table 2. The effect of storage conditions on seed germination and endophyte status.

Grass Variety	Months Stored	Storage Conditions		Seed Germination*	Endophyte Status**
		Temp.(^{o}C)	Rel. Hum.(%)		
Treazure/ Before Storage		NA	NA	**87** (15)	**60%** (20)
Treazure/	5 months	4	0	**90** (0)	**0%** (20)
		4	75	**93** (4)	**0%** (24)
		4	Variable	**78** (5)	**6%** (16)
		25	0	**82** (4)	**0%** (12)
		25	75	**57** (10)	**17%** (12)
		25	Variable	**90** (10)	**0%** (12)
		variable	Variable	**92** (4)	**25%** (12)
Scotts Peren. Rye/ Before Storage		NA	NA	**85**(5)	**31%** (32)
Scotts Peren. Rye/ after 5 months		4	0	**88** (10)	**25%** (28)
		4	75	**80** (20)	**31%** (16)
		4	Variable	**92** (11)	**8%** (12)
		25	0	**84** (9)	**8%** (12)
		25	75	**85** (4)	**16%** (12)
		25	Variable	**81** (5)	**50%** (12)
		variable	Variable	**84** (2)	**16%** (12)

*Numbers are the percent germination in bold, with the SD (n=40) in parenthesis.

** The number in bold is the percentage of plants with any endophyte. The number in parenthesis is the number of plants sampled.

The assays were done again after the seeds had been stored for an additional 5 months (June 2005). The results (not presented) showed that the endophyte had *completely disappeared* from both the Treazure and the Perenial Rye. Percent germination of the seeds was still quite high (>70%) except under conditions of high (75%) humidity and 25C. These results show clearly that the endophyte can die more rapidly than the seeds.

In July, a western US seed company sent samples of Tall Fescues, some of which were allegedly endophyte positive, and others negative. Although these were sent by Courier, they were in transit for over a week during which time they were probably stored at room temperature. Upon arrival at Maritime MicroBiologicals, they were placed in a cool (4°C) dry location. One week after arrival, they were planted in small pots of soil, and the percent germination was measured. After 4 wks growth, samples of the leaf sheaths were taken, stained and assessed for endophyte.

The results (Table 3) showed that very few of the plants had endophyte in them.

Table 3. Percent germination and endophyte status of grasses.

Cultivar	Alledged endophyte status	% Germination	Measured endophyte
Jasper E 017-9-ORF-2	?	18%	0/8 (0%)
Jasper E CRF 24-4-41	?	74%	0/8 (0%)
Grande II tall fescue a	90%	75%	4/32 12.5%)
Grande II tall fescue b	0%	88%	0/8 (0%)
Victory II M22-4-501	?	18%	1/8 (12.5%)
Victory II M31-4-112	?	90%	0/8 (0%)

The number of positive plants / number of plants assayed. This is expressed as a percentage in the parenthesis.

There were a few additional plants that could have been rated as "endophyte positive" but the hyphae were associated with Alternaria-like spores, or they appeared to be decay fungi, possibly degrading the older leaf sheath cells. Although these fungi might have technically been "endophyte" (i.e. "in-plant"), there is little reason to think they would inhibit chinch bugs.

Subsequent discussions with a staff member at the company indicated that the seeds' endophyte status was based on results obtained with an immunoassay. He indicated that it is often assumed that if a plant is positive, seeds taken from that plant will also be positive. This is probably not a valid assumption since i) the immunoassay will register positive even if the *Neotyphodium* is dead and ii) even if the *Neotyphodium* is alive, it will not necessarily grow completely through the plant and into the seeds. Information about efficacy of the endophytes with respect to chinch bug control was not available.

Conclusions from endophyte work (Section I). Although much research and commercial literature supports the idea of using endophyte-enhanced grasses as part of a chinch management strategy, it is not as simple as planting "endophyte-enhanced" seed. Our results indicate that many allegedly endophyte-enhanced seeds (e.g. Victory, many of the other fine fescues we tested) have little or no endophyte in them. Even when there is fungus in them, the amount of mycelium present, as judged by microscopy, is much smaller than in endophyte-containing grasses that have been shown to be chinch resistant. Our results contrast with the literature that shows it is necessary for a high proportion of the grass plants to be colonized if chinch control is to take place. Richmond and Shetlar (2000) showed good control required that <u>most</u> (e.g. 90%) of the grass was colonized. Use of the right cultivar with the right endophyte is also important. Mathias et al (1990) showed that the endophyte-containing perennial ryegrass "Repell" was good. Yue et al.'s data (2000) demonstrates the necessity of having the right cultivar-endophyte combination. It might also be necessary to have the plant-fungus combination growing in conditions where the compounds that are active against the chinch are produced. Little is known about this.

The endophyte in the colonized chewing fescue (cv Treazure) with which we worked may not be active against chinch. Indeed, we observed chinch bugs feeding on plants where the endophyyte was present. No effect on insect health was evident. It may be the intensity of colonization was not high enough. Our subjective observations showed that the density of hyphae in the leaf sheath and stem of colonized Treazure was much lower than in some endophyte containing tall fescues. In future work, it might possible to quantify the density of endophyte hyphae. This could be done microscopically (e.g. grid line intersect method) or chemically.

Good chinch control also depends upon the grass endophyte producing metabolites that are active against the insect (Wilkinson et al. 2000). These may only be produced under certain conditions. A good review, including more references and interesting information about the effects of the various alkaloids on various species of insects and mammals is presented at: http://www.plantphys.net/printer.php?ch=13&id=37. If the alkaloids that are active against chinch bugs are identified, it might be possible to assay fro these chemicals in "endophyte-enhanced" lawn grasses, thereby attaining evidence for their resistance to chinch.

In subsequent work, if endophyte-containing grasses are being assessed for chinch control, a positive control should be included so that the results build upon the positive results that others have obtained. For example, the perennial ryegrass "Affinity" has been shown to have an endophyte that is active against chinch and other insects. Another possibility would be "Repell". These cultivars might then be compared to fine fescues (such as Treazure) which are less well characterized. However, prior to conducting any tests, it would be cost-effective to confirm that the seeds batches that are being tested *actually contain a stable, viable endophyte association*. After this is established, the next step might be small scale (inexpensive) lab-scale assays such as those described by Yue et al (2000) or Mathias et al. (1990). These might serve to identify appropriate candidates for the subsequent, relatively labour intensive field trials.

Even when an active endophyte-grass combination with agronomic properties suitable for our region is identied, there is still some question as to whether it should be used as part of a chinch control program. Some reasons for this are discussed in an e-article by Dr. D. Patriquin (Dalhousie University) entitled "Endophytic grasses for turf. Full speed ahead or proceed with caution?. (www.versicolor.ca/lawns/docs/dpEndophytes.html.) It is pointed out there that some endophytic grasses can actually *attract* chinch, some can be toxic to birds or mice, some might cause trouble if the seeds spread into pastures, etc. In short, a good case is made for "proceeding with caution". Taken in combination with the information shortage about specific cultivar/endophyte/chinch bug relationships, it does not seem right that the New Brunswick landscape industry or homeowners should be paying a premium for "endophyte-enhanced" seeds. The onus should be on the seed industry to demonstrate that their seeds actually contain viable endophyte, that this does what "enhanced" implies (e.g. inhibits chinch) and that it does not pose a significant risk to non-target species.

Section II. Entomopathogenic Fungi and Charactization of Soils.

During late Fall (October, November), soils were collected from a number of sites (Angelview, various test plots, some manufactured topsoils, etc.). The samples were stored at 4°C in loosely closed plastic-coated bags until they were assayed 3 or 4 months later. A primary goal of these assays was to determine if entomopathogens such as *Beauveria* and *Metarhizium* were present in the soils. A secondary goal has been to characterize the soils for a few other parameters

(pH, bacteria numbers, FDA, moisture) that might be of general interest and might also relate to the chinch problem. To this end, we developed the assays described below.

To assess the prevalence of entomopathogenic fungi in soils, we used two assays. The first was a dilution plate count assay, based on use of a semi-selective medium. The second was a bioassay based on larvae of *Galleria*, the wax moth. We also did a number of assays to characterize the soils. The methods used for each of these is described below.

A. Methods. Selective plate count media for entomopathogens. Plate counts for soil microbes. *Galleria* bioassay for entomopathogens. pH, soil moisture and Fluorescein diacetate hydrolysing activity (FDA) . Soil respiratory activity.

Selective Media for Entomopathogenic Fungi. In an initial attempt to quantify entomopathogens in soil, we used some selective media described in the literature. These could detect the fungi, but only if the soil had been “spiked” with over 10^5 *Metarhizium* or *Beauveria* conidia per gram. When spore numbers were small, as we suspected they would be in some of our soils, other microbes from the soil out-competed the entomopathogens on the agar.

We therefore developed two new media. Both are modifications of the medium presented by Vaninen et al.(2000) using some information presented by Bruck (2004). Our EE series of media differ from Vaninen’s in that the amounts of antibiotics chloramphenicol and cycloheximide is altered, it contains rose Bengal, and it has more agar. EEF has the same basic composition as EEA but contains more chloramphenicol and cycloheximide, and also contains small quantities of the fungicides dodine and benomyl. EEA supports growth of *Beauveria* and *Metarhizium* (along with some other Hyphomycetes, Zygomycetes and some bacteria). EEJ is more stringently selective for *Metarhizium*.

It should be noted that neither medium (nor any of the others that we tested) is *completely* selective. It is necessary to microscopically confirm the identity of fungal colonies by aseptically transferring them to Sabouraud Dextrose agar. Both *Metarhizium* and *Beauveria* sporulate (make conidia) within about two weeks on Sabouroud. Microscopic examination of the spores and spore-bearing structures (conidiophores) are compared to those of reference cultures to ascertain fungal genus, and sometimes species.

The compositions and methods for making EE and EEJ are:

Distilled water	For 1 liter	For 250 ml
Glucose	5 grams	1.25 g
Yeast Extract	2 g	0.5 g
NaNO3	1 g	0.25
Anhydrous MgSO4	0.25g	0.06g
KH2PO4	1g	0.25g
Sodium Propionate	1g	0.25
Bile (Oxgall)	1g	0.25
Water	1 l	250 ml
Rose Bengal stock	2 mL	0.5
Agar	18 g	4.5g

Mix all ingredients (except antibiotics, stock 1 and 2, see below). Microwave to melt agar, then autoclave for 15 min at 121 C. Remove from autoclave and when cooled to ca 70C (still hot) add antibiotic stocks and mix well.

For EEA, add 200 µl l^{-1} of stock 1. For EEF, add 800 µl l^{-1} of stock 1 and 800 µl l^{-1} stock 2. In both cases, mix the agar well and then pour plates. After they are cooled, and before using them, the plates are opened and placed under a HEPA-filtered air stream for 30 min to dry their surface before inoculation.

> Stock 1 = 250 mg chloramphenicol + 1000 mg cycloheximide in 5 ml Ethanol. Stored in freezer.
> Stock 2= 5 mg benomyl and 38 mg dodine in 10 ml acetone. Stored in freezer.
> Rose Bengal 0.25 g in 25 mL water. Stored in freezer. Some of these are suspensions. They are heated in hot water, sonicated and mixed before use.

For identification, the entomopathogens are transferred to Sabouraud Dextrose Agar. A commercial preparation of this is available from Becton, Dickinson and Co. or Difco. Alternatively, it can be made by autoclaving 10 g casein hydrolysate, 40 g of dextrose and 15 g of agar in a liter of water.

We also wanted to be able to detect other microbes (e.g. bacteria) in the soil. To this end, we used Standard Methods Agar (SMA). It is made by adding 2.5 g yeast extract, 5 g of casein hydrolysate, 1 g of glucose and 18 g of agar to a liter of distilled water. The pH is adjusted to 7.0. The medium is then heated to dissolve the agar, autoclaved, and dispensed into Petri plates. As discussed in b below, we use it in our dilution assays to give an indicator how much microbial activity there is in the soils.

Quantification of entomopathogenic fungi and general bacterial populations in in soil.

For these measurements a dilution plate count is used. For the dilution, 4 g samples (fresh wt) of the soil are placed into vials containing 18 mL of 0.05% Tween 80 and 4, 1g steel balls. The vials are shaken at 250 rpm on a reciprocal shaker for 15 min. They are then sonicated (Sonic Dismembrator 300, Artek, NY) for 5 min, and then shaken again for 5 min. The goal is to dislodge the microbes from the soil. Samples of the suspension are then further diluted by shaking with 1 mm glass beads in vials containing sterile 0.05% Tween 80 to give 10X, 100X, 1000X etc. dilutions. Aliquots (20 or 100 µl) from these are then spread onto EEA, EEF or SMA plates using a bent glass rod ("hockey stick"). Plates are then incubated in a 25°C incubator until the propagules grow into visible (countable) colonies.

Colonies on the SMA plates (bacteria and fungi) are counted after 4 days. The number of colonies is mulitiplied by the dilution factor. It should be noted that the result is *not* a count of total bacteria or fungi, but only an index, since only a fraction of soil microbes grow on SMA (or any other agar). Note is also made of the number of colony types (distinct morphologies) on the highest dilution plates. This number serves as an index of microbial diversity.

Colonies on the EE and EEJ are counted after 7 days. Those that look like *Beauveria* or *Metarhizium* are transferred to Sabouraud agar where they produce conidia. Microscopy is used to confirm their identity. Mulitplying the number of *Beauveria* or *Metarhizium* colonies on the EE or EEJ plates gives an estimate of the number of propagules of *Beauveria* or *Metarhizium* in the soil.

If no entomopathogen-like colonies are observed, it is assumed there are fewer than the detection limit. This is calculated by considering the amount of soil used for the smallest dilution, assuming that one conidium would form one colony.

Qualitative bioassay for Entomopathogens. We also used a modification of the *Galleria* (wax moth larvae) bio-assay for entomopathogens (Zimmerman, 1986), since this baiting method is more sensitive than the plate count. For this, the test soils were sieved through a 1 cm mesh sieve and 10 g of moist soil placed into clear plastic 2 oz portion cups with lids (Sweetheart product UR2H, available at Scoop and Save, Fredericton). Ten pin-holes were put into each lid. Care was taken to use active, light coloured larvae, these being bought at Pets Unlimited, Fredericton. Four were placed into the soil in each cup. The containers with soil and larvae were kept in a 25°C, 70% Relative Humidity incubator. Each day the larvae are assessed for viability, and the containers were shaken gently so the larvae are re-covered with soil. In soils without any entomopathogenic fungi, the larvae often remain active for over 20 days, or until they started to pupate. As discussed in the results, they died much more rapidly if entomopathogens were present.

After death, larvae were kept in the containers, the soil being kept moist until the fungi emerged from the cadavers and sporulated. Microscopic examination served to show whether the fungus that caused death was *B. bassiana*, *M. anisopliae*, or some other factor. In some cases the dead insects were retained in the containers an additional month, during which time some interesting mushroom-like structures (synnemata) sometimes grew.

Soil pH was measured by making a slurry of 5 g of the soil with 5 g of distilled water. After 20 minutes the pH was measured after standardization of the electrode with pH 4 and pH 7 buffers.

Soil moisture was detemined by weighing sub-samples of the fresh soil, and then drying these to constant weight at ca 70°C. The weight lost upon drying (i.e. wt of water) was divided by the dry weight of the soil, and multiplied by 100% to give the soil moisture.

Fluorescein diacetate hydrolysing activity (FDA) was used as an indication of total microbiological activity in the soil. A wide variety of enzymes (e.g. proteases, esterases, etc.) cleave this colourless substrate into the green-coloured fluorescein, so the rate at which this colour appears can be used as an index of the total amount of microbial activity in the soil. (See Schnurer and Rosswall, 1982 or Boyle and Kropp, 1992 for a discussion of the attributes of this assay). In principle, the assay is similar to commercial assays used to test compost maturity.

In the version of the FDA assay we used, 10 g of the soil was shaken in pH 7.2, 95 mM phosphate buffer for 30 minutes. Steel balls were included to give thorough disruption of soil aggregates. One ml sub-samples of the suspension were placed into tubes with 9 ml of reagent (10 mM phosphate buffer containing 20 mg/l of the fluorescein diacetate) and shaken for three hours. The tubes were then centrifuged at 5000g for 1 min, and the amount of colour (i.e. flurescein) in the supernatant determined spectophotometrically by measuring the optical density at 490 nm. The OD of replicate samples that were not incubated for 3 hrs (reagent blank) was subtracted from the OD of the samples. By considering the incubation time (3 hrs), the amount of sample per tube (0.01g) and the moisture content of the soil, values were corrected to give the change in OD per hour per dry g of sample. This is an index of total microbiological activity.

Respiratory activity. In some cases we measured carbon dioxide production (respiration) by soil samples, this, like FDA being an indicator of metabolic activity. For this assay, we placed 100 g of the soil into a 1 liter mason jar. After 1 and 4 hours, the carbon dioxide concentration in the jar was measured using a GasTech model R1-411A gas analyser. The difference between the two values was used to calculate the respiratory rate.

B. Results

Assays # 1-5 were used to characterize the various soil samples collected during the first year. The results are presented in Table 4.

Briefly, the pH of most of the soils was somewhat acidic, typical for soils in the Maritimes. Conventional wisdom would suggest that adding lime might be of benefit for plant growth. However, to contradict conventional wisdom, the pH in the Angelview soils was relatively low, yet grass growth was good, and there is no chinch problem. The pH at the CEB plots was relatively high, but (not surprisingly) decreased when peat is added. It is possible that the higher chinch numbers attributable to peat in these plots may actually relate to pH effects.

The % moisture of the soils was probably not of particular interest. We simply used the values to correct data to a dry weight basis. None-the-less, they show a tendency to be higher in samples with more organic matter (e.g. compost) since this both holds more water, and has a relatively low density. It might be worthwhile to determine the water-holding capacity of chinch-prone and chinch-resistant lawns, since moisture content is known to affect chinch severity.

The FDA values are generally lower than what we have previously found with freshly-collected soils or composts. These soils were stored for a few months which may have caused many of the microbes to go dormant. In some cases, FDA was negligible, even though the microbial counts (next column) showed there were lots of bacteria present. This was also true with the composts. In previous work we have found immature composts have high FDA activity, but this droped markedly as the composts mature. The relatively high FDA of some soils (e.g. those from Angelview) may reflect the presence of root fragments. These may have served as a C substrate to maintain an active microbial population during storage.

The data under “microbes g^{-1}” are derived from the dilution plate counts on SMA. In general they are typical of soils. It is probably worth drawing attention to the fact that even these relatively low counts are *many orders of magnitude higher* than those for either the *Metarhizium* or the *Beauveria*. The index of microbial diversity, as indicated under “types” is generally lower for the manufactured topsoils or composts than it is for the natural soils, or soils like Angelview that have supported plant growth for some time. Healthy natural soils are known to contain literally thousands of microbial species, although only a small fraction of these will grow on SMA. Increasing the microbial diversity of manufactured soil might improve quality. This might be attained by adding natural topsoil, in addition to compost.

The data under the columns for *Metarhizium* and *Beauveria* requires some explanation to make it interpretable. Please look at the footnote at the bottom of the table. Briefly, the plate count only detected the fungi in a few soils, and their propagule numbers were low. It should be emphasized that *most* of the numbers in the columns are *not* the number of *Metarhizium* or *Beauveria* spores that are present, but rather the detection limit that we used when we assayed for them. In other words we are saying there are *fewer* propagules than the indicated number; possibly none.

It is of interest that *Beauveria* (and *Metarhizium*) were most consistently found in the Angelview soils – It seems likely this means their conidia were more common. *Metarhizium* was also present in soil #16 (Neils Old Pasture). Information about chinch in this soil would be of interest.

It is informative to compare the estimates of *Beauveria* and *Metarhizium* propagule numbers to the conidia numbers that are typically used in experiments to kill insects, including chinch. For example, as mentioned above, Samuels and Coricini (2004) found that chinch mortality was only consistent when they exposed the insects to suspensions containing ca 5×10^8 conidia per ml. In order to get 100% infection of chinch bugs Ramoska (1984) had to put 4.4×10^6 conidia onto each insect. At the conidial densities we detect in the soils, the incidence of infection might not be very high. Chinch control might be attained by inoculating soils with high numbers of entomopathogen conidia, even thought the fungi are considered to be ubiquitous. Increasing the conidia number in the soil should lead to an increase in infection rate (Krueger et al. 1991).

Table. 4. Characterization of soils and plate counts for *Beauveria* sp. and *Metarhizium* sp..

#	Soil Identity*	pH	% H_2O	FDA mean (sd)	Bacteria.g^{-1}	Bacterial types	*Beauveria* (Propagules g-1)	*Metarhizium* (Propagules g-1)
1	Angelview walkways	5.1	31	0.29 (.07)	5.3×10^7	13	2.5×10^3	1×10^4
2	Angelview plot, corn gluten,G3	5.7	29	0.350(.02)	nd			
3	Angelview plot, turkey comp.	5.2	26	0.27(.07)	1.4×10^7	10	2.6×10^3	$<0.5 \times 10^2$
4	Angelview plot, control C1	5.4	27	0.39(.02)	6.6×10^6		nd	2.3×10^3
5	Angelview plot, 40:0:0 M3	5.6	25	0.00(.02)				
6	Angelview plot, 34:0:0 A3	5.4	25	0.09(.04)				
7	Angelview plot, SCU 21:7:7 S3	5.8	25	0.00(.07)				
8	Urban LS. Mature Compost	5.5	48	0.45(.13)				
9	Urban LS. Lawn comp mix	5.9	22	0.00(.02)	2.3×10^7	3	$<1 \times 10^3$	$<1 \times 10^3$
10	Urban Organics, 50/50 soil/comp	6.3	18	0.02(.08)				
11	Urban Organics, 20/80 soil/comp	6	33	0.15(.05)				
12	Quispamsis front field, organic C	5.4	25	0.36(.16)	9.4×10^7		$<2 \times 10^2$	3.4×10^2
13	Quspamsis, main lawn, org A.	5.9	20	0.27(.04)	6.4×10^7	27	$<1 \times 10^3$	$<1 \times 10^3$
14	Quispamsis back lawn, herb, B	5	27	0.23(.07)				
14	Chapel Plaza, high chinch	4.3	20	0.46(.06)	9.1×10^6	12	$<1 \times 10^3$	$<10^3$
15	Elm, high chinch	5	27	0.21(.02)				
16	Neils Field, old Pasture	5	25	0.58(.04)	2.4×10^7	14	1.5×10^3	nd
17	Bark compost	6.9	46	0.00(.03)	4.6×10^7	8	$<1 \times 10^3$	$<10^3$
18	CEB 7, 8, Alt. Soils 2	7.2	21					
19	CEB 45, 46, Alt. Soils 2	7.1	20	0.25(.08)				
20	CEB, 29, 30 Alt. Soils 2	7.3	17					
21	CEB 47, 48, Fred Comp. 2	6.7	15					
22	CEB 1, 2, Fred Comp. 2.	6.2	16	0.00(.04)				
23	CEB, 9, 10, Fred Comp. 2	6.8	17					
24	CEB, 5, 6, Card. Comp.	6.4	18					
25	CEB 27, 28. Card. Comp. 2	6.8	19	0.00(.04)				
26	CEB 19, 20. Card Comp. 2	6	16					
27	CEB 21, 22 Control	6.2	16	0.260.06	2.8×10^8		$<0.5 \times 10^2$	$< 0.5 \times 10^2$

28	CEB 31, 32	6.1	17	0.260.06				
29	11, 12. Control, no peat or com	5.9	14	0.000.12				
30	CEB P2, 49, 50 2” peat	5.6	18	0.160.06	2.2×10^{7}		7.5×10^{2}	$< 2 \times 10^{2}$
31	CEB 17, 18 Peat.	5.4	18					
32	CEB 33, 34. Peat	5.2	16	0.180.10				
33	109 Winchester, Quis.	4.3	25					
34	Monct. Trial Fescues	5.7	21	0.280.06				
35	Monct. Trial Compost	5.4	22	0.170.06				

Important Note: If number has a “ <”prior to it, it indicates we have not found any colonies that look like *Beauveria* or *Metarhizium* at this detection level. ND= not determined. *Additional data available from Garth Nickerson, NBAFA

Galleria assays. Since the plate assay had limited sensitivity and had sometimes given ambivalent results, we decided to use the *Galleria* assay. In an initial experiment, we steamed soil #17 to remove any entomopathogens from it, and then added known numbers of conidia of either *Beauveria* sp. (BotiniGard’s GHA strain)or *Metarhizium* sp (Bid A strain). The results (Table 5) showed that without the conidia added, the larvae survived well in the soil. (They actually pupated at about day 11). However, when either *Beauveria* or *Metarhizium* conidia were added, the larvae died within a week. With both fungi, death occurred more rapidly when more conidia were added. Live and dead Galleria are shown in the accompanying photo.

Table 5. The death rate of *Galleria* on soil with different doses of *Beauveria sp.* or *Metarhizium* sp.

Treatment[1].	Days until *Galleria* dead [2].
Steamed soil 17 + no conidia	> 10
+ 10^3 Beauveria conidia per gram	4-5
+ 10^6 Beauveria conidia per gram	3-4
+ 10^3 Metarhizium conidia per gram	6-7
+10^6 Metarhizium conidia per gram	3-4

1. Soil steamed at 100°C for 1 hr. Assay system incubated at 30°C. Humidity 75%
2. Numbers are the day when *half* of the larvae were dead, followed by the day when *all* were dead.

Live *Galleria* larvae (left) or larvae about two weeks after conidia of either *Beauveria* (middle) or *Metarhizium* (right) were added to the soil.

We then used the *Galleria* assay with a variety of the soil samples. In each case, a negative control (steamed soil to verify that the soil was not toxic), and positive controls (spiked with either

Beauveria or *Metarhizium* conidia to verify that the entomopathogens were effective if they were in the soil) were included along with the sample.

The results (Table 6) showed that the assay worked well. In untreated soil 1 (Angelview soil) half the larvae were dead within 7 days, and all four were dead within 10 days. With steamed soil, the larvae survived over 21 days. In soil to which either *Beauveria* or *Metarhizium* conidia were added, the larvae died within about 4 to 6 days. When the dead larvae were maintained in the soils, about half of those in the untreated soil (50%) became covered with white, conidia-producing mycelial growth typical of *Beauveria*. The other half became covered in *Metarhizium* growth. In steamed soil with *Beauveria* added, all the cadavers became covered with *Beauveria,* while when *Metarhizium* was added, the cadavers became covered by *Metarhizium* growth. It could therefore be concluded that both *Beauveria* and *Metarhizium* were present in soil 1, but the conidial numbers were considerably smaller than the number of conidia used in the spike (10^5 per gram soil). This substantiates the plate count results that are presented above.

The results obtained with soil 9 differed in that no *Beauveria* was recovered, all of the *Galleria* deaths being attributable to *Metarhizium.* Survival was good in the steamed soil, and death was rapid in the soil to which either the *Beauveria* or the *Metarhizium* conidia were added. It seems likely that *Metarhizium* conidial numbers were too low to be detected by the plate count assay.

In soils 13 and 17, neither *Beauveria* or *Metarhizium* were present. The *Galleria* survived relatively long in these soils, regardless of whether they were steamed. In contrast, survival was not good if the soils were spiked, confirming that if the entomopathogens had been present in appreciable amounts, the *Galleria* would have died.

In soil 30, the *Galleria* in the untreated soil died, but death took a relatively long time. *Beauveria* conidia formed on the cadavers, suggesting this was the cause of death. It seems likely that this soil contains *Beauveria*, but propagule numbers are low.

Table 6. *Galleria* bioassay using representative soils, or soils "spiked" with entomopathogens.

Treatment [2]	Days till dead [1]	% of Cadavers with *Metarhizium* sp.	% of Cadavers with *Beauveria* sp.
Soil 1, no addition	7 to 10	50	50
Soil 1 + *Beauveria*	4 to 5	0	100
Soil 1 + Metarhizium	5 to 6	100	0
Soil 1, steamed	> 21	0	0
Soil 9, no addition	7to 10	100	0
Soil 9 + *Beauveria*	3 to 4	0	100
Soil 9 + *Metarhizium*	6 to 7	100	0
Soil 9 Steamed	> 21	0	0
Soil 13 no addition	12 to 25	0	0
Soil 13 + *Beauveria*	4 to 5		100
Soil 13 + *Metarhizium*	7 to 8	75	
Soil 13, Steamed	> 21	0	0
Soil 17 no addition	>21	0	0
Soil 17+ *Beauveria*	6to 7	0	100
Soil 17 + *Metarhizium*	7 to 8	100	0
Soil 17, Steamed	> 21	0	0
Soil 27 no addition	17 to 25	0	0
Soil 27 + *Beauveria*	4 to 5	0	100
Soil 27 + *Metarhizium*	5 to 6	100	0
Soil 27 Steamed	17 to 24	0	0
Soil 30 no addition	16 to 19	75	0
Soil 30 + *Beauveria*	4 to 5		100
Soil 30 + *Metarhizium*	4 to 7	50	
Soil 30 Steamed	>21	0	0

1.Numbers are the day when *half* of the larvae were dead, followed by the day when *all* were dead.
2. Where indicated, conidia were added for 10^5 per gram soil. The soil was steamed for 60 minutes, where indicated.
3. 25°C, variable humidity.

When the *Galleria* and soil in treatment 1 were maintained for an additional three weeks after larval death, a white mushroom-like structure began to grow from the cadaver. After 4 weeks, it had expanded and spore-producing structures (conidiophores) had formed over it's apex. After discussions with experts in the field (Dr. M. Goettel, Ag. Canada, Lethbridge, Dr. L. Hutchison, Lakehead University, Thunder Bay) it was concluded that these were probably synnematal structures (fascicles of spore-bearing hyphae). There was a suggestion that they could be *Isaria sp.*

When this test was re-done with a wider variety of soils, the synnemata that formed with soils # 1, 3 and 4 (all of which were from the Angelview plot) were white, and relatively large while those that formed on two other soils (#16 = old field, #34 = Moncton plot) were smaller and

pinkish in color. (See photo below. For scale, it should be noted that the font size in the photograph was 8). This suggests that either different species or strains of *Beauveria* may predominate in the soils. In soil number 34 no *Beauveria* was detected in the plate count assay, demonstrating the greater sensitivity (lower detection limit) of the *Galleria* assay.

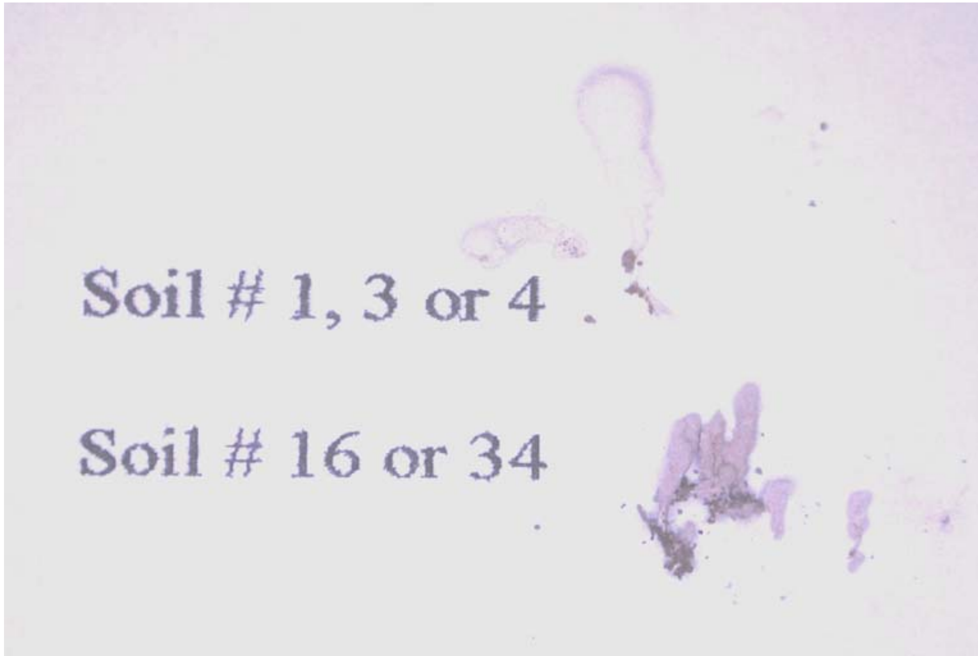


Photo. Typical synnemata that formed on Galleria larvae in soils # 1, 3 or 4 (Angelview soils) or in soils #16 (old field) or 34 (Moncton Trial). Font size used in photo is 8.

During year 2 we assayed more soils for entomopathogens. Initially, the plate count was used, but no *Beauveria* or *Metarhizium* colonies were detected, except in spiked soils. The detection limit was 4x 10^3 propagules per gram which is a bit higher than the limit used previously. This was necessary because the soils, having been recently collected, were microbiologically more active.

We re-assayed the soils using the *Galleria* bait method. We also measured the soils' pH and respiratory rate. The results (Table 7) showed that *some* entomopathogenic fungi (*Beauveria* and/or *Metarhizium*) were present in many of the soils. Where "microplots" is indicated, the soils were used in the microplot assays (see year 2 report). As reported above, both *Beauveria* and *Metarhizium* were detected in the Angelview soil. The Nashwaak River Soil ("natural soil, from Wetmore's) had only *Metarhizium* in it, while the Kynock soil only had *Beauveria*. In total, eight of the soils had *Beauveria* in them, while six had *Metarhizium*. Four had both. There was no clear relationship between the presence of the entomopathogens in the soils, and whether the site from which they were taken had a chinch problem. For example, the Rothsay Fire Hall site had extremely high chinch populations, and the soil from it had *Beauveria*. The volley ball court soil had both entomopathogenic fungi in it, and it had chinch bugs. The Angelview soil also had both fungi in it, but had no chinch problem. Interestingly, none of the compost samples (the last four entries in the table) had either fungus in them, probably due to the pasteurization phase the composts go through during preparation.

In all cases, it took quite a long time for the Galleria to die and show the infection. This, in conjunction with the negative plate count results indicates that even where propagules of the entomopathogens were present, they were not numerous.

TABLE 7. Characterization of year 2 soils with respect to entomopathogenic fungi, pH, and respiration.

Soil	pH	Respiration (µmol $CO2 \cdot hr^{-1} \cdot g^{-1}$)	Presence of Entomopathogens by Galleria Assay*	
			Beauveria sp.	*Metarhizium* sp.
Nashwaak River Soil (microplots)	5.9	13	-	+
Kynock Soil (microplots)	5.9	9	+	-
Angelview Soil (microplots)	6.1	10	+	+
Volleyball Court	4.8	10	+	+
Urban Landscaping M.T.S.	6.5	15	-	+
Neil Pond Residence	6.0	6	+	+
Rothesday Fire Station	5.2	10	+	-
Scotts Black Earth	4.7	7	+	-
Scotts Premium Soil	5.6	9	+	-
Scotts Lawn&Garden Soil	4.4	8	+	+
Envirem's Killarney Lake compost	6.6	22	-	-
Envirem's Miramichi Bark compost	6.7	38	-	-
Kynock's Compost	7.1	22	-	-
Crane Mountain Compost	7.7	57	-	-

*Results are a composite of two assays, each with 4 *Galleria* larvae per container.

The respiratory rates of the soils gives some confirmation that the soils were microbiologically active. The validity of this assay is supported by the relatively high data for the composts, which would be expected to contain lots of active microbes. The Crane Mountain compost was highest. The manufacturer told me it might not be completely mature, which would explain the high value.

There was however no clear relationship between the respiratory rate of the soils and the presence of chinch at the soils' sites of origin. For example, Angelview (with no chinch) and Rothesay (with abundant chinch) had about the same respiratory rates.

The pH values of the soils ranged widely, many being quite acidic. The pH of the volley ball court soil and that from the Rothesay Fire Station were both low. This may contribute to plant stress, that might in turn relate to the high chinch populations at these sites. In the same breath, many other soils with low pH (e.g. Angelview) do not have a chinch problem.

Entomopathogen persistence in soil. In year 1 we did some tests to assess persistence of conidia in two soils, amended in various ways. Tests were done with soil #1 (Angelview Walkways) and #9 (Urban Landscaping Lawn Soil). In these tests we added 2×10^6 conidia of per gram of the test soil. Where indicated we added 30% by volume of peat moss to the spiked soil. In other replicates a

mixture of lawn-care chemicals including diazanon, sevin, 2-4-D (Killex) and N:P:K fertilizer was added. For this, we added 15 µl per g soil of a "spiking" solution consisting of 37 µl of diazon. 37 µl of sevin, 37 µl of Killex and 112 mg of miracle gro (15:30:15) fertilizer in 2 ml of distilled water. This amount was chosen to simulate a high application rate, as calculated from directions on the chemical's label, or (for NPK) from recommended fertilizer rates for turf. At intervals, the number of viable conidia in the soil was measured using EEA (Beauveria) or EEF (Metarhizium) medium.

The results are presented in Table 8. The variation in the data for recovery after 24 hours may reflect differences in physical recovery of the conidia from the soil matrix. Subsequent decreases may represent decreased viability. However, in that values are for single determinations (no replicates), the measurement variability can not be estimated. - Only large differences should be considered significant. These do not seem to be present. It seems conidial numbers remain high and similar in all treatments. i.e. There is no evidence that either peat, pesticide or soil type has a strong influence on conidial survival, at least at low (4^oC) incubation temperature.

Table 8. Persistence of *M. anisopliae*. or *B. bassiana* conidia in Soil 1 (Angelview soil), Soil 1 amended with pesticides or peatmoss, or in Soil 9 (Urban Landscaping's Lawn soil mix) after various times of storage at 4° C.

Soil and Addition[1]	Incubation Time		
Soil 1, No addition	24 hours	2 weeks	12 weeks
+Metarhizium	3.6×10^5	1.4×10^5	2.2×10^5
+Beauveria	5.8×10^5	7.0×10^5	5.0×10^5
Soil 1 + peatmoss			
+Metarhizium	4.7×10^5	2.3×10^5	5.7×10^5
+ Beauveria	5.1×10^5	3.6×10^5	3.4×10^5
Soil 1 + pesticides			
+Metarhizium	6.6×10^5	1.2×10^5	1.8×10^5
+ Beauveria	8.3×10^5	7.2×10^5	5.4×10^5
Soil 9			
+ Metarhizium	2.8×10^5	1.4×10^5	2.3×10^5
+ Beauveria	5.7×10^5	3.3×10^5	2.2×10^5

Further tests were made during year 2 to assess survival in warmer soil, or in soil in which grass was growing. In addition, the effects of drying and freezing were measured. For these tests, *Beauveria* conidia (B2) were mixed into non-sterilized soil to give $1.5 \pm 0.4 \times 10^6$ conidia per gram. Where indicated the pesticide mix mentioned above was added to the soil. The soil was then placed into small (2" diam) plastic flower pots which were planted with ca 200 Kentucky bluegrass seeds. These pots had drainage holes in them. Other aliquots of the soil, with our without pesticide added were put into Petri dishes (no drainage) and stored at $4C^o$ (refrigerator), frozen ($-14C^o$) or on the window sill. The soil in the Petri dishes on the windowsill dried after about a week. It should be noted that the data in Table 9 are multiplied by10^{-6} and are on a fresh weight basis. Small - differences (e.g. +/- 2 fold) could be due to differences in moisture. Emphasis should be on big (e.g. order of magnitude) differences.

The results (Table 9) showed little effect of any of the treatments at four weeks, conidial numbers being similar regardless of the presence of pesticides, freezing temperatures, variable or dry

conditions (windowsill) or the presence of plants growing in the soil. By 7 weeks, the counts had gererally dropped, particularly in the treatments that contained plants. It is possible that this may have been due to the conidia being washed down through the soil out the bottom of the pots. There were no consistent pronounced differences between the treatment with or without pesticides.

Of the treatments where the soils were held in containers (no possibility of conidia leaching out), the counts may have been lower in the soils stored at 4C^o with or or without pesticides. However, none of the differences were very pronounced, particularly when considered in light of the relatively high standard deviations. In short, it seems that the conidia are remarkably robust, tolerating a wide range of conditions.

Table 9. Effect of plants, pesticides and storage conditions on survival of Beauveria conidia in soil.*

Treatment	2 weeks	4 weeks	7 weeks
+ plants	2.6 (0.6)	1.6 (0.6)	0.4 (0.2)
+ plants+pesticide	2.0 (0.5)	1.2 (0.3)	0.3 (0.02)
Soil stored at 4C^o		2.1 (0.9)	1.1 (0.3)
Soil at 4C^o+ pesticide		3.7 (0.3)	0.7 (0.3)
Soil on windowsill, dry		3.4 (0.3)	2.8 (0.2)
Soil on windowsill, dry+ pesticide		1.3 (0.2)	1.1 (0.1)
Soil at -14C^o		4.4 (0.5)	1.9 (0.4)

*Plate count data. Values are x 10^{-6} per gram with the standard deviation (n=3) in parenthesis.

Effects of pesticides on germination of *Metarhizium* and *Beauveria* spores. To investigate the effects of pesticides further, various concentrations of the pesticide mix mentioned above were established by adding 0, 10, 50 or 100 µl of the pesticide stock solution to 10 ml of hot EE agar medium, mixing this and pouring it into Petri plates. After the agar had cooled, 20 microliters of either *Beauveria* or *Metarhizium* conidia suspension (ca 200 conidia) were spread on the agar and the plates. These were then incubated for 5 days, when the colonies were counted.

The results (Table 10) showed little effect of the pesticides/ fertilizer mix on the germination of either fungus. The colonies of *Metarhizium* were actually larger on the plate with the pesticides, possibly because the fertilizer stimulated growth. Further testing might investigate the effects of longer exposure time, or might look for effects on fungal growth or conidia formation, however, these results do not support the idea that pesticide usage might in some manner interfere with chinch control by either *Beauveria* or *Metarhizium*.

Table 10. Number of colonies formed on agar containing indicated amount of pesticide.

	µl of pesticide stock solution*			
	0	10	50	100
Beauveria bassiana	105	266	203	224
Metarhizium anisopliae	112	102	98	95

* Values are single determinations.

Conclusions from Section II. (Entomopathogens in soils, characterization of soils).

It is generally reported that *Beauveria* and *Metarhizium* are ubiquitous, and indeed, this would seem likely since their conidia are easily distributed through the air. However, our results and those of others (e.g. Keller et al. 2003) indicate that although the fungi may be present, their propagule numbers (i.e. spores or other dissemination structures) in soil are generally quite low. There is a large disparity between the propagule numbers typical of a soil (10^2 or 10^3) and the 10^6 or so that might be required to give control of chinch (see e.g. Samuals and Coracini, 2004). This suggests that adding spores to the soil might decrease the chinch bug population. Our preliminary tests in this respect have indeed shown that some *Beauveria* strains can kill chinch (see photo below).

Some references and discussion concerning persistence of entomopathogenic fungi in soils is available in an article on the internet by McCoy, Quintela and Faria (see Environmental Persistence of Entomopathogenic Fungi. http://agctr.1su.edu/s265/mccoy.htm), and also from Vanninen et al (2000). Bruck's work (2004, 2005) also addresses conidial persistence, with particular reference to soils that contain peat. The consensus seems to be that spores persist reasonably well (at least many months) in most soils, although factors such as storage temperature and presence of pesticides, fertilizers, microorganisms, etc. can have some limited effect. Our results would substantiate this. If conidia were added to soils, it would be expected that they would remain active for at least some months, which should be long enough for them to infect the target insects. Pesticide presence should not have a marked effect on this.

From the perspective of understanding the chinch bug problem, it is interesting to ask what role *Beauveria* sp. might play in differentiating turf that is resistant to chinch from turf that is not resistant. It could be that strains of *Beauveria* that are more active against chinch occur in chinch-resistant turf. Such strains might be the Isaria-forming one from Angelview, or the one from the dead insects from Newfoundland. These will be tested next year.

An alternative hypothesis is that in chinch prone turf, there is some factor that interferes with secondary conidia production (conidia produced on the dead insect) by the *Beauveria*. When a chinch bug dies from *Beauveria*, very large numbers of conidia (about 10^9) can form on the cadaver. If these spread into the surrounding 1 kg of soil, the conidial count would be increased to 10^6 per gram - which is probably enough to kill chinch bugs. Possibly some factor (low moisture? pesticides? peat?) interferes with this secondary conidial production in chinch prone-turf. Ramoska et al. (1983) showed that Beauveria can kill insects even if the humidity is low, possibly due to the microhabitat on the insect cuticle being higher than conditions in the surrounding soil. However, subsequent conidial production on the dead insect (secondary spore production) is much higher under high humidity (Kreuger et al. 1991). Very little information is available concerning other factors that might affect conidial production in soils. Work during year 2 will address this.

Preliminary indications are that if chinch bugs are exposed to high numbers of *Beauveria* conidia, they will die within about a week. However, it is probable that the results will depend upon the strain of fungus, how it is formulated, etc. Another factor is whether the spores persist in the soil, and whether they get transferred to the insect. The results presented above in conjunction with those of others suggest that the spores will indeed persist in a soil to which they are added for at least a few months, even if the conditions are not ideal. During year 2 we will compare

virulence towards chinch bugs of the various strains, and look at their persistence under simulated field conditions.

Section III. Growing Chinch Bugs.

Early in the project we decided to grow chinch bugs, since it became evident that insects from the field were not going to fill our research needs. -Often the insects were not vigorous, and may have been pre-infected with disease, or covered with microbes that would affect their response to the *Beauveria*.

In our first attempt to grow the insects, we put insects onto turf taken from outdoors, and held in screen-covered boxes. However, if the screen was fine enough to retain chinch bugs, air circulation in the boxes was hampered, and fungal diseases and unwanted insects proliferated. It was also very difficult to keep the plants adequately watered since there was relatively little soil in the boxes. Although a few chinch bugs survived on the sod, there was no evidence that they reproduced.

We therefore changed our approach and started growing various grasses on steam-sterilized soils contained in closed, clear, plastic 1L containers. This worked after we improved our methods for controlling moisture in the containers and air circulation through them. However, after some time, disease symptoms became evident. Close inspection showed the plants also had a serious thrips problem. Some chinch did however persist on the grass.

Finally, following a suggestion of Wyatt Anderson, a graduate student of Dr. Baxendale, University of Nebraska, we used Sorghum, instead of turf grass as the host plant for the insects. Three seeds were planted in steam-sterilized sandy loam, contained in a 1 l clear plastic container. About 4 cm of vermiculite was placed in the bottom of the containers, with 10 cm of the soil on top. The bottom of the container had a screened, 1 cm diameter hole in it, so that when it was placed in shallow water or fertilizer solution, the liquid would flow into the vermiculite, but not the soil. A clear plastic tube with screened ports in its side and top to allow air movement was placed over the top of the plant, being sealed onto the container with latex caulking. These systems were kept in the growth box, and were watered twice a week. They were also fertilized every second week using a fertilizer solution (Plant Products, 10:52:10 used at ½ the recommended strength) in place of the water. About once every 2 months the systems were heavily watered from the top to rinse out accumulated salts.

Photo. Chambers with sorghum used to rear chinch.

When chinch bugs were placed into these systems, they would persist on the leaves, and eventually, they mated, and layed eggs. These hatched, and chinch nymphs developed.

Photo. Various stages of chinch clustered in crack in soil at base of sorghum. i.e. a small chinch "volcano"

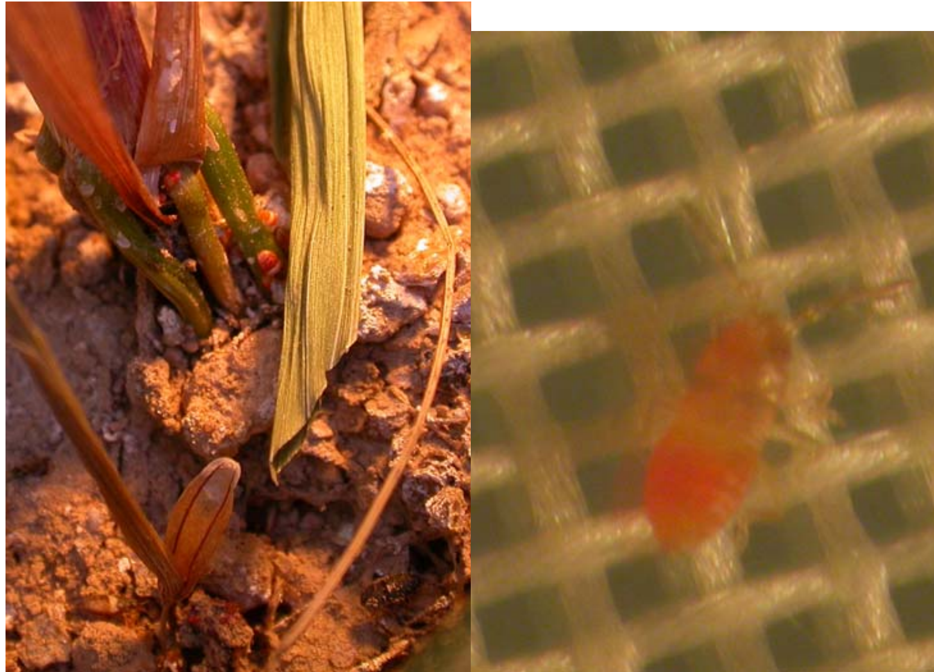


Photo. Left showing nymphs on sorghum leaf sheath. Right. Nymph on screen of chamber.

In some cases chinch bugs were found in the bottom of containers, near the drain hole. They have also been seen in cracks in the soil, both in the sorghum containing systems and in the fescue-containing rhizotrons. In short, it seems that chinch, at least sometimes go at least a few inches into the soil. These subterranean chinch might be difficult to collect with a vacuum detect by scrabbling, or to collect in a vacuum sampler.

Chinch rearing has been more difficult than anticipated. Part of the problem is simply that the chinch stresses the plants, making them more susceptible to other problems. It is well known that disease and thrips are difficult to control on stressed grasses. Some stress is almost inevitable since grasses are grown in closed containers like the ones we have been using. Moisture stress is a particular problem – we aim at maintaining low moisture since this seems to promote chinch development, but it is difficult to maintain at the right level without undue plant stress, particularly since our growth boxes lack humidity controls.

It has also proved difficult to contain the chinch. As is evident from the photo above, the nymphs are very small, and tend to move towards any openings in the chambers. We sometimes found chinch of various developmental stages clustered on the screen.The first instar nymphs may have been able to go through the mesh we were using over the vent holes. We now use a finer mesh mosquito netting.

We will continue with our chinch rearing efforts. Despite initial problems, we can now rear chinch bugs. However, limitations with our plant growing facilities make it impossible to grow the large number of insects that are needed for our experiments. Access to suitable greenhouse space would be of great benefit.

Acknowledgments

This project reflects the efforts of many participants. In particular, Julie Derrick provided excellent technical assistance. Garth Nickerson, Jack Wetmore and Neil Pond continue to play central roles by contributing advise and information to fill the major gaps in our understanding of the biology of chinch bug and grasses. Tim Jackson also offered many ideas. Thanks are extended to chinch researchers in Newfoundland (particularly to Dr. Peggy Dixon, Nancy Hudson and Robyn Auld), in Quebec (Sophie Rochefort) and the United States (Dr. Shetlar and students, Dr. Baxendale and students) for sharing their ideas… and sometimes their chinch bugs. We are very grateful for the financial (and other) support of the New Brunswick Horticultural Trades Association, NBAFA's RIP program, and of NRC's Industrial Research Assistance Program (IRAP).

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