

Biological Control of Chinch Bug Research Project, 2005-2006

**Report for Year 2.
May 2005 – April 2006**

(Note: some results from year 2 have been moved into the up-dated year 1 report, since they were continuations of work started then.)

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Year 2. Biological Control of Chinch Bug Research Project. 2005 – 2006.
(Some of results from moved to report for Year 1).

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Executive Summary for Year 2.

This report focuses on results obtained between April 2005 and March 2006 but also incorporates some related results obtained earlier in the project. Emphasis this year was on understanding the role that entomopathogenic fungi (*Beauveria* sp. and *Metarhizium* sp.) might play in the control of chinch bugs in lawns.

A number of fungal isolates were tested for their ability to kill adult chinch bugs. Two isolates, both purified from *Beauveria*-killed adult insects from Newfoundland were particularly active, with efficacy similar to that of the fungus used in the commercial product, Botinigard ESTM. These isolates were grown on rice, and the colonized rice grains, when added to soil in an *in-vitro* assay, killed a significant fraction of the insects within a week. Tests showed that death was more rapid when more pathogen spores were present. Rapid insect death (in less than e.g. 1 week) required a concentration of at least 10^7 conidia per gram soil.

Quantitative plate count assays for the fungi, used in conjunction with a qualitative *Galleria* (wax worm)-based bioassay confirmed the year 1 findings that *Beauveria* sp. and/or *Metarhizium* sp. are present in many, but not all New Brunswick soils. However, the number of propagules (e.g. spores) was always low ($< ca 10^4$ conidia per gram soil) and would therefore not be expected to have a short-term effect on the insects. There was no clear relationship between the presence of the fungi in a soil, and presence (or absence) of chinch bugs at the site. These results are presented in the year 1 report.

Tests showed that *Beauveria* can make very large numbers of spores on insect cadavers (“secondary conidia”). It was hypothesized that if a “starter population” of *Beauveria*-infected insects died in a soil, spores from them might infect other chinch bugs thereby starting an epizootic. To investigate this idea further, the number of spores forming on dead insects in various soils was measured. Interestingly, results differed by 1000X between the soils, ranging up to 10^9 conidia per insect. There was however no *simple* relationship between secondary conidia production and the prevalence of chinch at the site. The ability to support secondary spore production could none-the-less be an important attribute of soils. This should be explored further.

Persistence of conidia in soil was investigated. Last year, it was found that the spores survived well when in soils stored in the refrigerator. This year we monitored persistence in soils stored at various temperatures and humidities, with or without the addition of fertilizers and pesticides, and with or without grass growing on the soil. In all these lab tests, spore survival was good, there being no marked effect of humidity, pesticides, temperature or presence of plants. The results are presented in the year 1 report. We also looked at conidial persistence in the outdoor “microplots” (containers) with grass growing on on them. Here the spore numbers decreased by about 10X every three weeks. It seems likely that the microplot data differed from the lab results because the conidia were rinsed into the lower parts of the microplots during watering.

The outdoor microplots were built in the early summer of the year 2 growing season. They were designed to give insight into the effects of soil type, soil depth, moisture content, and *Beauveria* inoculation on chinch bug survival. Preliminary results collected after the first season show that the grasses (mainly Kentucky Blue) grew well in all the plots. Some soils supported more growth than others. The two moisture regimes were just starting to have an effect on growth at the end of the season. The plots will be re-assessed during the next growing season.

Of the 40 adult chinch that were added to each plot in early August, about 20 were recovered in a floatation assay done in October. This was about 10X more insects than were seen by simple inspection (i.e. “scrabble count”) showing that floatation assays are more efficient at detecting insects. There were however no statistically significant differences between the number of insects recovered from the various treatments. Nor was there any evidence of the insects reproducing in any of the treatments, in that only adult insects were recovered. Another possibility is that the photoperiod was too short later in the season. Consultation with other researchers suggests the chinch might require a cold treatment before they reproduce, so perhaps the insects will reproduce this spring.

The microplots were maintained over the winter. Prior to the up-coming growing season more chinch bugs and *Beauveria* spores will be added. The grasses should be better established, some thatch might be present and water and soil depth treatment differences will probably be more pronounced. We therefore anticipate being able to assess the effects of these factors on the chinch bug problem.

As reported in the year 1 report, we have now grown chinch bugs on a number of occasions, the whole life cycle of the insect having been completed. However, we have not been able to grow the large numbers of insects required for some tests. It seems the death rate in our growth chambers has always been equal to or higher than the birth rate. We have asked other researchers for advise and believe the problem is with our growing space. Those who report with growing the insects use essentially the same system (sorghum plants enclosed in a clear plastic cylinder), but the plants are maintained in a greenhouse. The light intensity in our chambers may not be high enough.

The consequent shortage of insects for experiments made it of particular interest when live chinch bugs were recovered from various field sites during the winter. Garth Nickerson first showed the insects could be recovered from the upper layers (e.g. leaf debris, plant crowns, thatch layer) on top of the frozen soil at the volley ball court, this being a chinch infested site in Fredericton. On later occasions in the winter we recovered about 100 insects per m² of lawn at the Rothesay fire hall site. Finding these insects during the winter not only gave us a source of insects for our work, it also demonstrated that at least some chinch bugs overwinter on-site, as opposed to moving to “hedgerows” or other surrounding vegetation, as is often suggested. This may have implications for design of chinch control strategies.

Introduction.

This report presents results of experiments that were carried out during year 2. Some additional results pertaining to parts of the project that were initiated last year are presented in the accompanying up-dated version of the year 1 report.

The goals for this year's work were outlined in the proposal, and also in the Contract/terms of reference document that was discussed during the meeting of June 29, 2005. The goals are addressed in the sections below. Additional background information and the methodologies that were used are in the report for year 1.

Methods. See Year 1. Report.

Results and Discussion

A. Assessing prevalence of entomopathogenic fungi in various soils. (Also see reports for years 1 and 3).

Most of the results from this part of the project are presented in the up-dated report for year 1. In year 1 the assays had been made on soils that had been stored for over six months. This year, fresh soils were used. Very briefly, this year's results confirmed that *Beauveria* and/or *Metarhizium* are present in many soils, but that the propagule numbers are low. Interestingly, the fungi were relatively rare in the composts or manufactured topsoils that were tested. This may be part of the reason for the relatively frequent chinch bug problems that are observed on lawns made on manufactured topsoils.

In addition, soil pH and respiratory activity was measured. Differences in these factors did not correlate in any meaningful way with prevalence of chinch bugs at the site where the soils originated. It did not seem that the chinch problem could be explained simply by e.g a pH problem or low microbial activity in the soil.

To further characterize the soils with respect to attributes that might be of relevance to the chinch problem, we tested for acute toxicity of the soils towards the insects. For example, it seemed possible that soils at chinch-free sites might contain some factor (residual insecticides? metals? phenolics?) that might kill the insects. The specific possibility had been mentioned that the Angelview soil might contain insecticides like DDT, or DDT metabolites.

To test this, 10 g moist soil was placed in 30 ml clear plastic containers along with adult chinch bugs. These were collected in September from the Rothesay Fire Hall site. The containers were incubated at 25°C, and survival of the insects was monitored for 30 days.

The results (Table 1), showed that chinch survival in all soils was fairly good, none showing evidence for acute toxicity. There was no clear correlation between survival of the insects and chinch populations at the site where the soils were collected. If there had been e.g. toxic levels of insecticides, one would have expected rapid insect death. There was no entomopathogenic fungal

growth on the insects that did die during the assay period. Survival was poorest in compost from Crane Mountain. This may relate to this compost having been immature. (It smelled like ammonia and also, as shown in the year 1 report had a high respiratory rate.

TABLE 1. Survival of chinch bugs in un-amended, damp soil samples*.

Soil	Day until 50% chinch dead.	% survival at 30 days
Nashwaak River Soil (microplots)	> 30	60
Kynock Soil M.T.S.(microplots)	> 30	60
Angelview Soil (microplots)	> 30	90
Volleyball Court	> 30	80
Urban Landscaping M.T.S.	> 30	90
Neil Pond Residence	> 30	60
Rotheday Fire Station	> 30	90
Scotts Black Earth	> 30	70
Scotts Premium Soil	> 30	70
Scotts Lawn&Garden Soil	> 30	80
Envirem’s Killarney Lake compost	27	50
Kynock’s Compost	> 30	70
Crane Mountain Compost	17	20

Values are based on results from one container containing 10 insects. M.T.S. = manufactured topsoil.

In short, the results did not support the idea that acute toxicity factors, either biotic or chemical served to differentiate the soils with respect to their ability to support adult chinch bugs.

B. Lab-scale testing of *Beauveria* sp. and *Metarhizium* sp. isolates for their suitability as chinch-control agents.

1. Selection criteria for isolates used in tests. During year 2 many *Beauveria* and *Metarhizium* isolates were tested for their ability to kill chinch bugs. A commercial *Beauveria bassiana* isolate (strain GHA) that we isolated from Botinigard ESTM was also included in the tests. The codes for the various fungi that were tested, and information about their origins are in Table 2. We have listed the species as *Beauveria* sp., or *Metarhizium* sp. where the identity is not positive beyond the generic level, however, it is likely the *Beauveria* species are *B. bassiana*.

Isolates from soil were purified by growing them on EEA (antibiotic-containing) agar medium (see Year 1 report for details) and sub-culturing from the advancing colony’s edge onto Sabouraud Agar (Difco). To ensure purity, all isolates were cultured on Sabouraud Agar and Standard Methods before stock cultures (slants on Sabouraud) were stored at 4°C. Working cultures were made on Sabouraud agar plates incubated at 25 °C. The number of transfers after isolation was minimized (< 4) to lessen the chance of decreased virulence associated with the fungus being away from its host insect.

Table 2. Entomopathogens used in tests.

Identity	MMBI Code	Conidia/g rice	Source
<i>Beauveria bassiana</i> .	B1*	2.9 x 10 ⁸	Dr. G. Thurston. 2004. CFS. Fredericton, NB
<i>Beauveria bassiana</i> GHA	B2*	2.1 x 10 ⁸	Purified fr. Botinigard ES TM
<i>Beauveria bassiana</i> .	B3*	3.2 x 10 ⁸	Isolated by Boyle fr. adult chinch Ax from Dr. Dixon and N. Hudson, St.J. NF. Mar. 05
<i>Beauveria bassiana</i> .	B4*	4.2 x 10 ⁸	Isolated by Boyle fr. adult chinch B1 from Dr. Dixon and N. Hudson, St.J. NF., Mar.05
<i>Beauveria bassiana</i>	B5	7.8 x 10 ⁷	Dr. Thurston, 2001, CFS, Fredericton, NB
<i>Beauveria</i> sp.	B6	6.7 x 10 ⁶	Fr. Moncton soil. (Makes a pink synnematal structure). May 05
<i>Beauveria</i> sp.	B7	1.0 x 10 ⁸	Isolated by Boyle fr. adult chinch BX fr. St.J. NF. Mar.05
<i>Beauveria</i> sp.	B8*	3.6 x 10 ⁸	Isolated by Boyle fr. adult chinch A1X from St.J. NF. Mar.05
<i>Beauveria</i> sp.	B9	1.4 x 10 ⁸	Fr. Angelview soil. (Forms white synnematal structure). May 05
<i>Beauveria</i> sp.	B10*	1.9 x 10 ⁸	Fr. Angelview soil. Apr. 05
<i>Beauveria bassiana</i>	B11	1.7 x 10 ⁷	Fr. Deb Morreau, Ag. Can. Kentville. 2001
<i>Beauveria bassiana</i>	B12*	8.5 x 10 ⁷	Fr. Dr. L. Hutchison. Lakehead University. Sept. 04 (#256)
<i>Beauveria bassiana</i>	B13*	6.0 x 10 ⁷	Fr. Dr. L. Hutchison. Lakehead University. Sept. 04(#102)
<i>Beauveria bassiana</i>	B14	2.3 x 10 ⁷	Fr. Dr. L. Hutchison. Lakehead University. Sept. 04(#291)
<i>Beauveria bassiana</i>	B15	4.7 x 10 ⁸	Fr. Dr. L. Hutchison. Lakehead University. Sept. 04(#028)
<i>Beauveria bassiana</i>	B16*	2.8 x 10 ⁸	Fr. Dr. J. Sweeney, CFS, Fredericton, N.B. May 05
<i>Beauveria</i> sp.	B17	nd x 10 ⁸	Fr. Angelview Soil, Aug. 05
<i>Beauveria</i> sp.	B 18	nd x 10 ⁸	Fr. Scotts Premium Soil. Aug. 05
<i>Metarhizium anisopliae</i>	Ma	3.5 x 10 ⁷	Dr. M. Bidochka, Jn. 04. SDB11ii
<i>Metarhizium anisopliae</i>	Mb*	5.6 x 10 ⁷	Dr. M. Bidochka, Jn. 04. HKBilb
<i>Metarhizium anisopliae</i>	Mc*	9.2 x 10 ⁷	Dr. M. Bidochka, Jn. 04. NL 121
<i>Metarhizium anisopliae</i>	Md	5.4 x 10 ⁷	Dr. M. Bidochka, Jn. 04. 36B2i
<i>Metarhizium anisopliae</i>	Me	8.0 x 10 ⁶	Dr. M. Bidochka, Jn. 04. CIA IIV
<i>Metarhizium anisopliae</i>	Mf	2.7 x 10 ⁶	Dr. M. Bidochka, Jn. 04. 43A21
<i>Metarhizium anisopliae</i>	Mm	6.8 x 10 ⁷	Deb Morreau. Ag. Can. Kentville. Apr. 04

As indicated in text, isolates with “*” have been chosen for further testing. All are being maintained at MMBI.

We also assessed the abilities of the isolates to produce conidia, this having been identified as a pre-requisite for the practical use of biocontrol fungi. To this end, the isolates were grown on rice, upon which these fungi are known to make conidia. Rice (100g), water (50ml) and corn oil (1ml) were simmered until the water was absorbed. About 5g of this medium was then placed into 30 ml glass vials that were closed with aluminium-covered foam caps. The vials were autoclaved for 10 min at 121°C, cooled, and inoculated with a piece of the colony cut from a working culture. Vials were incubated for 20 days at 20°C when the conidia were quantified. For this, they were shaken vigorously in 0.05% Tween 80 and conidia in the liquid counted using a haemocytometer. The number of conidia per gram of rice medium that each isolate made is presented in the third column of Table 2.

To confirm the isolates' entomopathogenic status, we assessed their abilities to infect *Galleria mellea* (purchased from Pet World, Fredericton) it being known that this insect larvae is susceptible to both *Beauveria* sp. and *Metarhizium* sp. For this, 5 g of damp, autoclaved (sterile) Nashwaak River soil was placed into 30 ml plastic containers with 5 grains of the rice upon which

conidia of the fungi had formed. Control containers received autoclaved grains of rice. Five *Galleria* larvae were added and survival of these was monitored. The results (not presented) showed that all the isolates killed at least 80% of the insects (≥ 4 of the 5 larvae) within 3 days. *Galleria* death took over 9 days in the controls. Mycelial growth and conidia formation characteristic of *Beauveria* or *Metarhizium* formed on the larvae a few days after they had died. In short, the isolates were entomopathogens. Subsequent testing for virulence against chinch bugs focussed on isolates that i) came from different habitats ii) formed abundant conidia, and iii) killed *Galleria* rapidly. These are indicated with an “*” in Table 2.

2. *In-vitro testing of isolates abilities to kill adult chinch bugs.* We tested these isolates for their abilities to kill chinch bugs. Unless specified otherwise, the tests were made on adult chinch bugs that were collected in late September from the Rothesay Fire Hall site.

The isolates of interest were grown on rice medium as described above. Ten colonized rice grains (ca 0.25g), or 10 colonized rice grains plus 1 g of moist steam-sterilized Nashwaak soil were placed into replicate 12 ml tubes for each isolate. Ten rice grains gave a conidial concentration of about 10^8 *Beauveria* or 10^7 *Metarhizium* conidia per tube. Control tubes received autoclaved, colonized rice inoculum, with or without steamed soil. Five adult chinch bugs were added to the tubes, and the tubes were closed with aluminium foil caps perforated with a pin hole for aeration.

For each isolate there were four tubes with five insects each. Two of the tubes had soil, and two did not. The tubes were incubated in a dark, humid chamber at 20°C, and insect death was monitored for 28 days, at which time the experiment was terminated. The results were corrected for death that occurred in the controls using the approach described by Abbott (1925), this being standard practice in entomology. Briefly, the percent death in a treatment was corrected by subtracting the percent death that occurred in the corresponding controls. (Death in controls with soil was used to correct treatments with soil; those without soil were used for treatments without soil).

This data was then used to identify the time each isolate took to kill 50% of the insect (LT50) or 100% of the insects (LT100). The results for the treatments with rice inoculum only are in Table 3A and those for rice plus soil are in 3B. A complete time course for the effects of the more active isolates (B2, B3, B4 and MC) on chinch death are presented graphically in Figure 4.

Table 3. Time required for select isolates to kill 50% (LT50) or 100% of chinch bugs with soil (Table A) or without soil (Table B) in the test system.

A. Rice	LT50	LT100
B1	7	>28
B2	7	7
B3	7	13
B4	4	13
B8	12	14
B10	21	22
B12	>28	>28
B13	>28	>28
B16	>28	>28
Mb	9	15
Mc	12	20
B. Rice and Soil		
B1	15	>28
B2	9	15
B3	12	15
B4	12	12
B8	12	14
B10	19	21
B12	>28	>28
B13	>28	>28
B16	>28	>28
Mb	7	22
Mc	16	20

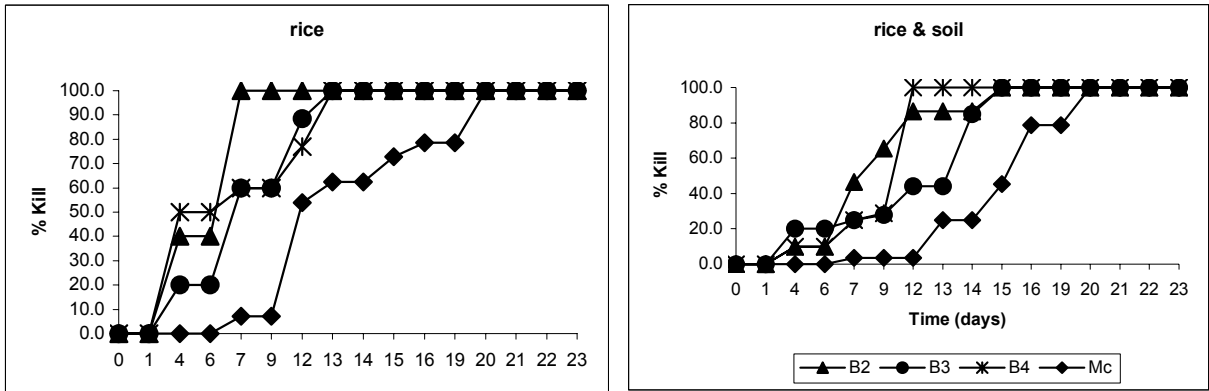


Fig. 4. Time course for killing of chinch by the most active isolates. Results for tubes with rice inoculum in the left figure while those with inoculum and soil are on the right.

The results in Table 3 show that some *Beauveria* sp. and *Metarhizium* sp. isolates can kill chinch bug adults quite rapidly. The three isolates that showed the highest virulence were B2, B3 and B4. Isolate B2 was from the Botinigard product, while B3 and B4 were both from dead chinch bugs that had been sent from Newfoundland. All three killed 100% of the chinch bugs within about two weeks. The *Metarhizium* took about three weeks.

In contrast to these results, some of the other *Beauveria* and *Metarhizium* isolates had very little effect. This underlines the importance of using the correct isolate. There is probably genetic

variability within the virulent isolates (B3 and B4) that we used. They were initiated from millions of conidia taken from the surface of insects. Although conidia, being asexual spores, might be considered genetically homogeneous, parasexual recombination and mutation can generate variability. It seems likely that this variability could be used to advantage to re-select sub-strains of B2 or B3 that were more virulent. It is likely that the commercial isolate (B1) has already been subjected to such a selection program.

It was interesting to observe the behaviour of the insects with respect to the *Beauveria*-covered rice grains. They seemed to be attracted to them. Some insects were observed to climb onto the grains, thereby getting themselves covered with conidia. They then sometimes contacted other insects transferring conidia to them. On the other hand, the insects were also observed to remove clumps of conidia from themselves (and also their neighbours) through grooming behaviour. Movement of conidia on and off of insects is currently being investigated.

In another experiment we examined the effects of *Beauveria* conidia on survival of chinch bugs in a manufactured topsoil. In this test we placed chinch bugs into containers of the soil, with or without conidia being added to the soil. The soils and insects were then incubated in the refrigerator (4°C) for three weeks to simulate an overwintering period. We then moved the containers to a 20 °C incubator and persistence of the insects was monitored.

Kynock manufactured topsoil (the same soil used in our microplot experiment) was used in the test. This was sieved through a 1 mm sieve (ie. fine) and then *Beauveria* conidia (isolate 2) in 0.05 % Tween 80 were mixed into the soil to give a about 10^8 conidia per gram soil. Control soil received an equal amount of autoclaved conidial suspension. About 1.5 g of soil and 5 insects were used in each replicate, there being 3 containers (15 insects) for each treatment.

The results (Figure 5) show quite clearly that the insects died more rapidly in the treatments to which the *Beauveria* had been added.

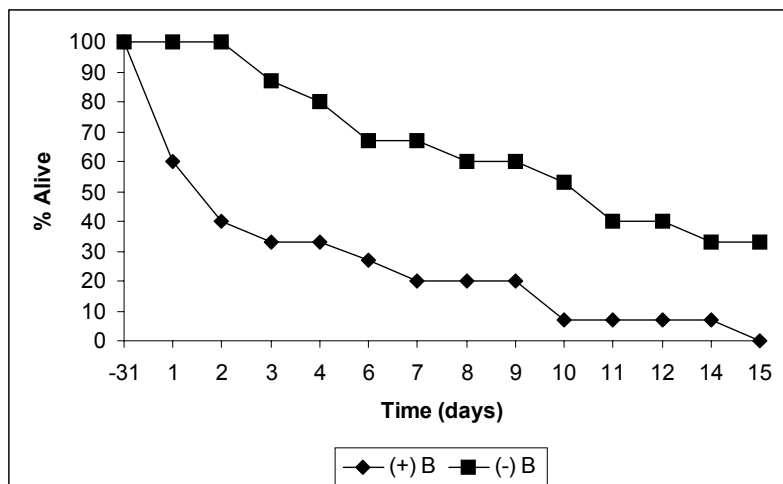


Figure 5. Persistence of chinch bugs in manufactured topsoils with or without the additions of *Beauveria bassiana* conidia.

Chinch death due to the *Beauveria* was evident within two days of moving the containers to the warmer temperature. After 10 days, essentially all the insects in the *Beauveria-treated* replicates were dead, while only about half those in the controls were. Differences between the two treatments remained evident until the experiment was terminated at 15 days.

In another experiment we looked at the effect of conidia concentration on chinch bug death. For this, the insects were dipped briefly into aqueous conidia suspensions of strain B2, adjusted to various concentrations. The insects were then moved to small test tubes held in a humid, 25C incubator and monitored. The results collected on day 7 are presented in Figure 6. A clear dependence of insect death on conidial concentration is evident. At the spore concentration of 10^8 per ml almost all the insects were killed by day 7, while at 10^5 very few were.

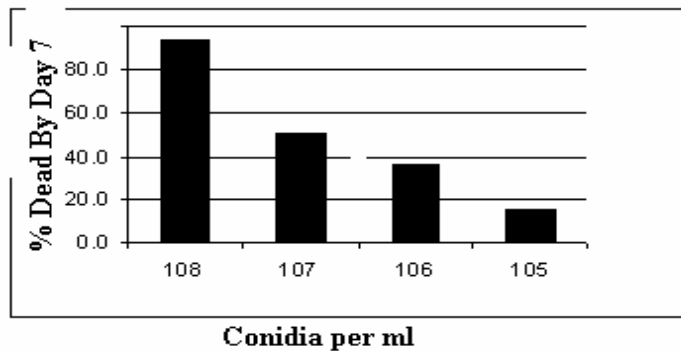


Figure 6. Effect of *Beauveria* concentration on chinch bug death.

We sent our two most virulent isolates (B3 and B4) to Dr. Harry Kope, Contact Biologicals, Victoria BC for morphological characterization. Some characteristics and measurements for two entomopathogenic *Beauveria* species (*B. bassiana* and *B. brongniartii*) with the corresponding information for B3 and B4 are tabulated below.

Species	Conidiogenous apparatus	Colony diameter (cm)	Conidial size (μ m)	Conidial shape
<i>B. bassiana</i>	up to 30 μ m long	0.6 – 2.3	2 - 3	hyaline smooth-walled globose
<i>B. brongniartii</i>	up to 40 μ m long	1.0 – 1.6	2.5 – 4.5 x 2.0 – 2.5	hyaline smooth-walled ellipsoidal
#B3 and #B4	up to 60 μ m long	1.5 – 2.5	2 – 4	hyaline smooth-walled globose

Dr. Kope said the characteristics of B3 and B4 fit well with the descriptions given for *B. bassiana* (colony growth rate, conidia size and shape), however the over all length of the conidiogenous apparatus (the flask shaped phialide with the long denticulate rhachis) of our isolates was 60 μ m, as opposed to 30 or 40 μ m for better known strains. This morphological difference, although

minor, may be sufficient to merit a variety designation for our isolates. It could also facilitate tracking the isolates in the field.

The isolates were also sent to Dr. Tharcisse Barasubiye, Manager, National Fungal Identification Service, Agriculture and Agri-Food Canada for further characterization using molecular biological techniques. He recently wrote back and confirmed they were both *Beauveria bassiana* (Bals.-Criv.) Vuill.

C. Effects of various factors on secondary conidia production.

The number of *Beauveria* conidia in a soil will increase as a result of *Beauveria*-infected chinch bugs dying in it. It could be hypothesized that when the chinch bug population reached a threshold level, a few of the insects would become infected by *Beauveria* and die. Formation of high numbers of conidia on their cadavers (“secondary conidia”) might then initiate an epizootic. It might be that the chinch problem results from something interfering with “secondary conidia” production.

As an inroad into this idea, we counted the number of conidia on some *Beauveria*-killed chinch bugs that had been incubated in a humid chamber for about two weeks after death. Haemocytometer counts showed one of the insects had 10^8 conidia on it. This suggested that one *Beauveria*-killed insect might infect over 100g of soil with 10^6 conidia/gram.

We then compared secondary conidia formation on *Beauveria*-killed chinch bugs between various soils. For this, we placed adult chinch bugs and *Beauveria* (isolate B2)-colonized rice grains in tubes as described above. As soon as the insects died we held them at 4°C until enough dead insects had been collected for the experiment. This took 3 days. Samples of the soils to be tested were then moistened and placed into 30 ml clear plastic containers. Five of the *Beauveria*-killed insects were then buried 1 mm below the surface of the soil. The control soil did not receive any dead insects. The containers were then incubated at 20°C for 3 weeks. There were three replicates for each soil. The soil from each individual container were then vigorously shaken with 0.05% Tween 80, and the number of conidia in it measured by dilution plate count. Results were corrected to represent the conidia per insect, or conidia per gram soil. (The values are the same since there were 5 insects in 5 g soil).

The results (Figure 7) show that there were very large differences between the soils. Not surprisingly, no *Beauveria* was detected in the control treatment. The highest number of conidia (an average of 10^9 conidia per gram soil, or per insect) formed in the Nashwaak River Soil. Numbers were lower, but still very significant in many of the other soils.

Interestingly, one of the lowest values was obtained with the Rothesay Fire Station soil where the chinch problem was very bad. One might hypothesize there is something blocking *Beauveria* conidial formation at this site so the *Beauveria* can not control the chinch. However, before a simple cause-and-effect relationship like this is proposed, it should be noted that conidial production was *high* on the volley ball court soil, where the chinch problem was also bad. In short, the results show that secondary conidial production can differ greatly on different soils, but (unfortunately) it is not possible to draw a simple relationship between this, and the severity of a chinch problem at the site from which the soil came. It does seem logical that secondary conidia

production would be important for *Beauveria* to be effective in nature, since it is the only way (other than inoculation) by which the conidial numbers might become high enough to kill the insects.

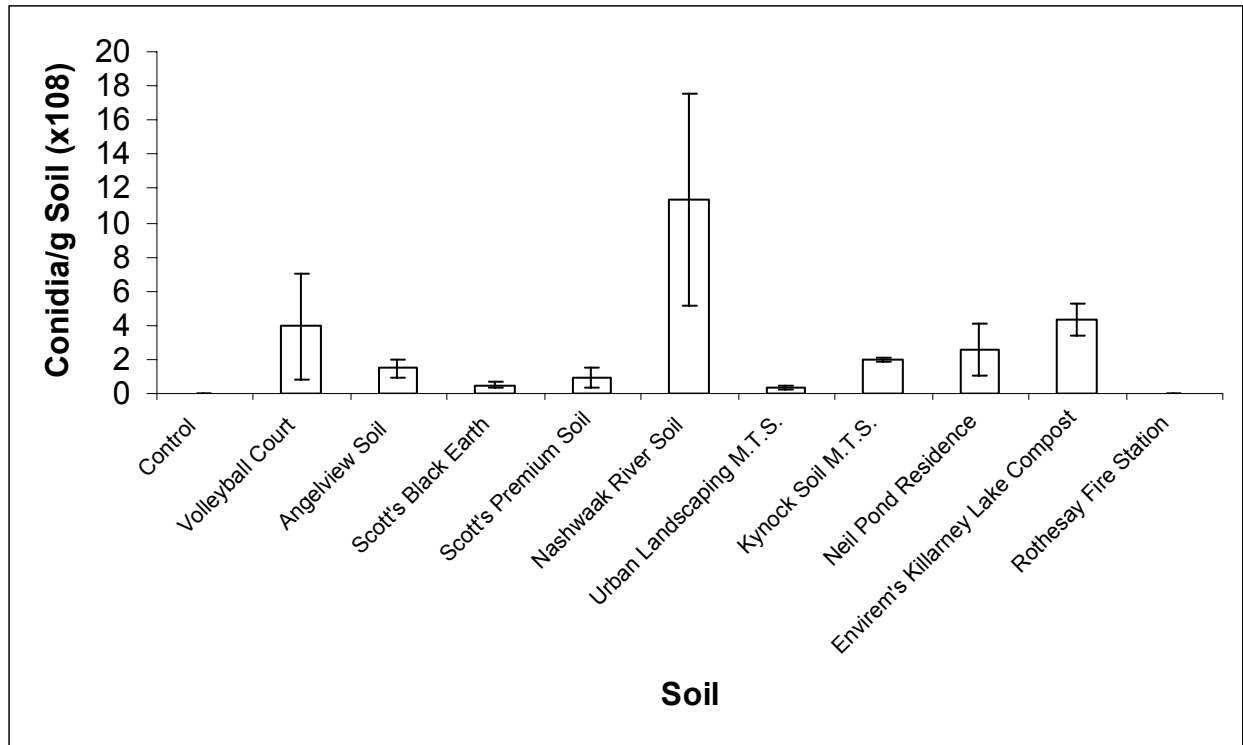


Figure 7. Production of conidia on *Beauveria*-killed chinch bugs in various soils (i.e. “Secondary” conidial production. Values are means for the three replicates, with the bars representing the standard deviation.

We investigated secondary conidia production further using *Galleria mellea* (Greater Wax Moth) instead of chinch bugs as the test insect. Observations made during the *Galleria* baiting assays of the various soils (see year 1 report) supported the data from above, showing that different soils support different amounts of secondary conidia production on the *Galleria*. - In some soils, the *Beauveria*-killed *Galleria* soon turned white with conidia, while in other soils only small patches of white conidia formed on them.

We suspected that secondary conidia production on the dead chinch or *Galleria* might relate to difference in the populations of other microbes in the soil, these in some manner interfering with conidia production by the entomopathogen. To explore this possibility, we pre-incubated “clean” *Galleria* larvae (just collected from the pet food store) for 48 hours in natural soil, steamed natural soil (microbe population depleted) or in natural soil that had been “spiked” with chloramphenicol and cycloheximide to deplete the microbes. These antibiotics were chosen since they inhibit a wide spectrum of bacteria while having relatively little effect on *Beauveria*. (They are what we use in our semi-selective EEA medium). After the pre-incubation, the larvae were moved to steamed

soil inoculated with 10^7 conidia per gram of *Beauveria*. After about 4 days in the *Beauveria*-containing soil the *Galleria* died and the fungus sporulated.

As can be seen in Figure 5, the number of conidia that formed on the larvae differed considerably between the three treatments, being most abundant on the larvae that had been pre-incubated in the steamed (low microbe population) soil. Conidia formation was intermediate on the antibiotic-treated soil and lowest on the untreated soil. These results strongly suggest that the presence of antagonistic bacteria in a soil may control the number of secondary conidia that *Beauveria* may form on insects that die in that soil.



Figure 8. Effect of pre-treatment of *Galleria* on secondary conidial production by *Beauveria bassiana*.

D. Outdoor Microplot testing at Maritime MicroBiologicals Inc.

The effects of soil type, topsoil depth, soil moisture, and Beauveria inoculation on chinch damage, turf performance and on chinch bug numbers and Beauveria persistence.

1. Methods for building, maintaining and assaying microplots. These were constructed from 20 l white plastic buckets, drainage holes being drilled in the bottom. The tops were covered by fine mesh to prevent insect escape or entry. The lower few inches of the container was filled with gravel, and this was covered by landscape fabric, sealed to the wall of the container. The fabric served to stop chinch bugs and soil from moving down into gravel. The soils were placed on top of this fabric. The soil was either 4" or 6" deep, complementary depths of gravel being used so the top surface of the soil was always about 5" below the rim of the bucket (see figure 9).

The containers are being held in a cold frame, with a translucent roof and open sides so the plants are exposed to ambient temperature and light, but water can be controlled. (i.e. there is no rain input). The roof panels are generally folded back (opened) if no rain is anticipated to maximize ventilation around the buckets. Bark mulch is packed around the buckets for insulation. During the

winter, the roof panels are removed, so that snow falls onto and between the buckets to further insulate them.



Fig. 9. A microplot before soil added, and cold frames with microplots.

The factors that are being tested in these systems include:

- a) Soil Depth (4" vs 6")
- b) Soil moisture (no water stress vs some stress)
- c) Soil Type (Three soils, see below).
- d) Microbes (*Beauveria sp.*, steamed or non steamed soil).
- e) Chinch bugs (40 added vs none added).

In most cases 5 replicate microplots were established. Tests of the above factors are being made individually, or sometimes in combinations.

a) Soil preparation.

Soils were prepared for use in the test systems on June 7-9, 2005. The soils included in the tests were:

A) Soil from Wetmore's nursery. This was described as Nashwack River Valley Soil, and is a natural soil.

B). Soil from Kynock resources. This is a manufactured topsoil obtained from Halifax, consisting of 25% compost, the balance being a sandy silt subsoil.

C. Angelview soil. This was collected from the Angelview plot in Fredericton.

Soil samples were taken and analysed by the NB DAFA soil lab. The results are available from either Garth Nickerson (NBAFA) or from MMBI on request, and will be presented with the final report. Some additional information about the soils is also presented in Table 7 of the up-dated year 1 report.

Lime was added at 10 g per kg to soil A to raise the pH from 5.5. to 6.3. (Preliminary lab tests allowed calculation of the required amount of lime). Soil B has a pH of 6.2 and soil C, 6.6. No lime was added to either soil B or C.

For a few replicates, sub-lots of soils A and B were steamed at 100°C for 2 hrs (pasteurized) to remove most indigenous microbes.

The soils were then mixed, sieved (1 cm diam) and placed on top of the landscape fabric. In most treatments, the soil was 4” deep, but in indicated replicates it was 6” deep. Fertilizer (Agromart 6.5:26:26) was then added at a rate of 1.5 g per container this being mixed into the top 2” of soil. The containers were then heavily watered.

b. Seeding. On June 9, after the water had soaked into the soil, the containers were seeded. An attempt was made to get endophyte-free seeds since it was thought endophytes might confound experimental results. A seed mix from Maritime Turf Supplies was used. This consisted of 40% Kentucky Bluegrass, 40% Creeping Red Fescue and 20% Perennial Ryegrass. Seeds (1.75 g) were evenly dispersed over the soil.

On June 4, before planting these seeds in the containers, we measured their percent germination using a standard assay on wet filter paper. By June 14, only 5% of the Kentucky Bluegrass germinated, none of the Creeping Red Fescue did, and about 30% of the Perennial Rye did. We therefore decided to re-seed the containers.

On June 23, we re-seeded the containers with Scotts Bluegrass 100 mixture donated by Maritime Turf Supplies. This mix consisted of 1/3 Abbey, 1/3 Evicta and 1/3 Serene cultivars. The percent germination of these seeds in the standard assay was 68%. We used 1 g per container.

Compositing the information for the two seeding dates, the best estimate is that the containers received a mix of about 90% (by volume) viable Kentucky Bluegrass seeds and 10% viable Perennial Rye seeds. Based on the lab germination tests, about 1000 viable seeds were added to each container, most of these being Kentucky Bluegrass.

After seeding the second time, a thin layer (ca 0.1”) of silica sand was placed over the seeds. The containers were then heavily misted every day for the next two weeks, at which time most seeds had germinated.

d) Plot maintenance.

On July 8 and at approximately 1 wk intervals after that, the grass in the plots was cut to a height of 3” using a device made by mounting a 3 cm long cutting blade into a hand-held high speed (15,000 rpm) rotary tool. (Similar to a Dremmel tool). This cut the grass into small pieces (like a mulch mower) and the clippings were left in the containers to decompose.

The amount of growth was also assessed at each cutting. For this, two measurements were taken. In the first, the average length of the cut grass blades was measured. For the second, the percent cover of the soil was subjectively assessed; A value of “0” was assigned if all the soil was visible

(i.e. essentially 0% coverage), a “1” if about 20% of the soil was covered, “2” if 40% was covered, etc. up to a “5” where no soil was visible.

On July 15 the different watering regimes for the “high” and “low” water treatments were started. High water treatments received 1.5 liters of water per container on Tuesdays and Fridays., applied via a watering can. Low water treatments received 1.5 liters of water only on Fridays. A measurement was made of soil water content on October 10, using a meter provided by Garth Nickerson. The two watering regimes were followed until October 16 when all of the buckets were flooded to facilitate chinch bug collection.

On July 29 all of the buckets were photographed. Two example photographs are below. Growth in the one on the left (rated at about 4.5) is clearly better than that on the right (rated at 2.5).



Figure 10. Two microplots showing growth rated as 4.5 (left) or 2.5 (right).

e) *Addition of chinch bugs.* Between about August 3rd and August 10th chinch bugs were collected by vacuum from various locations around Fredericton. These were individually picked from the other debris that the vacuum collected, and 40 insects were added to the microplots where “+chinch” is indicated. Most of the chinch bugs were adults or fifth stage nymphs. After the insects were added, all the containers (including those to which no chinch was added) were closed with a fine mesh white nylon material, this being affixed over the rim of the container with an elastic band.

f). *Addition of Beauveria bassiana conidia.* Two strains of *Beauveria bassiana* (MSA isolated from Botinigard ES and A1X isolated from a dead chinch bug) were grown on sterilized rice in sealed plastic bags equipped with microporous breather patches following standard procedures. After 3 weeks, conidia were collected from the rice in sterile 0.01% Tween 80. The conidial concentration was 1.9×10^8 conidia per ml for the MSA and 3.1×10^8 conidia per ml for the A1X. Equal volumes of the two conidial suspensions were mixed together, and half of the mixture was sterilized by autoclaving.

On August 3, 2005 100 mL these suspensions were added to the containers so that the “+ *Beauveria*” treatments received about 2.5×10^{10} conidia per container, and the controls received an equal quantity of dead conidia. Plate counts of these preparations confirmed that over 90% of the

conidia were viable in the + *Beauveria* treatments, while none were where they had been autoclaved. It can be estimated from the quantity of soil in the containers (ca 3 kg) that there were approximately 8×10^6 conidia per gram soil immediately after conidia addition.

The number of *Beauveria* conidia persisting in the soils of representative inoculated and non-inoculated buckets was measured after 3, 6 and 10 weeks. For this, three 1 cm x 10 cm cores of soil were withdrawn from representative buckets, mixed together, and assayed by dilution plate count.

g). Counting of chinch bugs. On a few occasions during the growing season, the grass was parted, and the soil surface was carefully examined to determine if the chinch bugs were still present. Only a few insects could generally be seen. At the end of the growing season (October 16) all the microplots were assessed for chinch using a floatation assay. Each container was lifted from its location in the coldframe, and placed into a larger container filled with 25°C water. The water from the larger container percolated up through the drainage hole, through the gravel and then through the soil and grass, carrying the chinch bugs with it. After about 10 minutes, the chinch bugs were recovered from the debris, or grass blades at the surface of the water using an aspirator (Fig. 11). They were counted, and their developmental state (age) assessed. (All were adults.) The chinch bugs from each container were stored in a small container at 4 °C. The microplots were placed in the coldframe to drain for two days, when the chinch bugs were replaced, and the mesh lids re-affixed. In November bark mulch was packed around the microplots for insulation. In December, the roof panels were removed so snow would insulate the surface. The microplots were then left to overwinter.

In January, one of the containers that had chinch in it was brought inside to thaw. Interestingly, within a few days active chinch bugs were observed. The container was returned to the outdoors.



Figure 11. Assay of chinch bugs at end of growing season. Note insects clinging to grass.

2. Initial results from microplots. The results that have been collected have been collated, and some of them are presented below. It should be emphasized that the results are preliminary. The grasses have just barely had time to get established, and the chinch populations have not yet grown. Treatment differences were just beginning to show when the final measurements were made. The results show that the test system is working well, but the important data has yet to be collected. This will happen next year.

a. Grass growth. About 5 weeks after the seeds were planted, (July 26) the grass was ready for the first cut. The amount that needed to be cut in each plot to reduce its height to ca 5 cm above the soil surface was measured and recorded. Subjective estimates were also made of the percent soil cover in each plot. These measurements were repeated each week until October 16. The data for each week is available, but will not be presented here. Briefly they showed that plant height continued to increase at a more-or-less regular rate throughout the season, until the end of October.

In Table 4 the average results for the three main soil treatments, under the high and low moisture regimes are presented. It should be emphasized that these are averaged over the entire season. Differences tended to become more pronounced later in the season. The data (and subjective observation) shows that plant performance, measured as either the cumulative amount cut or the average soil coverage was best in the Nashwaak soil. Results were similar in the Kynock and Angelview soils, although soil cover was better in the latter. By the end of the season, cover in most replicates in all the soils was approaching 100% as indicated by the subjective measurement approaching 5. There was not much difference between the results with the high or low watering regimes in any of the soils, although as mentioned above differences were starting to become apparent at the end of the season - The different watering regimes were not imposed until after the first cut, and it would have time for the water reservoir in the containers to become depleted. The soil moisture values measured on October 10 confirmed that the different watering regimes did result in different soil moistures, at least by the end of the growing season.

Table 4. Growth of grasses in the microplots as affected by soil type and watering regime.

Soil Type and Moisture Regime	Total cut during season (cm)*	Average percent soil coverage (Subjective, 0-5)*	Soil moisture (%)
Nashwaak Low water	58	4.4	14
High water	66	4.4	34
Kynock Low water	28	3.6	11
High water	33	3.7	27
Angelview Low water	28	4.0	25

*Values are averages for all cuts made during the growing season. (N= 5).

At various times during the season we inspected the soil surface and counted the number of chinch bugs. In general, there were only a few in any particular microplot, and there did not seem to be any relationship between the number observed and the treatment.

b. Persistence of *Beauveria conidia*. At three, six and ten weeks after August 3 when the *Beauveria* had been added, soil samples were taken from representative treatments and *Beauveria*

propagules (conidia) assessed by plate count. The results showed there were about 4.3×10^6 conidia recovered from the soil at the time of application. After 3 weeks, only 7.5 (sd = 7) $\times 10^5$ were detected. After 6 weeks, this dropped to 1.0 (sd = 0.7) $\times 10^5$. After 10 weeks, it decreased again to 4.0 (sd = 3.9) $\times 10^4$ conidia. When the data is presented on a log scale (Figure 12) the numbers are seen to drop in an almost linear fashion. It is interesting that these results differ from those obtained under lab conditions. It could be that the conidia were rinsed into the sub-soil area in the microplots. Unfortunately, we did not compare conidial numbers in the “low” and “high” water regimes.

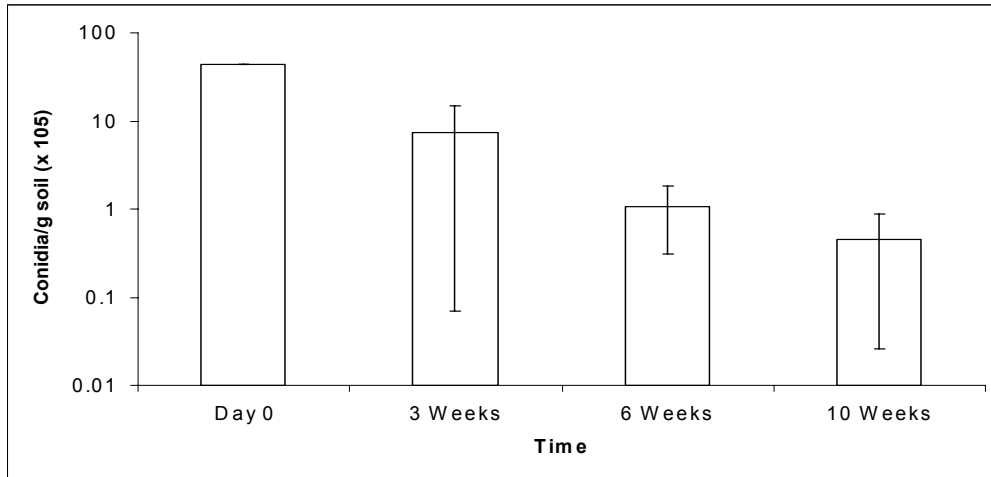


Figure 12. *Beauveria* propagules (plate count) in the microplots various times after addition of *Beauveria* inoculum on August 3. Note that the Y axis is logarithmic.

These results, in conjunction with the results suggesting that high conidial numbers are needed to attain chinch control, suggest there might be a need for repeated application of the conidia.

c. Chinch bug survival. At the end of the growing season, we floated the chinch bugs out of the soils in all the microplots and counted them. We also did floatation assays on the treatments that had not received chinch bugs so they would remain comparable with respect to soil moisture for subsequent work. The results were somewhat disappointing, in that treatment effects were not (yet) evident (Table 5). There had been 40 adult insects added to each microplot. On the average, 12 were recovered from the Nashwaak soil, and 21 from the Kynock soil. This suggests that the insects persisted better on the Kynock soil, but the relatively high standard deviation makes the difference statistically insignificant. There was no evidence that inoculation with *Beauveria* decreased the survival of chinch.

Table 5. Number of chinch bugs recovered by floatation from soils with or without *Beauveria*.

Soil	<i>Beauveria</i> added	Not inoculated	Average for soil
Nashwaak	12.5 (2.4)	12 (9)	12 (6)
Kynock MTS	23 (13)	19 (6)	21 (9)

* Values are means number of insects recovered with the sd in parenthesis. N = 4.

Nor were there any statistically significant differences between the number of chinch bugs recovered from Nashwaak soil, Kynock soil or Angelview soil in the part of the trial investigating the effects of soil moisture (Table 6).

Table 6. Number of chinch bugs recovered from various soils under low or high water regimes.

Soil	Low Water	High Water
Nashwaak	20 (11)	18 (8)
Kynock MTS	19 (3)	21 (10)
Angelview	26 (5)	Not determined

* Values are means with the sd in parenthesis. N = 4

In all cases the insects that were recovered were adults, so they were probably the same insects that were added two months earlier. They either did not reproduce in any of the treatments, or the survival of their offspring was negligible. We once saw some insects mating, but never saw any eggs. It is possible that results would have been more interesting if the insects had been added earlier in the season to established turf.

3. Preliminary conclusions from microplot tests.

The main goal for the tests was to look at the effects of various factors on chinch bug survival. The variability in the data was high, but certain conclusions, largely of a “no significant effect” can be drawn. For example, even though the “high” and “low” watering regimes resulted in soil moistures that differed by two fold, this had no marked effect on the chinch bugs. It is sometimes speculated that the chinch problem might be controlled by watering. The microplot results make this seem unlikely. Similarly, much speculation has been done about why no chinch bugs are present at Angelview park. One hypothesis was that there is some factor in the soil there that kills the chinch bugs. The microplot results showing similar survival in the plots with Angelview soil to that with the other soils (and also the results in Table 1, above) do not support that hypothesis.

The “no-effect” results obtained with the *Beauveria* testing were disappointing, but can not be discounted. – Despite conidia of two virulent strains having been added to the soils, there was no clear-cut effect on chinch bug survival. The lab test results contradicted this, suggesting that adding *Beauveria* would kill most of the chinch bugs within a few weeks. This obviously did not happen in the microplots. Unlike the lab situation, the conidial numbers in the microplots dropped logarithmically with time, this being attributed to them moving down into the lower soil horizons. None-the-less, the chinch bugs should have been exposed to significant numbers of the conidia, for at least a few weeks. It is not clear why these did not kill them. If this test is repeated, it might be worthwhile to use repeated inoculation with the *Beauveria*. It would also be worthwhile to look at the effects of the *Beauveria* on younger chinch bugs, and on reproductive rates (fecundity) of the insects. These could be subjects for subsequent work.

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