INDIRECT EFFECTS OF GARLIC MUSTARD ON THE BLACKLEGGED TICK?

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Abstract. The blacklegged tick, *Ixodes scapularis*, is the predominant transmitter of Lyme disease to humans. Finding methods to reduce the abundance of ticks is an important area of study, and the use of entomopathogenic fungus as a biocontrol has shown promise. One such entomopathogen, *Beauveria bassiana*, occurs naturally in the soil, although further study needs to be done to determine its prevalence. Control of the ticks by *B. bassiana* may be blocked to some degree by the invasive weed garlic mustard, *Alliaria petiolata*. Garlic mustard has been shown to secrete allelopathic chemicals from its roots that interfere with the growth of mycorrhizal fungus, so it follows that these chemicals may also affect the growth of *B. bassiana*. Four different studies were conducted to examine the effect of garlic mustard on *B. bassiana*, and indirectly on the blacklegged tick. *B. bassiana* distribution was found to be sporadic in the soil, which may indicate that the contact that it has with ticks in nature may be minimal, but that relationship has yet to be determined. Experiments testing the inhibition of *B. bassiana* growth by garlic mustard did show some conclusive results. In one experiment where different concentrations of *B. bassiana* were added to soil from garlic mustard and non-garlic mustard areas, garlic mustard showed a significant effect of inhibiting the infection of wax worms with *B. bassiana* (P=0.035). When testing the effects of garlic mustard chemicals directly on *B. bassiana* through the use of garlic mustard leachate incorporated into petri plates on which the fungus was grown, results showed that garlic mustard leachate greatly inhibits the germination of *B. bassiana* (P=<0.001), with higher concentrations of garlic mustard leachate needed to inhibit the growth of higher concentrations of *B. bassiana*. These experiments have established that garlic mustard does inhibit the growth of *B. bassiana*, which further study may show indirectly aids in the survival of blacklegged ticks.

The blacklegged tick, Ixodes scapularis, is a vector for several diseases that affect humans and livestock and is the predominant transmitter of Borrelia bergdorferi, the spirochete that causes Lyme disease (Bergdorfer et al. 1982). In 2005, the Centers for Disease Control and Prevention reported 23,305 cases of human infection with Lyme disease (CDC 2006). Increases in human cases of Lyme disease usually coincide with an increase in tick population (Ostfeld 1997), making controlling populations of ticks a matter of the utmost importance.

Several methods have been used in an attempt to keep blacklegged tick populations small, including treatment with pesticides (Schulze et al. 1994), management of white-tailed deer populations (Daniels and Fish 1993), and introduction of tick predators (Knipling and Steelman 2000). These means of control often have high ecological costs, including damage to non-target organisms (Schulze et al. 2001) and the development of pesticide resistance. Given the limitations of these methods, other means of controlling tick populations are being looked at, including the use of entomopathogenic fungi. According to studies, coming in contact with the fungi (through spraying or rinsing ticks with a solution containing spores) Beauveria bassiana has been shown to have a large affect on blacklegged ticks, resulting in 65% mortality for unfed adult ticks after 28 days, and nearly 100% mortality for unfed nymphal ticks after 21 days (Kirkland et al. 2004).

In nature, some varieties of entomopathogenic fungus have been shown to be the most important factor controlling a population of arthropod pests. For example, in Iowa the population of the twospotted spider mite is kept in check by a fungus occurring in the soil called *Neozygites floridana*. When the amount of fungus in the soil decreases due to environmental conditions or chemicals, the spider mite populations grow rapidly, causing serious impacts on crops (Morjan et al. 2002). The entomopathogenic fungus *B. bassiana* may have a similar effect on *Ixodes scapularis*, but the relationship needs to be studied to a greater degree.

Poor growth conditions and the spraying of chemical have been shown to decrease the presence of *Neozygites floridana* in the soil, and it is likely that the same would be true for *B. bassiana*, being that it too is an entomopathogenic fungus. One factor that has been shown to increase the presence of fungicidal chemicals into the soil in areas where *Beauveria bassiana* is found is the presence of garlic mustard.

Garlic mustard, *Alliaria petiolata*, is an invasive weed originating in Europe that has become established in the forests of the Midwestern and Northeastern United States and Canada (Roberts and Anderson 2001). The takeover of the understory by garlic mustard displaces native species and ultimately decreases the species diversity in the areas where it is found, threatening native plant communities (Meekins and McCarthy 1999). Garlic mustard also inhibits the germination of the arbuscular mycorrhizal fungus *Glomus etunicatum*, which colonizes the plant roots of 71-84% of herbaceous deciduous forest understory species and substantially increases the nutrient uptake of the host plants (Roberts and Anderson 2001). This inhibitory effect seems to be caused by the secretion of allelopathic chemicals from the roots and tissues of garlic mustard plants (Vaughn and Berhow 1999).

Being that garlic mustard secretes chemicals into the soil, it is likely that these chemicals would come in contact with and affect other species present in the soil besides mycorrhizal fungi. The growth and germination of spores of the entomopathogenic fungus *B. bassiana* could also potentially be inhibited. As was mentioned previously, *B. bassiana* has been shown to be an effective, natural biocontrol of blacklegged ticks, a function which the presence of garlic mustard may hinder.

The question then arises: How does the presence of garlic mustard affect the survival of blacklegged ticks? It seems likely that the presence of garlic mustard enables the survival of blacklegged ticks in areas surrounding the plants by inhibiting the growth of *B. bassiana* in the soil. Thus, garlic mustard, an invasive weed that has already been shown to have many negative ecological effects on the areas it overruns would potentially have another negative effect added to its list, this one having connotations for humans and for Lyme disease. If it is established that *B. bassiana* does frequently occur in nature and keep populations of blacklegged ticks in check, garlic mustard will be aiding ticks in their survival and indirectly increasing their population size. An increase in tick population means more Lyme disease cases for humans.

MATERIALS AND METHODS

Engorged I. scapularis nymphs deployed into the field in mesh bags

The first experiment involved putting engorged nymphal ticks into organza mesh bags held closed with draw strings. These ticks had been surface sterilized with 10% bleach solution. The bags containing the ticks were placed in plots that had been established in the field, half containing garlic mustard and half without garlic mustard (Bertrand and Wilson 1997). Ten plots, five with extensive cover by garlic mustard and five with garlic mustard completely absent were marked off, with each plot measuring approximately 5 meters by 5 meters and being at least 10 meters from any adjacent plots. On the plots where garlic mustard was present, the stand of garlic mustard extended past each marked plot area by at least one meter in every direction. Twenty blacklegged ticks were placed in each plot, spaced out so that each occupied approximately one square meter. Ticks were examined once each week for a month, and those that were found unmoving during the checks were removed from the plots and taken back to the laboratory. After determining that the ticks were deceased, they were rinsed with dH_2O to remove dirt, and were then placed in a 70% ethanol solution for ~30 minutes. Ticks were then placed on petri plates with Sabouraud Dextrose Agar medium and incubated. Plates that grew fungus were sent to an entomopathogenic fungus expert to identify whether any of colonies were *B. bassiana* or other entomopathogenic species. The week of tick mortality was also recorded to see if disparity existed between tick survival on garlic mustard and non-garlic mustard plots.

Engorged I. scapularis nymphs placed on garlic mustard and control soil in the laboratory

The second experiment was similar to the first experiment described, but it took place within the confines of the laboratory, removing several environmental variables that were present in the field experiment. Soil was collected from all five garlic mustard plots and mixed together, and the same was done with soil from the five control plots. The homogenized garlic mustard soil was then placed in 25-4oz. plastic containers that were filled with soil up to the 1 inch mark. Twenty-five containers were also filled with the homogenized control soil. Once the containers had been filled, one engorged nymphal tick was placed in each. These ticks had been previously surface sterilized with 10% bleach solution. On days 7, 14, and 21 the soil in the containers was watered with 5 milliliters of dH_2O to keep the soil moist. On day 28, the ticks were removed from the soil and rinsed with dH_2O to remove dirt. Then ticks were placed in a 70% ethanol solution for ~30 minutes. Ticks were then placed on SDA plates and incubated. Plates that grew fungus were sent to an entomopathogenic fungus expert to identify whether any of the colonies were *B. bassiana*.

Engorged I. scapularis nymphs and wax worms placed on garlic mustard and control soil that has B. bassiana added

The third experiment took into consideration the fact that it is unknown how much *B. bassiana* is present naturally in the soil. For this experiment, homogenized garlic mustard soil was mixed with varying concentrations of a liquid suspension of *B. bassiana* spores, ranging from 0 to $2.3x10^7$ spores/ml, and ten plastic containers were filled with each concentration. Homogenized control soil was also mixed with the same concentrations of *B. bassiana* and five plastic containers were filled with each concentration (Benjamin et al. 2002). A tick that had been surface sterilized with 10% bleach solution was then placed on the soil surface of each of the containers. These containers were checked on days 7, 14, and 21 and the number of ticks infected with fungus was recorded for each day.

Five additional containers were filled for each concentration of garlic mustard and control soil. A wax worm was added to each of these fifty containers. Wax worms are known to be especially sensitive to *B. bassiana* and using them will indicate if the fungus is present in the soil, even at levels that may be too low to affect the blacklegged ticks. The wax worms did not survive surface sterilization with 10% bleach solution, so they were rinsed with dH_2O to remove surface contaminants. Wax worm containers were examined every other day for 10 days, and the number of worms showing fungal infection was recorded for each day.

Growth of B. bassiana on media with garlic mustard leachate added

The fourth experiment was conducted to test what direct effects garlic mustard chemicals had on the growth of *B. bassiana*. For this experiment, a garlic mustard leachate was prepared by soaking 50g of air-dried whole garlic mustard plant material in 1000ml sterile distilled water for 24 hours. The leachate was then passed through filter paper to remove large debris. Fifteen 10ml plastic Petri dishes were prepared with SDA medium containing only water, 15 plates had media that was prepared with half water and half garlic mustard leachate, and 15 plates were prepared with only garlic mustard leachate. To five of the plates in each of these groups, 5- 5microliter drops containing *B. bassiana* at a concentration of $2.3x10^3$ spores/ml were added. The same procedure was done to five plates from each group with drops containing *B. bassiana* concentration of $2.3x10^2$ spores/ml. The final fifteen plates were inoculated with 5-5microliter drops per plate of dH_2O. The plates were incubated at 30EC for 5 days and removed. The number of drops per plate that grew fungus was recorded (Roberts and Anderson 2001).

RESULTS

Engorged I. scapularis nymphs deployed into the field in mesh bags

The ticks in the bags were checked on days 7, 14, 21, and 28, and the number of deceased ticks was recorded. On day 7, 100% of the ticks on both garlic mustard and control plots survived. On day 14, 43% of the ticks on garlic mustard plots survived and 41% of the ticks on control plots were still alive. On day 21, 21% of ticks on the garlic mustard plots and 12% of ticks on the control plots were still alive, and during the last check (day 28), 4% of ticks on both the garlic mustard and control plots were still alive. Being that both garlic mustard and control plots yielded the same tick mortality after 28 days, it does not appear that there was a significant difference in the survival of ticks dependent on what type of plot they were on (Figure 1).

Engorged I. scapularis nymphs placed on garlic mustard and control soil in the laboratory

At the end of 28 days, 55% of the ticks on garlic mustard soil and 80% of the ticks on control soil were infected with some sort of fungus. 60% of the ticks on garlic mustard soil were still alive after this time period, with 30% of the ticks on control soil surviving. Ticks on garlic mustard soil did show lower fungal infection rates and lower mortality than ticks on control soil after 28 days, but the difference is not statistically significant (p=0.256) (Figure 2).

Engorged I. scapularis nymphs and wax worms placed on garlic mustard and control soil that has B. bassiana added

After 21 days of being in contact with soil that had been inoculated with *B. bassiana*, several ticks became infected with fungus. For ticks on garlic mustard soil, 20% were infected with fungus when no *B. bassiana* was added to the soil, indicating that this soil likely had some sort of fungus present when it was collected or the soil somehow became contaminated with *B. bassiana* as it was being mixed with other soil. When *B. bassiana* was added at a spore concentration of $2.3x10^4$ spores/ml, 40% of ticks on control soil became infected with fungus and 80% of ticks on garlic mustard soil were infected. With a spore concentration of $2.3x10^5$ spores/ml, 40% of ticks on control soil were infected with fungus ad 100% of ticks on garlic mustard soil were infected. With *B. bassiana* added at a concentration of $2.3x10^6$ and $2.3x10^7$ spores/ml, 100% of ticks on both types of soil were infected with fungus. Interestingly, in this experiment more ticks became infected with fungus when they were placed on garlic mustard soil over control soil. Analysis showed that the difference in fungal infection on the two different soils were not statistically significant (p=0.109) (Figure 3).

When the same experiment was conducted using wax worms, however, worms on garlic mustard soil were infected with fungus less than those on control soil (p=0.035). When concentrations of *B. bassiana* were added at concentrations of 0, $2.3x10^4$, $2.3x10^5$, $2.3x10^6$ and $2.3x10^7$ spores/ml, wax worms became infected with fungus at 20, 80, 60, 80, and 100% respectively. Wax worms on garlic mustard soil with the same concentrations of *B. bassiana* added were infected with fungus at 0, 40, 60, 20, and 60% respectively. This experiment showed that wax worms on control soil were infected with fungus more than those on garlic mustard soil, showing that garlic mustard inhibited the ability of *B. bassiana* to infect arthropods (Figure 3b).

Growth of B. bassiana on media with garlic mustard leachate added

When no *B. bassiana* was added to the plates, there were no colonies of fungus that grew, indicating that the plates were not previously contaminated. When there was no garlic mustard leachate present in the media, all five of the *B. bassiana* drops grew fungus independent of the spore concentration. When the media contained 50% garlic mustard leachate, 8% of the drops of *B. bassiana* with a $2.3x10^2$ spores/ml concentration grew fungus, while 76% of the drops with a $2.3x10^3$ spore/ml concentration grew fungus. When the media contained 100%

garlic mustard leachate, 8% of the drops of *B. bassiana* with a $2.3x10^2$ spores/ml concentration grew fungus, with 48% of the drops with a $2.3x10^3$ spore/ml concentration growing fungus. These results show that as the concentration of *B. bassiana* in the drops increased, the number of drops that grew fungus increased ($p<0.001$), and as the percent garlic mustard leachate in the media increased, the number of drops that grew fungus decreased ($p<0.001$). The interaction between the *B. bassiana* concentration and percent garlic mustard leachate was also highly significant ($p<0.001$), indicating that higher concentrations of *B. bassiana* require a higher concentration of garlic mustard leachate to inhibit growth (Figure 4).

DISCUSSION

Entomopathogenic fungi have been shown in numerous studies to be effective controls of blacklegged ticks. *Metarhizium anisopliae*, a fungus that has similar tick virulence to *B. bassiana*, has been more thoroughly examined, but both are able to induce high tick mortality when directly applied or sprayed on ticks (Kirkland, et al. 2004, Benjamin, et al. 2002). Little research has been done to examine how such fungi affect ticks when they are not directly applied, as was the case in this experiment.

A goal of this study was to see if tick mortalities or tick infection with fungus was different if they were placed on garlic mustard soil or control soil. It was found that if ticks were put out in bags onto these different types of soil, there was no difference in their survival rates. Also, although many ticks became infected with fungus, most likely postmortem, none of them were infected with *B. bassiana*. This may indicate that *B. bassiana occurs* only sporadically in the soil, or that for some reason the area where the plots were placed could not support *B. bassiana* growth. Either way, this experiment did not have any results that could help determine if garlic mustard soil affects *B. bassiana* infection of ticks because there was no fungus present to begin with in control or garlic mustard plots.

When *B. bassiana* was added at different concentrations to soil from garlic mustard and control areas, there was no significant difference in the fungal infection of ticks based on what type of soil they were placed on, but wax worms did show significant differences in infection rates. Wax worms on garlic mustard soil were infected with *B. bassiana* less than those that were on control soil, indicating that the garlic mustard soil was able to inhibit the growth of the fungus. It is unclear why the experiment with wax worms yielded significant results while the one conducted with ticks did not, but additional experimentation on a larger scale would likely yield more unambiguous results.

When the effect of garlic mustard chemicals on the growth of *B. bassiana* was tested directly by adding garlic mustard leachate to media on which *B. bassiana* was being grown, the chemicals were shown to greatly inhibit the growth of the fungus. Higher spore concentrations required higher concentrations of garlic mustard leachate to inhibit growth. These results would be expected to hold true in nature as well, with large numbers of garlic mustard plants being required to inhibit the growth of *B. bassiana* if it is present at high concentrations in the soil. The prevalence of *B. bassiana* in the soil has not yet been studied, so it is difficult to say how much of an effect large stands of garlic mustard would have on the fungus' ability to thrive in the soil. Further study will be able to determine this relationship and how much garlic mustard is actually required to inhibit *B. bassiana* growth in the soil.

This study showed that garlic mustard does have an inhibitory effect on the growth of *B. bassiana*. Being that this fungus occurs to some degree naturally in the soil and is a potential controller of blacklegged tick populations, the presence of garlic mustard may be indirectly aiding in the survival of these ticks. Although it is uncertain how much blacklegged ticks come in contact with entomopathogenic fungi in the soil, it seems possible that these fungi would play a part in keeping tick populations low. By inhibiting the growth of a natural enemy, garlic mustard is indirectly allowing tick populations to grow larger. Larger blacklegged tick populations lead to more cases of tick-borne illness in humans and livestock. It is imperative that effective ways of controlling ticks be found, so the interactions of blacklegged ticks, entomopathogenic fungi, and garlic mustard should be studied more thoroughly.

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APPENDIX

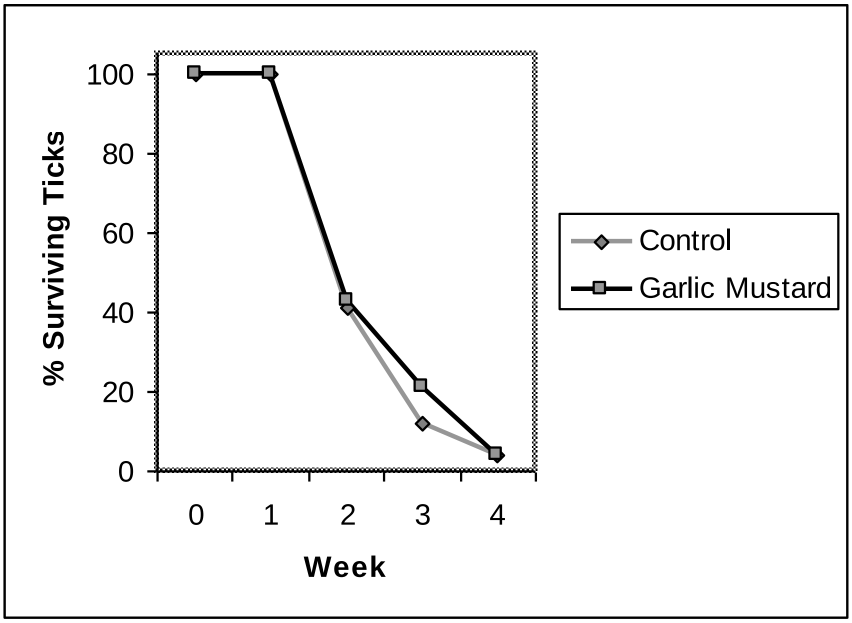

FIGURE 1. Weekly mortality rates for engorged *I. scapularis* nymphs on control and garlic mustard plots.

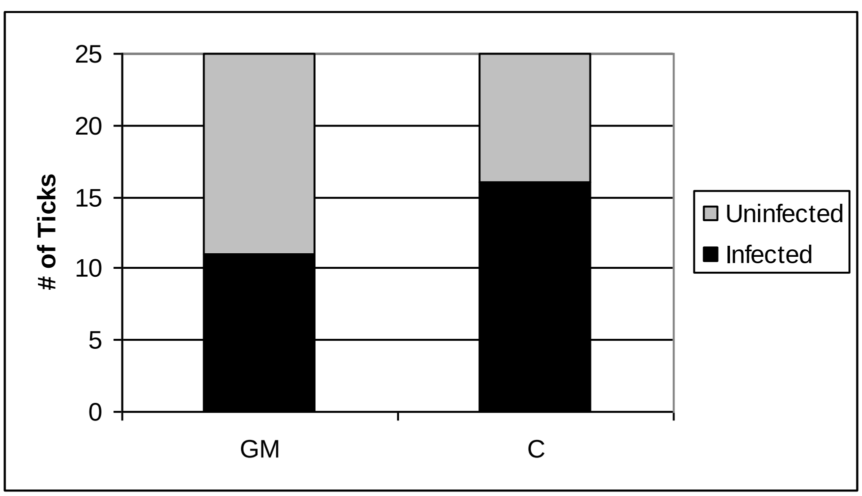

FIGURE 2. Fungal-infected and uninfected engorged *I. scapularis* nymphs after 28 days on control and garlic mustard soil. X^2= 1.288, DF=1, P=0.256

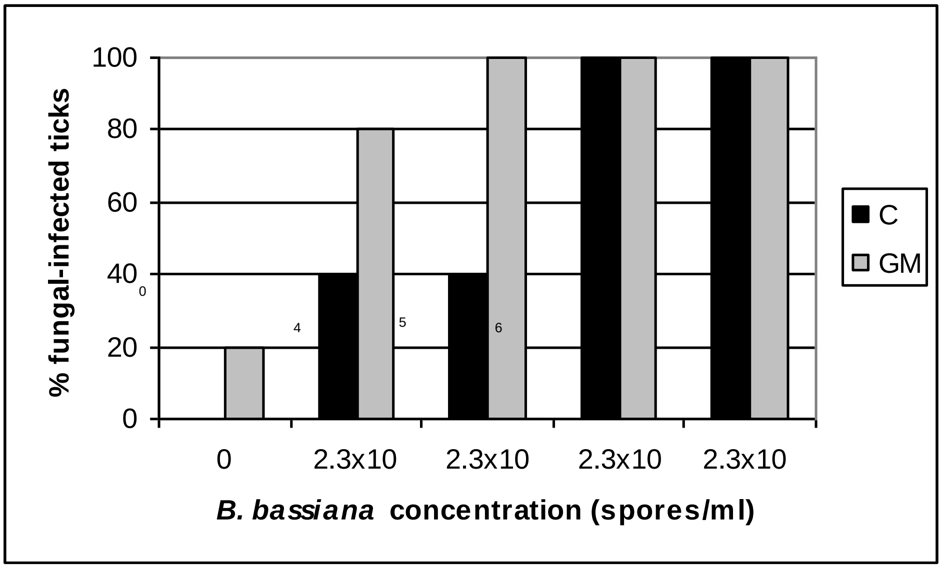

FIGURE 3A. Percentage of fungal-infected ticks with different concentrations of *B. bassiana* added to garlic mustard and control soil. *B. bassiana* concentraion: F=8.059, DF=4, P=0.034; Control vs. garlic mustard: F=4.235, DF=1, P=0.109.

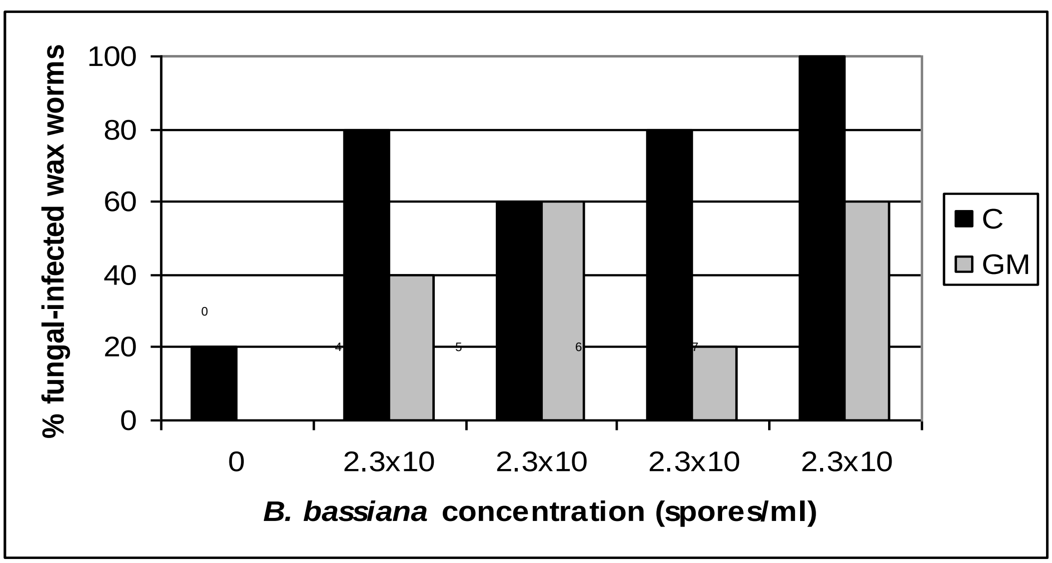

FIGURE 3B. Percentage of fungal-infected wax worms with different concentrations of *B. bassiana* added to garlic mustard and control soil. *B. bassiana* concentration: F=5.154, DF=4, P=0.071; Control vs. garlic mustard: F=9.846, DF=1, P=0.035.

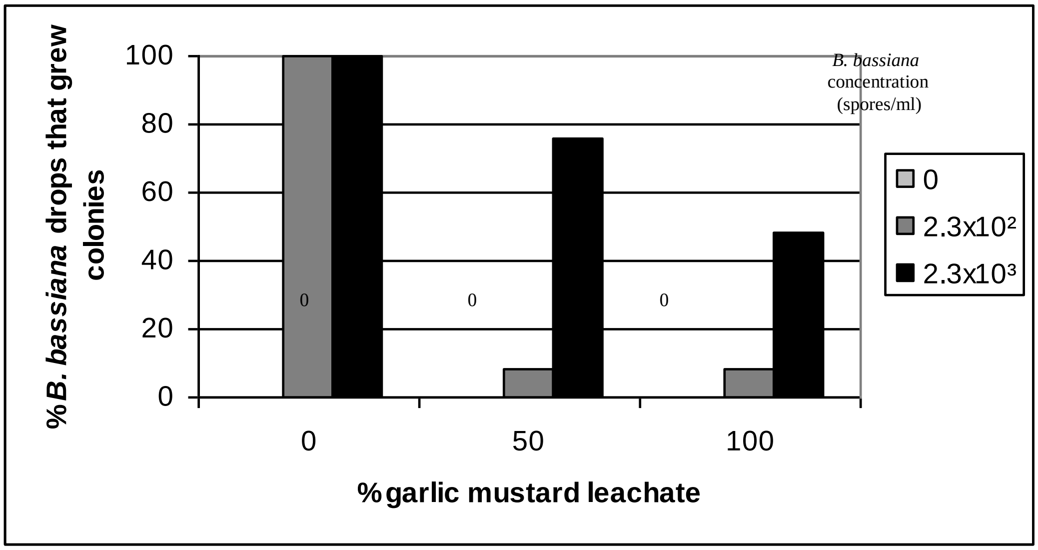

FIGURE 4. Percentage of *B. bassiana* drops that grew fungal colonies with varying concentrations of fungus and garlic mustard leachate. *B. bassiana* concentration: F=49.021, DF=2, P<0.001, % garlic mustard leachate: F=22.771, DF=2, P<0.001, Interaction term: F=9.115, DF=4, P<0.001