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Whey-Based Fungal Microfactories for In Situ Production of Entomopathogenic Fungi

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WHEY-BASED FUNGAL MICROFACTORIES

FOR *IN SITU* PRODUCTION OF

ENTOMOPATHOGENIC FUNGI

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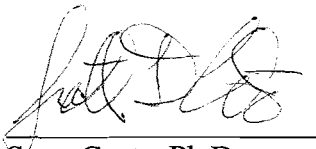

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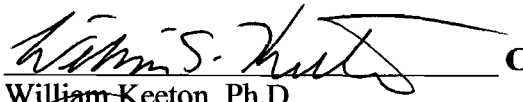

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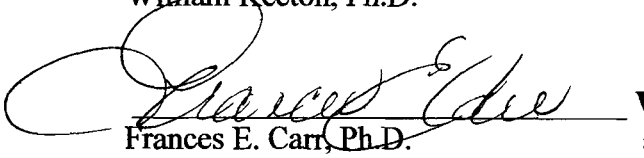

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Abstract

The eastern hemlock is a late-successional conifer species that is valued for its ecological functions, recreational importance, aesthetic beauty, and economic value. The hemlock woolly adelgid (HWA, *Adelges tsugae* Annand, Homoptera: Adelgidae) is an invasive aphid-like insect from Asia that is causing serious damage to the eastern and Carolina hemlock. HWA was first introduced to the Eastern United States to Virginia in the 1950's and has since moved along the east coast from Georgia to Maine. It can kill a healthy tree in three to seven years depending on many environmental factors. Native predators have not been successful in reducing damage to hemlock trees or limiting the spread of HWA.

Chemical, cultural, and biological control efforts have been implemented in states with high populations of HWA. Chemical applications are costly and current formulations are difficult to apply in large stands and in forested areas. Salvage cutting is a method to control the spread of adelgid and recover some economic value of the wood. Predatory beetles native to Asia, where HWA is present but not a severe problem, have been researched, reared, and released into infested stands in the Eastern United States. Their success for management of HWA has been difficult to ascertain. The entomopathogenic fungus *Lecanicillium muscarium* ((Petch) Zares & Gams) is another biological control agent with potential to manage and suppress adelgid populations. *L. muscarium* (Mycotal™ technical powder, Koppert Biological Systems) plus the nutritive base sweet whey, may promote conidia production without contact with a host and this means of increasing conidia is called a whey-based fungal microfactory. The whey would act as a nutritive substrate for fungi sprayed into hemlock forests. Formulation droplets deposited on hemlock needles should support fungal growth and serve as tiny factories for conidia production.

Microfactory production was characterized in different combinations of sweet whey (0, 5, 10, and 15%) and conidia concentration (1×10^6, 1×10^7, and 1×10^8 conidia/ml) applied to lids of Petri dishes. A dramatic 42- and 29-fold increase in conidia production occurred with the addition of 10% sweet whey to 1×10^6 and 1×10^7 conidia/ml, respectively. Increasing whey concentration increased the number of conidia that were recovered. Conidia production was also obtained on hemlock foliage, with similar trends in influence of conidia and whey concentration. The hemlock branches also contained HWA and their mortality was evaluated. Adelgid mortality was highest in formulations containing sweet whey, but whey had an independent effect on mortality. Several antimicrobials were evaluated for compatibility with *L. muscarium* in the microfactory formulation. Nisin did not inhibit conidia production. The addition of antimicrobials to the whey-based formulations may limit the competition between the fungus and other microbes present in the spray tank and on foliage.

Whey-based fungal microfactory technology is intended to facilitate multiplication of fungi in the natural environment. Transferring a portion of fungal mass-production into the treatment environment could reduce application costs and increase the feasibility of using fungi for biological control of HWA and other pest species.

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Literature Review

Introduction

Integrated pest management is a method of pest control that utilizes ecologically based practices including cultural, physical, biological, and chemical applications, which are employed to optimize plant health and promote ecological processes. Biological control is the establishment or promotion of naturally occurring predators, parasitoids, and pathogens (bacteria, viruses, fungi, and protozoa) for management of a target pest. Pesticides can be used in addition to biological methods for optimum suppression of pest populations.

Urban, forest, and agricultural ecosystems are all susceptible to invasive species. Invasive species such as the hemlock woolly adelgid (*Adelges tsugae*), chestnut blight (*Chryphonectria parasitica)*, and emerald ash borer (*Agrilus planipennis*) have been responsible for significant declines and changes in ecosystem functions and processes throughout the United States (Orwig *et al.*, 2002; Paillet, 2002; Poland & McCullough, 2006). Naturally occurring plant resistance and control agents have not been able to reduce the impacts of these invasive insects and pathogens.

The hemlock woolly adelgid (HWA) is an introduced pest of eastern hemlock (*Tsuga canadensis,* L. Carriere) and Carolina hemlock (*T. caroliniana*, Engelm.). The adelgid removes nutrients and tree death can occur in three to seven years (McClure *et*

al., 2001). The loss of these dominant late-successional species has major impacts on species composition of hemlock forests (Orwig *et al*., 2002).

Predatory beetles have been introduced as part of a 'classical biological control' scheme for suppression of hemlock woolly adelgid (Cheah *et al*., 2004). Chemical controls are also being implemented but are of limited utility in forest settings due to concerns over environmental impacts. The use of insect-killing fungi, or entomopathogenic fungi, is also being actively researched (Cheah *et al*., 2004). These fungi are reducing adelgid populations in small field trials but their use is limited by constraints of formulation and economics and the lack of registered products (Costa *et al.*, 2005).

The entomopathogenic fungus *Lecanicillium muscarium*, in the mycoinsecticide Mycotal™ (Koppert Biological Systems, The Netherlands) may have the potential to suppress adelgid populations. The combination of Mycotal and the nutrients found in sweet whey may form whey-based fungal microfactories where conidia production is increased without direct contact with the adelgid. The goals of this study were to evaluate the formation of whey-based fungal microfactories on Petri dish lids and on HWA infested branch tips, assess adelgid mortality, and to select antimicrobials as a formulation additive that may reduce competition between *L. muscarium* and other microbes. The combination of Mycotal, sweet whey, and an antimicrobial as a biological control formulation may have the potential to decrease HWA populations.

Hemlock Woolly Adelgid

Hemlock woolly adelgid (*Adelges tsugae*, Annand, Homoptera: Adelgidae) is an introduced pest that causes severe damage to the eastern and Carolina hemlock. HWA was first introduced to the Eastern United States during the 1950's in Virginia. It has since moved along the east coast and is present from Georgia north to Maine (McClure *et al.*, 2001). The degree of infestation varies for each state and within each hemlock stand.

The adelgid is an aphid-like insect introduced from Asia. HWA is not considered a serious pest in Japan, likely because of unidentified host plant resistance and naturally occurring predators (McClure *et al.,* 2001), which apparently are not present in the United States. The adelgid feeds at the base of hemlock needles using a piercing-sucking stylet. The feeding tube is composed of two outer mandibular stylets and two inner maxillary stylets. The sites of insertion are primarily current year growth indicating adelgid prefer immature tissues (Young *et al.*, 1995).

Feeding diverts nutrients from the needles, causing them to dry and stopping bud growth (McClure *et al.,* 2001). Infested trees lose their dark green color and turn grey and eventually needles drop off and entire branches die back. A tree can die in three to seven years depending on the degree of infestation and other stressors, although some trees survive longer. Weakened trees infested with HWA are more susceptible to other pests such as elongate scale (*Fiorinia externa,* Ferris) and hemlock borer (*Melanophila fulvoguttata,* Harris) (Cheah *et al.*, 2004).

Hemlock woolly adelgid has a polymorphic life cycle that includes the progredien (short) and sisten (long) generation each year (McClure, 1989). The sisten generation is

present from July to April and in late winter, it is easily seen due to its cottony ovisac on the underside of the needle at the base. Depending on temperature, the sisten adults lay eggs from March to April. Hatching nymphs are mobile, but soon insert their feeding tube and then remain stationary throughout their life. The progredien generation usually results in wingless, female adults that lay eggs that give rise to the sisten generation. However, if progrediens are over crowded as nymphs they may develop into winged sexupara (McClure, 1989). The sexupara leave hemlock in search of a suitable spruce tree to complete their life cycle. The sexupara only feed on *Picea*, but no suitable species are present in the Eastern United States for them to complete their life cycle (McClure, 1989).

Sisten nymphs move onto new growth after hatching and then go into aestivation during the summer months. Nymphs do not develop the typical covering of cottony wool during the aestivation period. Aestivation allows HWA to resist the extreme summer temperatures and is induced by long days (Salom *et al.*, 2001). As the weather cools in the fall, the sisten nymphs begin actively feeding and resume development, including accumulation of their woolly covering. They feed throughout the winter, develop into adults by early spring, and the cycle begins again.

Low temperatures in Northern New England states appear to be affecting HWA population dynamics and range expansion (Costa, unpublished data). Temperatures below -25^{o}C cause high levels of adelgid mortality, though they may loose their cold tolerance in late winter months (Skinner *et al.*, 2003). However, even with possible

impacts of low temperature, HWA continues to have profound influences on hemlock forests in the Northeastern United States.

Eastern Hemlock

The eastern hemlock is found on moist acidic soils in many topographic regions ranging from the Canadian Maritimes to Georgia and west to Minnesota (Ward *et al.*, 2004). Hemlocks provide shade and create cooler microenvironments in wetlands and riparian habitats (Orwig *et al.*, 2002). As a late-successional, long-lived species, hemlock provides important habitats for wildlife, plant, and aquatic populations. Mammals associated with hemlock-dominated forests include porcupines (*Erethizon dorsatum*) and white tailed deer (*Odocolileus virginianus)*. Ninety species of birds, including the black throated green warbler (*Dendroica virens)* and the Acadian flycatcher (*Empidonax virescens)* are strongly associated with hemlock forests and populations are significantly reduced with the loss of hemlocks (Tingley *et al.*, 2002).

The biodiversity of streams is also altered with the decline of the hemlock tree component of forests. Brook trout (*Salvelinus fontinalis)* populations are three times greater in hemlock-dominated streams. Piscoivore populations are higher then insectivore populations in hemlock-dominated streams (Ross *et al.*, 2003). Amphibian populations are higher in coniferous forests and the populations of redback salamanders (*Plethodon cinereus*) decrease with the removal of hemlock overstory (Brooks, 2001).

The eastern hemlock has a multilayered dense canopy, a shallow root system, and is extremely shade tolerant. Hemlock seedlings grow slowly and most readily establish in moist areas with little competition and adequate light (Mladenoff & Stearns, 1993).

The structure, function, and composition of the forest changes as hemlocks decline (Stadler *et al.*, 2005). Healthy hemlock stands usually resist vegetation invasion due to acidic and nutrient poor soils that contain a thick humus layer and receive little light (Rogers, 1978). With the decline of hemlock due to the adelgid the temperature of the forest floor increases, light availability increases, and nitrate exports are accelerated (Stadler *et al.*, 2005). These changes lead to establishment of less desirable woody species, such as black birch (*Betula lenta*), and intrusion of invasive species into the ground cover (Orwig & Foster, 1998).

Entomopathogenic and Biological Control Fungi

Entomopathogenic or insect-killing fungi are found in the phyla: Zygomycota, Ascomycota, and Deuteromycota, and have long been recognized for their potential use as insect biological control agents. The majority of the fungi belong to the class Hyphomycetes of the phylum Deuteromycota (Hajek, 1997). To be effective, the infective unit or conidia must disperse and achieve contact with the insect host (Hall, 1981). The life cycle of entomopathogenic fungi involves conidia adherence to the insect, its germination, penetration through the insect cuticle, colonization of insect hemolymph and body tissues, sporulation usually outside the host, and dispersal of the next generation of conidia (Hajek, 1997).

Environmental factors and nutrition control the infection cycle of fungi. Optimal moisture, temperature, available nutrients, and inoculum number influence germination, colony expansion, and infection rates of biological control fungi. The nearly ubiquitous entomopathogenic fungus, *Beauveria bassiana*, has a wide host range and can infect

beetles, caterpillars, and aphids. *B. bassiana* readily germinates at relative humidity (RH) levels above 95% but does not germinate below 90%. Germination rates are optimal at temperatures between 25-30^{o}C (Luz & Fargues, 1997). *B. bassiana* can not germinate in distilled water but, with the addition of glucose as a carbon source and ammonium chloride as a nitrogen source, germination and colony expansion are promoted (Smith & Grula, 1981).

Metarhizium anisopliae is a soil fungus that is active against approximately 200 insects including Japanese beetles, mosquitoes, and the spittlebug. The highest germination and sporulation rates for *M. anisopliae* occur at 100% RH and between 20-30^{o}C. Reduction in germination, sporulation, and virulence occurs as the RH and temperature drop (El Damir, 2005). Growth and sporulation increases through the addition of carbohydrates (glucose, fructose, maltose, mannose, soluble starch, sucrose, and xylose) to nitrate-based media. The addition of vitamins has no effect on the germination and growth of *M. anisopliae* (Li & Holdom, 1995).

Penicillium oxalicum is a biological control agent active against the soil fungus *Fusarium oysporum* (Pascual *et al.*, 1997a). Pascual *et al.* (1997a) found that the growth rate of *P. oxalicum* is highest at 25^{o}C, but sporulation is achieved at a much broader range of temperatures (10-25^{o}C). The rate of *P. oxalicum* germination is stimulated by xylose, mannose, sucrose, and fructose as carbon sources, and arabinose and mannose influence sporulation. Peptone and tryptone as protein sources promoted colony expansion and peptone also promoted sporulation.

Conidia production can also be influenced by the amount of inoculum within a formulation. When *in-situ* inoculum numbers are high the amount of nutrients provided to the conidia and lack of oxygen may become limiting factors in germination (Allen, 1976; Pascual *et al.*, 1997b). However, oxygen depletion at atmospheric levels in an open environment should not prohibit germination (Cochrane, 1958).

Several species of the fungi (*Colletotrichum* and *Phoma*) have decreased germination at formulation rates higher then 2.5 x 10^6 conidia/ml (Zhang *et al.*, 2003). Overcrowding at 1 x 10^7 conidia/ml produces self-inhibition with the fungus *C. gloeosporioides* f. sp. *jussiaes* due to exudates (Lax *et al.*, 1985). Self-inhibition due to germination inhibitors in the form of alkaloids is present in the fungus *Glomerella cingulata* (Lingappa & Lingappa, 1967). The effects of self-inhibition may be overcome by adequate moisture through dilution of exudates (Gottlieb, 1973).

Lecanicillium muscarium

Lecanicillium muscarium (Petch) Zares & Gams [formerly *Lecanicillium lecanii* (Zimm.) Zare. & Gams, formerly *Verticillium lecanii* (Zimm.) Viegas] (Zare & Gams, 2001) has a wide host range including aphids, scales, and phytopathogenic fungi (Askary *et al.*, 1997; Hall & Burges, 1979). It belongs to the class Hyphomycetes and reproduces asexually (anamorphic stage) by conidia without fruiting bodies (Hajek, 1997).

Askary *et al*. (1999) characterized aphid invasion by *L. muscarium* (*V. lecanii)* and observed the conidia adhering to all parts of the aphid using a mucilaginous matrix. The conidia were ovoid and the hyphae measured 1.8-2.6 μm in diameter. Germination and the development of hyphae took place approximately 24 hours after inoculation and

the hyphal bodies formed dense colonies. Penetration of the cuticle and detection within the cuticle occurred after 48 hours. No appressorial structures were present in the penetration of the epicuticle and the germ tubes entered through direct penetration. After 72 hours, the procuticle was severely damaged and after 120 hours, the hyphae had almost completely invaded and digested the integument. The blastospores that originated from the hyphae on the procuticle had colonized the hemocoel at the same time the integument had been invaded. All of the aphid's cavities were occupied by *L. muscarium* after 96 hours and death of the aphid occurred within five days.

In controlled environments and field trials, *L. muscarium* is antagonistic against powdery mildew. On cucumber, leaves infected with cucumber powdery mildew (*Sphaerotheca fuligine*) *L. muscarium* formed a mucilaginous matrix and sporulation occurred between 96 and 120 hours (Askary & Yarmand, 2007). Strawberry powdery mildew (*S. macularis*) levels were reduced in the field when *L. muscarium* was applied repeatedly above 1 x 10^{13} conidia/ha (Miller *et al.*, 2004). *L. muscarium* released lytic enzymes into growth medium that inhibited the growth of *Mucor mucedo, Botrytis cinerea, Pythium aphanidermatum,* and *Phytophthora palmivora* and then *L. muscarium* continued to grow in the presence of microorganisms (Fenice & Gooday, 2006).

For insect-killing fungi to be considered effective they must be able to control adelgid populations, be safe, easily mass-produced, and be compatible with predators that have been released and established in infested stands. *L. muscarium* is active against aphids and virulence against non-target pests has been tested. Non-target invertebrates in the orders Coleoptera, Hymenoptera, Collembola, and aphid species susceptible to

infection were studied (Sitch & Jackson, 1997; Wang *et al.*, 2005). No signs of infection occurred with non-target invertebrates and post-inoculum conidia numbers were lower on non-target insects than on aphids, the target host (Sitch & Jackson, 1997).

Non-target effects of toxins extracted from two strains of *L. muscarium* were tested on the ladybird beetle (*Delphastus catalinae*, Horn), a predator of whiteflies. Mortality and foraging behavior of adults and larvae were studied. The effects of the toxins were greater to larvae causing significant decreases in food intake (Wang *et al.*, 2005). Lab and field trails have shown that *L. muscarium* is compatible with the ladybird beetle (*Sasajiscymnus tsugae*, Sasaji & McClure), a predator of HWA (Costa *et al.*, 2005).

L. muscarium has potential to suppress the hemlock woolly adelgid in forest settings (Costa *et al.*, 2005). It is naturally occurring in the field and has been isolated as *Verticillium lecanii* on HWA at Mount Tom, MA (Reid, 2003). However, physical constraints such as penetration of aerial sprays into the hemlock canopy to obtain fungal contact with HWA and high formulation costs because of the expense of fungal mass-production need to be overcome for successful deployment of *L. muscarium*.

These constraints may be overcome through the addition of nutrients to the formulation mix to allow fungi to mass-produce after treatment application. This may increase inoculum numbers in the spray residue without direct contact with the insect host. The amount of conidia added to the formulation could then be decreased because of the ability of *L. muscarium* to mass-produce in the formulation.

Sweet Whey

Sweet whey, also known as cheese whey, is a byproduct from the production of Edam and Cheddar cheese. It is produced during the process of separating liquid from curd and is made up of lactose, protein, minerals, fat, and moisture (Shon & Haque, 2007). The nutritional composition of sweet whey varies slightly by manufacturing distributor. On average dried sweet whey contains 72% lactose, 1.2% fat, 13% protein, 2% moisture, and 3% salt (Sithole *et al.*, 2006). In the production of sweet whey, the liquid is removed and the product is a cream colored powder. The color of the whey depends on particle size that is determined by the process of water removal. Particle size can range from 10-1200 µl (Banavara *et al.*, 2003). Sweet whey is characterized by its solubility, oil-holding capacity, and available nutrients.

Sweet whey was selected as a nutrient source for fungal development due to the low protein and high carbohydrate levels that result in a carbon to nitrogen ratio of approximately 5:1. In preliminary trials of research toward this thesis whey protein concentrate, a 35-80% protein powder (Onwulata *et al.*, 2004), was evaluated as an additive but did not promote germination of *L. muscarium*, therefore no increase in conidia occurred (Grassano and Costa unpublished data). Initial trials with the addition of sweet whey to the technical powder of the bioinsecticide Mycotal that is formulated with *L. muscarium* promoted germination, growth, and sporulation of the fungus. The combination of sweet whey and Mycotal, or other biological control fungi resulted in a patent pending technology (Costa *et al.*, 2006) that has been named whey-based fungal microfactory technology, where fungi is mass-produced in spray application residue. If

properly developed, this technology should increase the number of conidia available to infect a host.

Antimicrobials

The growth and survival of competitive microbes in the whey formulation, spray tank, and on hemlock foliage may be reduced by the addition of antimicrobials to the sweet whey suspension. Fungus can grow in the presence of bacteria but fungal biomass will be higher when it is not competing with bacteria for nutrients (Mille-Lindblom *et al.*, 2006). It is important to find a concentration of an antimicrobial that will not inhibit *L. muscarium* yet still reduce the competition between the growth of this fungus and other microbes.

For this research the following antimicrobials were selected because they are considered "generally recognized as safe" GRAS: Nipacide Bit 20, sorbic acid, acetic acid, and nisin (Davidson *et al.*, 2005). They were also selected due to their activity spectrums and the ease at which they dissolved in the whey-based fungal microfactory formulation. Nipicide Bit 20 is similar to parabens, both have an inhibitory effect on fungi and bacteria and interfere with the metabolic processes of cell division (Shiralkar *et al.*, 1976).

Sorbic acid is a weak acid with a broad range of antimicrobial properties including yeasts and molds (Inglis & Cohen, 2004). Sorbic acid levels of 0.1123 mg/ml and 0.006 mg/ml delay the germination and growth of *Aspergillius niger* at different rates, however, *A. niger* has the ability to breakdown sorbic acid (Plumridge *et al.*, 2004). Sorbic acid at 8.0 mg/ml will inhibit the germination, hyphal expansion, and sporulation

of *L. muscarium* (Sun & Liu, 2006). *Metarhizium anisopliae* growth was completely suppressed by the addition of sorbic acid to basal media (Li & Holdom, 1995). The ability of sorbic acid to inhibit fungal growth also increases as the pH decreases for some fungi (Fujita & Kubo, 2005).

Acetic acid is a broad spectrum antimicrobial and is effective against both bacteria and fungi. Acetic acid is highly soluble in water and the concentration of acetic acid depends on the pH of the formulation and target microbe. Growth of the fungus *Colletotrichum gloeosporioides* was decreased by acetic acid at 1.8 mg/ml and completely inhibited at 3 mg/ml (Han-Chul *et al.*, 2003). Acetic acid added to basal media had no impact on the growth of the fungus *M. anisopliae* (Li & Holdom, 1995).

Nisin is not effective against fungi but is a bactericide active against gram-positive bacteria and some gram-negative bacteria (Dielbandhoesing *et al.*, 1998). It is extremely stable at temperatures not exceeding 25^{o}C and at the pH range of 3 to 3.5 (Davidson *et al.*, 2005). The minimum inhibitor concentration (MIC_{50}) of nisin for the lactic acid bacteria *Oenococcus oeni* is 2.4 x 10^{-5} mg/ml, the MIC_{50} for acetic acid bacteria is 0.2 mg/ml, and at $\leq$ 0.4 mg/ml nisin had no effect on yeasts (Rojo-Bezares *et al.*, 2007).

Conclusion

Hemlock wooly adelgid is a pest of the eastern and Carolina hemlock and can cause extensive tree mortality. Native predators and host plant resistance have not been able to stop the spread of the adelgid. Chemical, cultural, and biological control efforts have been implemented in states with high populations of HWA.

The entomopathogenic fungus *L. muscarium* is a naturally occurring fungus with activity against the woolly adelgid. Entomopathogenic fungi have a history of use for insect control. The use of insect-killing fungus for the control of HWA is being researched in the lab and field.

The objective of this study was to optimize the concentration of sweet whey and *L. muscarium* conidia for sporulation in whey-based fungal microfactories. The sporulation response was evaluated on Petri dish lids and on hemlock foliage. The implications this may have for hemlock woolly adelgid mortality were also evaluated. Additionally, the sensitivity of *L. muscarium* to several antimicrobial compounds was examined to determine their compatibility as formulation adjuvants.

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Chapter II

Article 1

Whey-based fungal microfactories for *in situ* production of *Lecanicillium muscarium* (Hyphomycetes: Moniliales) targeting hemlock woolly adelgid (Homoptera: Adelgidae)

by

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Whey-based fungal microfactories for *in situ* production of *L. muscarium* targeting hemlock woolly adelgid (Homoptera: Adelgidae)

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Abstract

The combination of sweet whey and Mycotal™ technical powder (*Lecanicillium muscarium)* generates conidia production on an inert surface without direct contact of an insect host, such as might occur with a spray deposit on foliage. We call production of conidia in this manner a whey-based fungal microfactory. Microfactory production was initially characterized at factorial combinations of sweet whey (0, 5, 10, and 15%) and conidia concentration (1×10^6, 1×10^7, and 1×10^8 conidia/ml) applied to lids of Petri dishes. A dramatic 42- and 29-fold increase in conidia production occurred with the addition of 10% sweet whey to 1×10^6 and 1×10^7 conidia/ml, respectively. The influence of whey on conidia production was less pronounced at 1×10^8 conidia/ml suggesting inhibition at higher conidia concentrations. Increasing whey concentration increased the number of conidia that were recovered. Conidia production was also obtained from hemlock foliage infested with hemlock woolly adelgid (*Adelges tsugae*).

Similar trends in the influence of conidia and whey concentration were observed. Adelgid mortality tended to be highest in fungal formulations containing sweet whey, but whey had an independent effect on mortality. Antimicrobials may have value as a formulation constituent to decrease competition for the whey nutrient resource that might occur between the insect-killing fungus and microbes present in the whey, spray units, and on foliage. Nisin, sorbic acid, acetic acid, and Nipacide Bit 20 were evaluated at a range of concentrations to determine if they inhibited conidia production in microfactories. Nisin and sorbic acid were the most likely formulation candidates because of their lack of negative effects. Whey-based fungal microfactory technology has promise for enhancing the potential of *L. muscarium* and other biological control fungi as pest management alternatives.

Keywords: *Lecanicillium muscarium*, Mycotal, entomopathogenic fungi, sweet whey, sporulation, hemlock woolly adelgid

Introduction

Entomopathogenic fungi are important biological control agents (Hall, 1981; Hall & Burges, 1979). Factors limiting their implementation include the cost of formulated materials and the effective distribution of the fungi throughout the treatment environment. Formulations adapted to microfactory technology that allows fungi to grow and sporulate in post-treatment residues may help overcome these limitations by decreasing costs and increasing the opportunity of fungal host contact. Essentially, a portion of the cost for fungal mass-production would be eliminated by shifting conidia production into the field, thus allowing reduction of the initial load of fungal inoculum in the formulation. Reducing treatment costs is particularly important when managing pests infesting lower value resources such as forests and rangelands.

Hemlock woolly adelgid (HWA, *Adelges tsugae,* Annand, Homoptera: Adelgidae) is an introduced pest of the eastern hemlock (*Tsuga canadensis,* L. Carriere) and the Carolina hemlock (*T. caroliniana,* Engelm.). The structure, function, and composition of the forest changes as hemlocks decline after HWA establishment (Stadler *et al.*, 2005). For instance, healthy hemlock stands usually resist vegetative invasion due to factors such as minimal light availability, and soils that are acidic, nutrient poor, and contain a thick humus layer (Rogers, 1978). With the decline of hemlock due to the adelgid, the temperature of the forest floor increases, light availability increases, and nitrate exports are accelerated (Stadler *et al.*, 2005).

Naturally occurring predators and host plant resistance have not been successful at limiting the impact of the woolly adelgid (McClure *et al.*, 2001). A variety of insect-

killing fungi, including *Lecanicillium muscarium* (*Verticillium lecanii*), have been isolated from forest populations of hemlock woolly adelgid (Reid, 2003). *Lecanicillium muscarium* (Petch) Zares & Gams (formerly *L. lecanii* (Zimm.) Zare. & Gams, formerly *V. lecanii* (Zimm.) Viegas) (Zare & Gams, 2001) has a wide host range that includes aphids, scales, and phytopathogenic fungi (Askary *et al.*, 1997; Hall & Burges, 1979). *L. muscarium* can penetrate the cuticle of aphids within 48 hours and complete invasion of the host occurs by 72 hours. Death of the insect host and sporulation occur by 96 hours (Askary *et al.*, 1999; Askary & Yarmand, 2007).

The success of entomopathogenic fungi depends on contact between the conidia and insect host (Hajek & Eastburn, 2003). Formulations of mycoinsecticdes contain fungal inoculum and inert ingredients that may be mixed with water before spray application (Butt *et al.*, 2000). Manipulation of the formulation by the addition of nutrients might generate fungal microfactory production where the conidia germinate, grow, and sporulate after being applied. Formulations based on microfactory technology containing *L. muscarium* as the biological control agent could increase the number of conidia available in hemlock canopies for managing the woolly adelgid.

Additives containing varying nitrogen and carbon sources have been evaluated for their effects on germination, sporulation, and/or virulence of entomopathogenic fungi (Curtis *et al.*, 2003; James, 2001; Mo *et al.*, 2005; Sun & Liu, 2006; Williams De Courcy *et al.*, 2000). Whey proteins and sugars are an available nutrient source from cheese production. The characteristics of the whey produced depend on type of cheese manufactured and further processing of the whey byproduct separation process (Sithole *et*

al., 2006). Sweet whey is composed of approximately 72% lactose, 1.2% fat, 13% protein, 2% moisture, and 3% salt with a carbon to nitrogen ratio of 5:1. Sweet whey was selected because of the lower protein and higher carbohydrates levels that give a C:N ratio favorable for fungal growth (Mo *et al.*, 2005).

Various microbes are present in whey, on spray equipment, and resident in the field that might compete with *L. muscarium* for nutrients added to formulation mixtures. Antimicrobials added to the formulation might reduce possible competition and further enhance conidia production. The antimicrobials nisin, sorbic acid, acetic acid, and Nipacide Bit 20 were selected for their various activity spectrums against bacteria and fungi. Nisin and acetic acid are antibacterial agents with limited activity to fungi and molds (Dielbandhoesing *et al.*, 1998; Han-Chul *et al.*, 2003). Nipacide Bit 20 and sorbic acid are bactericides and fungicides (Fujita & Kubo, 2005; Plumridge *et al.*, 2004).

The objective of this study was to optimize the concentration of sweet whey and *L. muscarium* for conidia production in whey-based fungal microfactories. This was evaluated on Petri dish lids and on hemlock foliage. The effects of whey-based formulations on hemlock woolly adelgid mortality were also evaluated. Additionally, the compatibility of several antimicrobial compounds as formulation adjuvants was evaluated based on their inhibitory effect on *L. muscarium* conidia production.

Materials and Methods

Microfactory Production

Whey-based fungal microfactory production was optimized through a factorial design where the concentrations of sweet whey and *L. muscarium* conidia were varied in formulations sprayed onto dry lids of 60 x 15 mm Petri dishes (Becton Dickinson Labware, Franklin Lakes, NJ). Treatments contained different concentrations of whey (0, 5, 10, and 15% w/v) and (Mycotal™ technical powder; Koppert Biological Systems, The Netherlands) *L. muscarium* at the following concentrations (1×10^6, 1×10^7, and 1×10^8 conidia/ml). Treatments without whey were considered controls. Treatments were replicated four times within a trial and the trails were repeated 5 times.

An initial suspension containing approximately 1×10^9 conidia/ml of the fungal isolate *L. muscarium* was prepared by mixing of 0.8 grams of Mycotal™ technical powder with 10 ml of sterile distilled water in a test tube containing small glass beads, which was vortexed for approximately 1 minute to disperse the suspension (Goettel & Inglis, 1997). The suspension was held at room temperature for one hour in a laminar flow hood and re-vortexed to form a homogenous suspension before conidia enumeration and further dilution. Direct counts of conidia were made microscopically using a hemacytometer (Hausser Scientific, Blue Bell, PA) to examine the initial suspension (Goettel & Inglis, 1997). Based on conidia counts the suspension was then further diluted to achieve the treatment stock suspensions 1×10^7, 1×10^8 and 1×10^9 conidia/ml.

Final treatment concentration of the conidia suspension was achieved by adding 0.50 ml of the stock suspensions to 4.5 ml whey suspensions. Sweet whey powder (Empire Cheese, Cuba, NY) was added to sterile distilled water to prepare suspensions that would yield final concentrations of 0, 5, 10, and 15% (w/v) whey after addition of the conidia suspensions. Treatment suspensions were sprayed approximately two hours after the Mycotal™ was initially added to water. This was done to allow the conidia to re-hydrate, as specified by the manufactures label.

Each treatment was sprayed onto four dry 60 x 15 mm Petri dish lids using an airbrush sprayer (Scorpion II, Silent Aire Technology, Houston, TX). Each lid was sprayed individually at a distance of approximately 10 cm and each lid was passed over twice with the spray mist to ensure complete coverage. The treatment volume was estimated to be 0.01 ml/lid. Immediately after a lid was treated it was placed on its base (see below). After all four lids/treatment were sprayed individual plates were sealed with laboratory film and positioned randomly in a sealed container. The plates were incubated in the dark at 23^{o}C +/- 1^{o}C for one week.

The base of each plate contained 1.5% water agar to provide 100% humidity during incubation (Beyer *et al.*, 2004). Plates were prepared at least one day prior to application and held in sealed bags. The plates and lids were dried in a laminar flow hood to remove condensation a half hour before spray application. All spray suspensions were produced using sterile techniques and sterile distilled water however, sweet whey naturally contains low levels of bacterial contamination (Sithole *et al.*, 2006).

Microfactory production was assessed on day 7 using direct counts of conidia removed from the plastic lids. One ml of 0.02% Tween 80 (Fischer Biotech, Fair Lawn, NJ) in water solution was pippetted onto each lid and the lid was scraped with a plastic spatula. The suspension was removed using a pipette and placed in a test tube containing 3-5 glass beads. An additional 1 ml of Tween 80 solution was then added to each lid. The lids were re-scraped and the remaining suspension removed. Each test tube was vortexed for 0.5-1 minute to break up the mycelium and dislodge conidia. The number of conidia was determined without dilution using a hemacytometer (Goettel & Inglis, 1997).

Hemlock Woolly Adelgid Assay

The production of microfactories on hemlock foliage and woolly adelgid mortality was evaluated in two replicated lab trials. A hemlock stand infested with HWA was selected as a collection site in Holyoke, Massachutes at Mount Tom State Reservation. In October 2006 and January 2007, branches approximately 15 to 30 cm long were cut, bagged, and transported in a cooler with ice to the lab. The branches were selected based on branch tips containing an HWA sisten population of greater then 10 individuals on the growth produced in 2006. The branches were stored in the refrigerator for ≤ 2 days before being used in assays.

In the October trial, for each run of the assay, 60 infested branch tips (3-6 cm, with ~ 20 HWA/tip) were cut and 20 were randomly selected to obtain an average adelgid mortality count prior to application of the formulations. Mortality was assessed stereo-microscopically by probing each nymph with an insect pin. Adelgid that lacked turgor and/or did not have hemolymph that was reddish in color and liquid, but had thick,

dark or clear hemolymph were considered dead. The same method was used post-treatment to determine mortality on the treated branch tips. The remaining 40 tips were randomly allocated into eight treatments so that five tips were sprayed for each treatment. The assay was repeated three times. The treatments were 2 controls (water and 10% whey suspension (w/v)) and combinations of 1 x 10^5, 1 x 10^6, and 1 x 10^7 conidia/ml in either water or 10% (w/v) whey formulation.

Suspensions were prepared just as in the microfactory production assay and applied using the airbrush sprayer. Both the upper and lower surfaces of each tip were treated and then placed individually, right side up in 100 x 15 mm Petri dishes (Fisher Brand, Canada) that were lined with filter paper (Whatman International Ltd., England) Sterile distilled water (300 µl) was pipetted onto the filter paper to create a humidified environment. The experimental plates were sealed with laboratory film and incubated at room temperature in a light chamber (16:8 L:D) for twelve days.

The second trial took place in January 2007 using the same treatments, but only four-branch tips/treatment were used and each assay was repeated four times. Twenty branch tips were again randomly selected for mortality pre-counts but the pre-counts were shared for assays one and two and for assays three and four because the assays were run concurrently in pairs -- branch tips for treatments and for mortality pre-counts were randomly selected at the same time for each pair of assays. In this trial, the tip of each branch was placed into wet floral foam and held in a 50 ml plastic centrifuge test tube (Corning Incorporated, Corning, NY) after treatment. The moist floral foam served to

humidify the bioassay environment. The tubes were held standing straight up in a light box at room temperature (16:8 L:D) for twelve days.

To examine microfactory production at day twelve the branch tips from both trails were removed and conidia counts were taken using the tape method (Gouli *et al.*, 2003). Clear plastic tape (4.78 cm Scotch Packaging Tape, 3M, St. Paul, MN) was placed onto 2.5 cm wide pieces of paper and cut into 1.9 cm wide pieces. The adhesive side of a piece was placed on the top and bottom of each branch tip and pressed with the blunt side of forceps so that an impression of the needles was made in the tape. Conidia present on the foliage should adhere to the tape. The paper was used as a label and the tape was then pulled off the tips. If needles remained on the tape, they were removed using forceps. The tape was then placed on a glass slide treated with a streak of lactophenol cotton blue stain (Bennex Limited, Shannon County, Clare, Ireland) and held at room temperature in the dark until counts were made. Counts were made stereo-microscopically at 400x and each slide was counted in five different areas. Conidia counts focused on the needle impression in the tape so that the optical field was made half on the needle and half off. Counts were handled in this manner due to the nature of fungal outgrowth from the needle. If a needle imprint could not be located, a count would be taken along the center of the slide moving from left to right.

Addition of Antimicrobials

Antimicrobials were added to the whey conidia suspensions to assess the inhibitory effects of different concentrations on *L. muscarium* conidia production in whey-based fungal microfactories. The following antimicrobials were evaluated and five

concentrations (Table 1) were selected based on their activity spectrum against bacteria and fungi: nisin M.W. 3354.3 (MP Biomedical Inc., Solon, OH), sorbic acid potassium salt M.W. 150.2 (MP Biomedical, Solon, OH), acetic acid (Thermo Sci Fisher Products Group, Waltman, MA), and Nipacide Bit 20 (Clariant Corporations, Charlotte, NC). The selected concentrations of antimicrobials were established by dilutions to the 10% whey (w/v) plus 1 x10^6 conidia/ml microfactory suspension. For each trial, seven treatments were assessed, which included two controls (water and 10% whey with the addition of 1 x 10^6 conidia/ml) and the five selected concentrations of an antimicrobial in microfactory suspension.

Each antimicrobial trail was conducted separately. The Petri dish method outlined for the microfactory production assay was used and each treatment suspension was applied using the airbrush sprayer. Each treatment was applied to four dishes and each antimicrobial trail was repeated four times. All experimental dishes were incubated for 7 days at 23^{o}C +/- 1^{o}C and the whey based fungal microfactory production was assessed by enumerating conidia as described earlier.

Statistical Analysis

All data were analysized with SAS GLM-ANOVA (SAS, 2005). The conidia counts to determine whey-based fungal microfactory production were averaged by treatment and the mean for all replicate trials were then analyzed (n = 5). The counts of recovered conidia were converted from conidia/ml to conidia/mm^2 to characterize conidia production based on the area of the growing surface. The results were then log 10 transformed based on the distribution of the residual plots. Data were back transformed

when calculating the relative increase in conidia production between treatments, e.g. a 10-fold increase in production. Individual contrasts were set up to compare conidia production between treatments. Both the concentration of whey and conidia were treated as class variables and an alpha = 0.05 was used in all analyses performed.

To quantify conidia production on hemlock branch tips the number of conidia recovered per branch top and bottom were summed and then averaged. The recovered conidia per treatment per trail were then averaged and the mean of the replicates for the trails were used for analysis of microfactory production (October assay n = 3 , January assay n = 4). The mean number of conidia per branch were log 10 transformed based on the distribution of the residual plots. Data were back transformed when calculating the relative increase in conidia production between treatments, e.g. 10-fold increase. Mortality data were also averaged by trail and the mean for replicates were used for analysis. The October HWA assay (n = 3) and January HWA assay (n = 4) were corrected to control for mortality pre-counts using Abbott's formula (Abbott, 1925) and then arcsine transformed using an adjustment based on the square root of actual proportions (Zar, 1974) as outlined by Anscombe (1948). Pre-planned comparisons for mortality assessment were made among least square means using P-diff (SAS, 2005) when treatment effects were significant.

Linear regression analysis was used to evaluate the effect of the antimicrobials on conidia production by treatment concentration as a continuous variable in the GLM-ANOVA analysis. The number of conidia recovered from each plate per trial was averaged and the mean of the replicates used in the analysis (n = 3). The mean conidia

recovered were converted from conidia/ml to conidia/mm^2 and then log 10 transformed based on examination of the residual plots. The data for each antimicrobial were examined for linear, quadratic, and cubic effects as appropriate. If high order effects, quadratic or cubic, were insignificant the analysis was re-run without them.

Results

Microfactory Production

Whey-based fungal microfactory production (Figure 1) was influenced by the concentration of whey (0, 5, 10, and 15%) and conidia (1 x 10^6, 1 x 10^7, and 1 x 10^8 conidia/ml) in the formulation suspension and their effects interacted significantly ($P = 0.002$, $F = 4.26$, $df = 6,48$). Dramatic increases in conidia production occurred in formulations containing whey and 1 x 10^6 or 1 x 10^7 conidia/ml, but the influence of whey was less pronounced at 1 x 10^8 conidia/ml. A 24-fold increase in number of conidia was produced at the lowest whey and conidia concentration and a 76-fold increase was produced with 15% whey at the lowest conidia concentration compared to the corresponding no whey treatment. At the highest conidia concentration, only a 4-fold increase occurred with 5% whey and a mere 6-fold increase occurred with 15% whey.

Individual contrasts (Tables 2 and 3) were used to compare conidia production among the whey and conidia treatments. When evaluating the differences in production between 0% whey and the 5, 10, and 15% whey formulations all increases were significant ($P \leq 0.05$) at all conidia concentrations. Although the increase in conidia density was not significant between 5 and 10% whey for 1 x 10^6 and 1x 10^7 conidia/ml,

or between 10 and 15% whey, the difference between 5 and 15% whey was significant and substantiates the observed trend in the effect of whey. When conidia production is compared among fungal treatments the results are similar at different levels of whey in that conidia density did not significantly increase between 1 x 10^6 and 1 x 10^7 conidia/ml treatments, but decreases in most cases when either compared to the 1 x 10^8 conidia/ml treatment. Also note that when no whey was included the residue in the 1 x 10^8 conidia/ml treatment was significantly greater then the 1 x 10^6 conidia/ml treatment, as would be expected.

Microfactory Production and Adelgid Mortality on Hemlock Foliage

Microfactory production on foliage was also significantly influenced by the concentration of whey and conidia in the formulation (Figures 2 and 3), but these factors interacted significantly (October: $P = 0.009$, $F = 5.65$, $df = 3,15$; January: $P = 0.001$, $F = 7.61$, $df = 3,22$). The addition of whey promoted conidia production compared to the corresponding 0% whey treatments except when conidia were at the highest treatment concentration (1 x 10^7 conidia/ml).

In the October trial (Figure 2), the addition of whey (10%) produced 81-, 234-, and 2-fold increases in conidia density at 1 x 10^5, 1 x 10^6, and 1 x 10^7 conidia/ml, respectively. In the January trial (Figure 3), the response was similar but the lowest concentration (1 x 10^5 conidia/ml) produced a 530-fold increase in conidia, 1 x 10^6 conidia/ml produced a 42-fold increase, and 1 x 10^7 conidia/ml had only a 5-fold increase.

In the October trial (Figure 4), hemlock woolly adelgid mortality was significantly influenced by the addition of whey ($P = 0.003$, $F = 12.5$, $df = 1,16$) and conidia ($P = 0.04$, $F = 3.57$, $df = 3,16$) concentration, but there was no interaction. The combination of conidia and whey appeared to increase mortality as the number of conidia inoculum increased. The effect of conidia on mortality increased between the control (0 conidia/ml) and both the 1×10^6 and 1×10^7 inoculum levels. The low and high conidia treatments also differed significantly. In the January trial (Figure 5), only the addition of whey ($P = 0.0001$, $F = 33.92$, $df = 1,24$) had a significant effect on mortality. However, mortality tended to increase with increased treatment conidia. The mortality in the 0% whey control for both the October and January trials was approximately 50%, which is not unusual for overwintering HWA populations (personal observation). The 0% controls plus fungi had HWA mortality ranging from 60-75% with increased mortality corresponding to increased conidia concentrations. The whey alone treatment had a mortality response with approximately 80% mortality in October and 89% for January. The high mortality associated with whey alone may have precluded ascertaining significant mortality effects of *L. muscarium* conidia as total mortality approached 100%. Adelgid treated in October had not yet developed their woolly coat where as those collected in January where covered with wool.

Antimicrobial Evaluation

Lecanicillium muscarium differed in its sensitivity to the antimicrobials tested. For nisin (Figure 6) and sorbic acid (Figure 7), there was no significant fit of the data to the linear model indicating a lack of effect on conidia production ($P = 0.27$, $F = 1.3$,

df = 1,22, r^2 = 0.06 and P = 0.07, F = 3.53, df = 1,22, r^2 = 0.14, respectively). Acetic acid (Figure 8) caused a reduction in conidia production characterized by quadratic effects of increasing concentration (P = 0.0003, F = 18.46, df = 1,21, r^2 = 0.96). Nipacide Bit 20 (Figure 9) caused a dramatic reduction in conidia production that was characterized by cubic effects (P = 0.003, F = 11.76, df = 1,20, r^2 = 0.82).

Discussion

The possibility of foliage covered with tiny microfactories manufacturing fungal conidia for biological pest control has considerable appeal. Spray deposits of fungal formulations containing sweet whey produced increased numbers of conidia, both on sterile Petri dish lids and field collected hemlock foliage. Increasing the concentration of whey increased the number of conidia recovered, with the maximum change being 76-fold for lids and 534-fold for foliage (Figures 1 & 3). These levels of conidia production could substantially increase the field titer of a biological control fungi applied for pest management or conversely allow significant reductions in the number of conidia applied, with concurrent reduction in management costs.

Humidity, temperature, and nutrition are key components to germination, growth, and sporulation of fungi (Hall, 1981). Humidity and temperature were experimentally optimized in the current study to highlight the effects of nutrient availability on conidia production. Carbon is a primary component of nutrition for colony expansion and sporulation (Sun & Liu, 2006). Sweet whey is made up of approximately 72.18% lactose, a disaccharide that is a preferred sugar source for conidia production of *L.*

muscarium. Sweet whey has a C:N ratio of 5:1, which is similar to the optimum level of ≤ 10:1 (C:N) found for conidia production of Hyphomycete fungi (Mo *et al.*, 2005). Prior to the selection of sweet whey preliminary trials using whey protein concentrate, a high protein (95%) nutrient source, did not generate conidia production (Grassano and Costa unpublished data). Likely, both the availability of nutrients in sweet whey and a favorable C:N ratio promoted conidia production when sweet whey was used.

The production response of microfactories to increasing concentration of conidia in the test formulation provides useful information concerning possible dynamics of fungal nutrition. Using higher concentrations of conidia in the initial formulation did not increase production. On both lids and foliage test surfaces, the two lowest concentrations of conidia assayed resulted in similar levels of conidia recovery within each system (Figures 1, 2, & 3). Further increase of initial conidia concentration led to a reduction in the number of conidia recovered when compared with the lower inoculum levels. On hemlock foliage, reduced production of conidia occurred at a 10-fold lower concentration of treatment conidia than occurred with production on plastic lids.

The reduced production of conidia and germination inhibition with high fungal concentrations has been noted in several fungi (Lax *et al.*, 1985; Lingappa & Lingappa, 1967; Zhang *et al.*, 2003). When inoculum levels are high nutrient and oxygen availability may become limiting factors to conidia germination (Allen, 1976; Pascual *et al.*, 1997). The reduced microfactory production we observed at high inoculum levels suggests the production system had become saturated due to either self-inhibition, competition for nutrients or moisture, or possibly the depletion of oxygen. Although,

oxygen depletion at atmospheric levels on hemlock branches in a forest should not prohibit germination (Cochrane, 1958), in our trial using a sealed bioassay environment, oxygen availability could have been limiting. In the hemlock foliage assays, competition for nutrients between *L. muscarium* conidia and resident epiphytes or additional oxygen stresses due to foliar respiration in the sealed container could have been involved in lowering the concentration at which inhibition occurred.

In evaluating microfactory production, the relative increase in conidia number may be economically important if the initial level of conidia inoculum can be reduced while maintaining equivalent or higher post-production conidia deposits. Interestingly, the two lowest levels of conidia inoculum provided similarly high levels of conidia recovery in the presence of whey, even though there was a 10-fold difference in inoculum level. On plastic lids, the addition of 10% whey to the lowest level of conidia inoculum gave a post-production conidia deposit that was 23-fold higher than if 10 times more conidia had been applied without the added whey (Figure 1). On foliage, the increase was 153-fold with 10% whey at the lowest level of conidia inoculum compared to the next highest inoculum level without added whey (Figure 2). Thus, the level of inoculum in a whey-based microfactory formulation might be greatly reduced providing a significant counterbalance to the high costs of mass-producing biological control fungi, which is currently limiting their wider adoption for pest management (Kunimi, 2005). However, factors such as degradation of conidia viability by UV radiation (Butt *et al.*, 2000; Cochrane, 1958) in the natural environment need to be considered and researched

when selecting inoculum concentrations optimally effective for microfactory production and pest management in the field.

For *L. muscarium* to reproduce successfully in microfactories, conidia need to be able to freely utilize the nutrients provided by sweet whey. Competition for these nutrients could occur in the spray tank after the formulation is mixed with water, especially if a waiting period for conidia hydration is used. Various epiphytes colonizing hemlock foliage (personal observation) could also compete for the nutrients after the spray is applied. Competitors may also include endemic populations of entomopathogens. For instance, the reported presence of *L. muscarium* (*V. lecanii*) isolated from HWA in the area of our collection sites (Reid, 2003) may explain why low numbers of conidia were recovered from foliage only sprayed with whey, e.g., no conidia controls. Another concern is that adding nutrients to foliage might release the growth of undesirable organisms such as phytopathogens. Although fungus can grow in the presence of bacteria, fungal biomass will be higher when it is not competing with bacteria for nutrients (Mille-Lindblom *et al*., 2006). The antimicrobials evaluated in this trail differed in their reported spectrum of biological activity and inhibitory effects on *L. muscarium* conidia production.

Acetic acid and Nipacide Bit 20 are not suitable candidates for inclusion in whey formulations of *L. muscarium*. These antimicrobials are broad spectrum and effective against both bacteria and fungi (Arora, 2004). Growth of the fungus *Colletotrichum gloeosporioides* was decreased by acetic acid at 1.8 mg/ml and completely inhibited at 3 mg/ml (Han-Chul *et al.*, 2003). Although a low range of concentrations was selected in

our research to target bacteria and not fungi, both still had a negative effect on *L. muscarium* conidia production (Figures 8 & 9). Possibly, the drying of spray deposits after application concentrated the antimicrobial in the formulation and enhanced their activity.

Nisin had no effect on the production of *L. muscarium* conidia (Figure 6). It is a fermentation product that is approved by the United States Food and Drug Administration for various food uses and is generally recognized as safe (GRAS listed). As a bactericide it is used to suppress gram-positive and some gram-negative bacteria (Dielbandhoesing *et al.*, 1998) and concentrations specifically inhibitory to bacteria were selected for testing. The minimum inhibitor concentration (MIC_{50}) of nisin for the lactic acid bacteria *Oenococcus oeni* is 2.4×10^{-5}, the MIC_{50} for acetic acid bacteria is 0.2 mg/ml, and within the ranges tested nisin had no effect on yeasts (Rojo-Bezares *et al*., 2007). The maximum concentration tested against *L. muscarium* in our trial was 0.1mg/ml and no deleterious effects were observed. The lack of effect on *L. muscarium* and favorable toxicity profile for human consumption give nisin good potential as a formulation adjuvant.

Sorbic acid potassium salt is a fungicide that is active against yeasts and molds but has limited activity against bacteria (Inglis & Cohen, 2004). In the current trial, it had no significant effect on the production of conidia. However, Sun and Liu (2006) reported that using 8mg/ml of sorbic acid as a carbon source, which was included in the range tested herein, inhibits *L. muscarium* germination, hyphal expansion, and sporulation. Inhibition in the fungal microfactory may have been overcome due to the carbon available in the sweet whey formulation. The ability of sorbic acid to inhibit

growth of some fungi also increases as the pH decreases (Fujita & Kubo, 2005) which may have been a factor in the current trial. Sorbic acid levels of 0.1123 mg/ml and 0.336 mg/ml delay the germination and growth of *Aspergillius niger* at different rates; however, *A. niger* has the ability to breakdown sorbic acid (Plumridge *et al*., 2004). Nisin and sorbic acid are food additives and sorbic acid is listed as a formulation additive by the United States Environmental Protection Agency (Arora, 2004).

A formulation augmented with a combination of nisin and sorbic acid might provide broad protection of the whey resource for utilization by *L. muscarium* -- their suitability for other biological control fungi would require individual evaluations. Additional research is needed to determine if adoption of antimicrobial adjuvants is actually necessary. Personal observation suggests that at least on hemlock foliage *L. muscarium* can compete for the whey resource. In various trials, we observed growth of a gray fungus on field collected foliage sprayed with whey alone, i.e., no *L. muscarium* added. However, in the same trials when *L. muscarium* was included in the formulation, no such growth occurred. If formulated biological control fungi can out compete resident epiphytes this may reduce concern over whey releasing endemic phytopathogens. Only further investigation in a variety of cropping systems will establish the necessity of an antimicrobial adjuvant.

With microfactory production it is essential for formulation conidia and secondary conidia to reach the adelgid located on the underside of the branches. Research using *Verticillium lecanii* has found that conidia can move through dense foliage and produce mortality in an insect target located on the leaf underside (Hall & Burges, 1979). Ultra

low volume applications in a coniferous forest has also achieved coverage within the canopy (Richard Reardon, personal communication). Hall and Burges (1979) found that *V. lecanii* conidia caused infection in aphids exposed to cadavers having secondary conidia production from an initial spray. Our assessment of conidia production on hemlock foliage at day 12 demonstrated that, in a laboratory setting, secondary conidia are abundant on foliage.

Evaluation of HWA survival in the October trial found that mortality increased with conidia concentration with or without the addition of sweet whey (Figure 4). In the January trial, mortality tended to increase with conidia in water and sweet whey formulations' however, the independent interaction between whey and conidia was not significant (Figure 5). The high mortality caused by whey alone may have masked the effect of the increased inoculum levels and account for why there was a significant response to conidia concentration in the October trial when the woolly coat was not present. In addition, the woolly covering was present on the adelgid during the January trial and this may have hindered the infection process. Mortality in the water treated control was also relatively high, however, substantial mortality is not unusual in the sisten generation of HWA (personal observation). Whey alone also caused mortality, which is interesting in itself. Additionally, fungal growth identified as *L. muscarium* was observed on foliage treated with whey alone suggesting that the whey caused endemic fungus to reproduce. Regardless, microfactory treatments resulted in approximately 89-99% mortality depending on the initial conidia concentration (Figures 2 and 3). Evaluation of the mortality results for synergism (Benz, 1971) suggested that the

observed responses were more than additive and points to the possibility that microfactory production of new conidia played a role in increasing mortality.

Hemlock woolly adelgid populations are not noticeably impacted by natural enemies or host plant resistance of hemlock trees (McClure *et al.*, 2001). The adoption of *L. muscarium* in combination with whey-based fungal microfactory technology may serve as a viable management approach for protection of hemlock forest resources from this invasive pest. However, our trails optimized the humidity for growth and sporulation of *L. muscarium.* This microclimate may not be regularly present within forest hemlock stands and further research is required to identify additives that may enhance moisture availability within the microfactory residue. Pilot studies targeting HWA in forested stands using aerial applications of microfactory formulations is also a next step in the evaluation process.

Entomopathogenic and other biological control fungi have been widely evaluated in their use as biological control agents, but adoption has been lacking because of poor efficacy and economic constraints (Butt *et al.*, 2000; Fuxa, 1995). Sweet whey provides a nutritive base that allows *L. muscarium* to germinate, grow, and sporulate without reaching an insect host, thus substantially increasing the effective inoculum level based solely on formulation constituents. This microfactory formulation technology (Costa *et al.*, 2006) might be applied to other fungal agents. The evaluation of microfactory technology with the myriad of biological control fungi being investigated to suppress various types of pests offers rich opportunities for future investigation.

Table 1. Antimicrobial concentrations selected for addition to 10% whey plus 1 x 10^6 conidia/ml formulations for evaluation of inhibitory effects to *L. muscarium* conidia production.

antimicrobial	concentration (mg/ml)				
Nisin	0.0014	0.0041	0.0123	0.037	0.1
Sorbic acid	0.21	0.617	1.85	5.5	16.66
Acetic Acid	3.125	6.25	12.25	25.0	50.0
Nipacide Bit 20	0.0187	0.375	0.75	1.5	3.0

Table 2. Contrasts analyzing the significant interaction ($P \leq 0.05$) between the 5, 10, and 15% whey concentrations and the conidia/ml treatment concentrations for effects on *L. muscarium* conidia production.

	conidia/ml		
% whey	1 x 10^6	1 x 10^7	1 x 10^8
0 vs. 5	< 0.0001	< 0.0001	0.0013
0 vs. 10	< 0.0001	< 0.0001	0.0003
0 vs. 15	< 0.0001	< 0.0001	< 0.0001
5 vs. 10	0.2052	0.1629	0.0295
5 vs. 15	0.0072	0.0269	0.2441
10 vs. 15	0.1349	0.3907	0.4912

Table 3. Contrasts comparing the significant interaction ($P \leq 0.05$) between treatment conidia/ml and the % whey in the treatment formulation for effects on *L. muscarium* conidia production.

	% whey			
conidia/ml	0	5	10	15
1 x 10^6 vs. 1 x 10^7	0.1498	0.6549	0.5629	0.9427
1 x 10^6 vs. 1 x 10^8	0.0121	0.0564	0.0083	0.0008
1 x 10^7 vs. 1 x 10^8	0.2575	0.0201	0.0016	0.0010

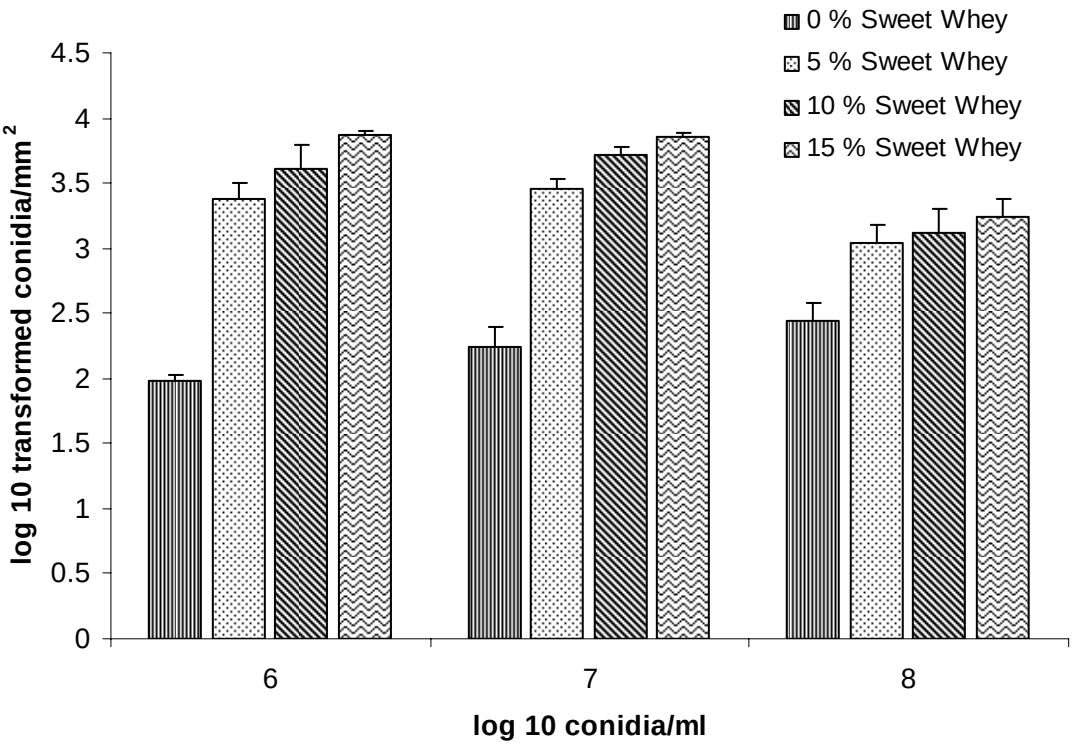

Figure 1. Whey-based fungal microfactory production (conidia/mm^2) recovered from Petri dish lids incubated for 7 days at 23 +/- 1^{o}C. As the whey concentration increases there is a significant ($P \leq 0.05$) increase in *L. muscarium* conidia production at 1 x 10^6 and 1 x 10^7 conidia/ml. There was inhibition of microfactory production at the 1 x 10^8 conidia/ml treatment concentration and the increase in *L. muscarium* conidia number was not considered significant compared to the two lower conidia inoculum levels

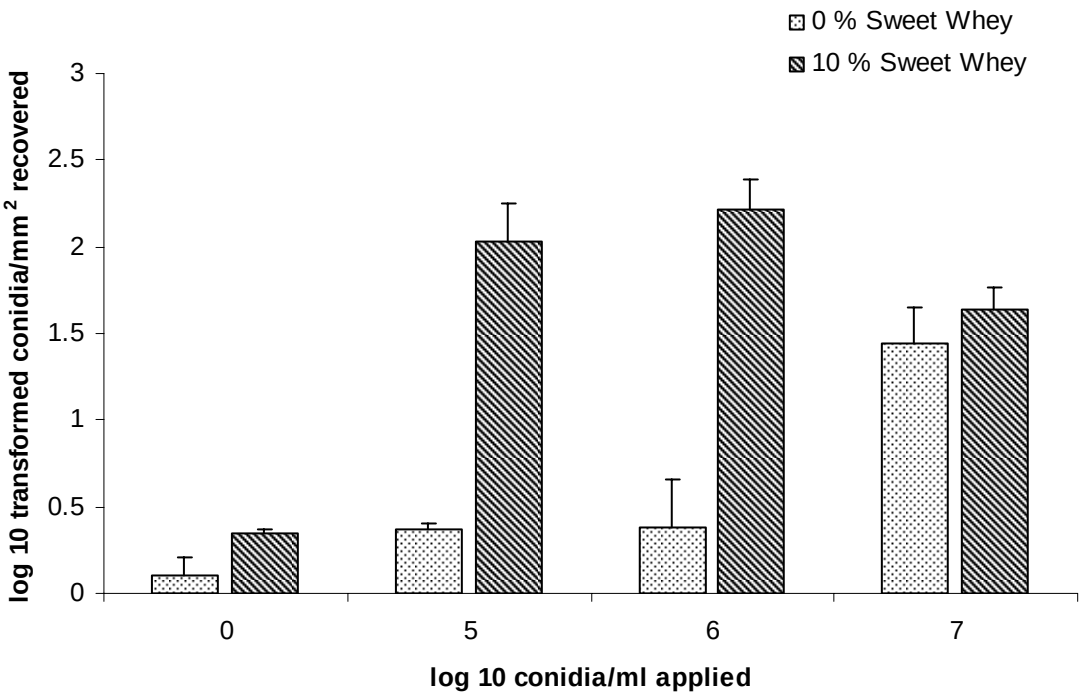

Figure 2. Microfactory production (conidia/mm^2) on October collected hemlock branches that were treated in the laboratory with various concentrations of *L. muscarium* in formulations with and without 10% whey. Whey and conidia concentrations interacted significantly except for formulations containing 1 x 10^7 conidia/ml where inhibition of conidia recovered occurred.

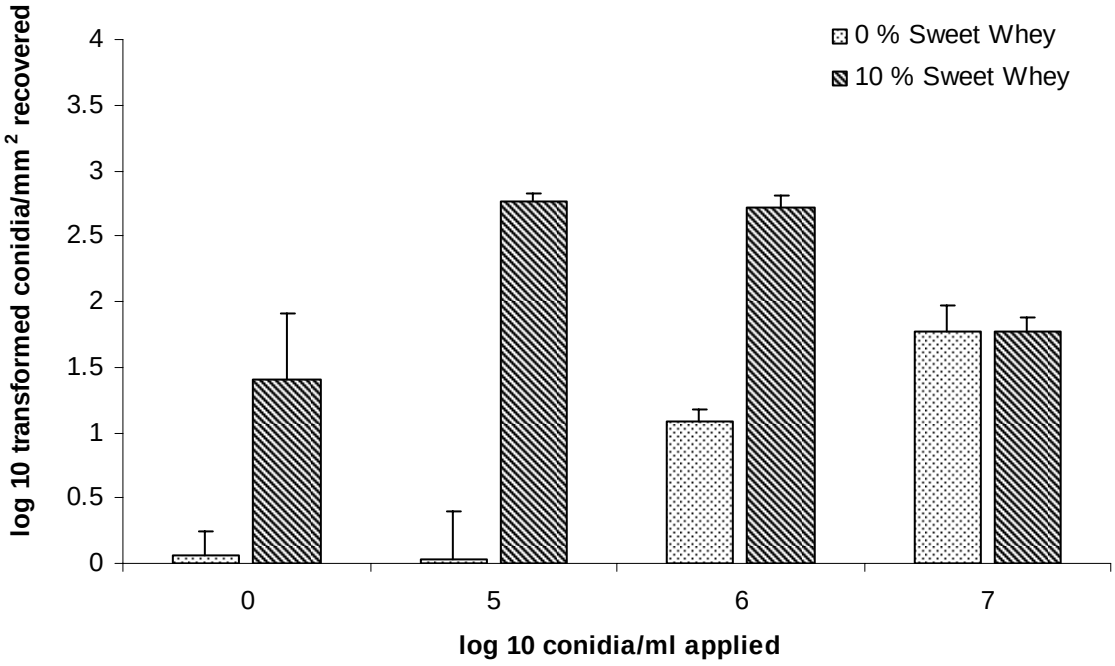

Figure 3. Microfactory production (conidia/mm^2) on January collected hemlock branches that were treated in the laboratory with various concentrations of *L. muscarium* in formulations with and without 10% whey. Whey and conidia concentrations interacted significantly except for formulations containing1 x 10^7 conidia/ml where inhibition of conidia recovered occurred.

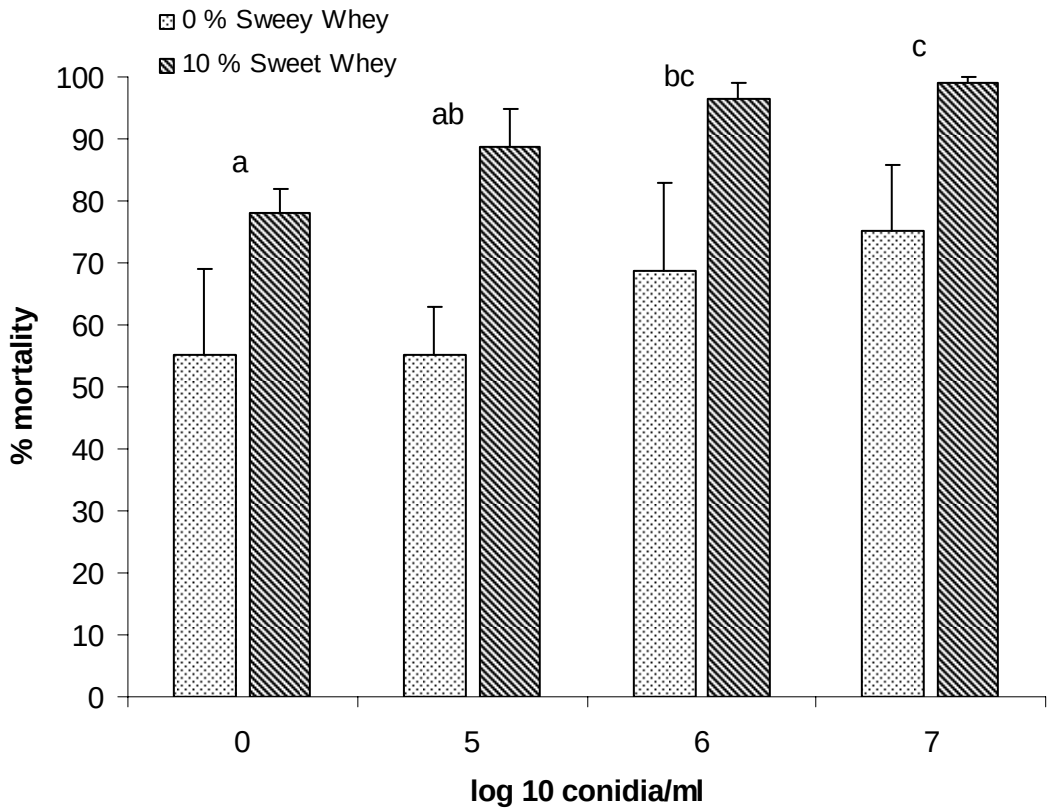

Figure 4. Hemlock woolly adelgid mortality assessed in the laboratory at day 12 post-treatment (October collected foliage). The branches were treated with various concentrations of *L. muscarium* conidia with and without 10% whey. Both whey and conidia concentrations have a significant ($P \leq 0.05$) effect on mortality but they did not interact significantly. Mortality was greatest at the highest concentration of conidia and when whey was added. Different letters indicate significant differences among conidia concentration.

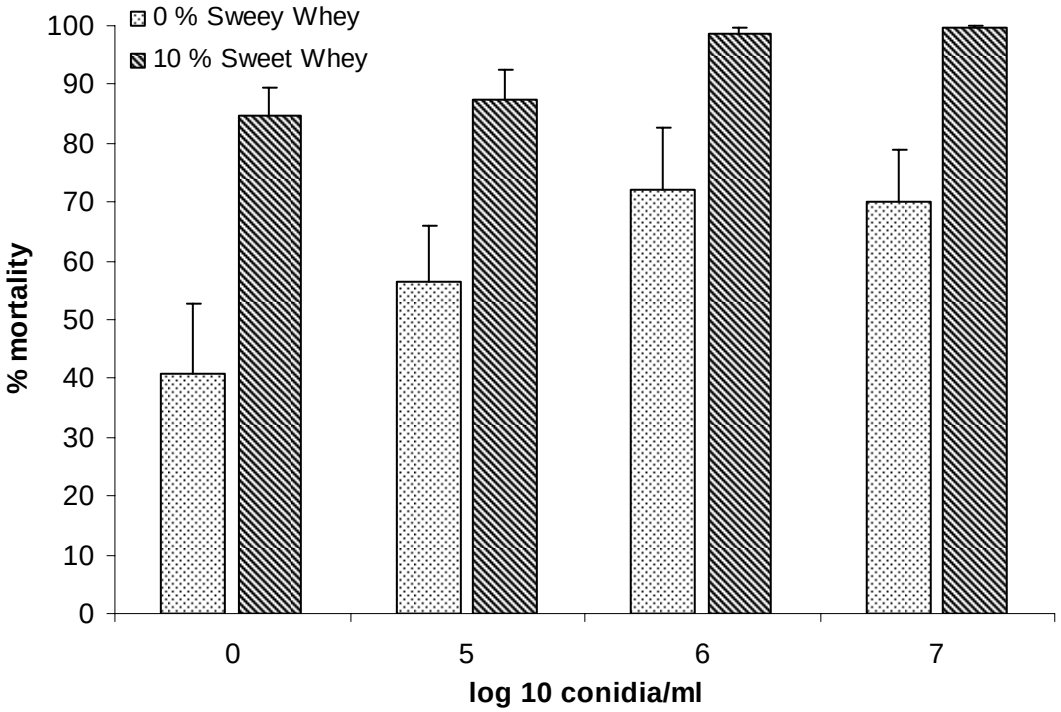

Figure 5. Hemlock woolly adelgid mortality assessed in the laboratory at day 12 post-treatment (January collected foliage). The branches were treated with various concentrations of *L. muscarium* conidia with and without 10% whey. Addition of whey had a significant effect on mortality ($P \leq 0.05$). Although, treatment mortality tended to increase as the conidia concentration increased the difference was not significant ($P > 0.05$).

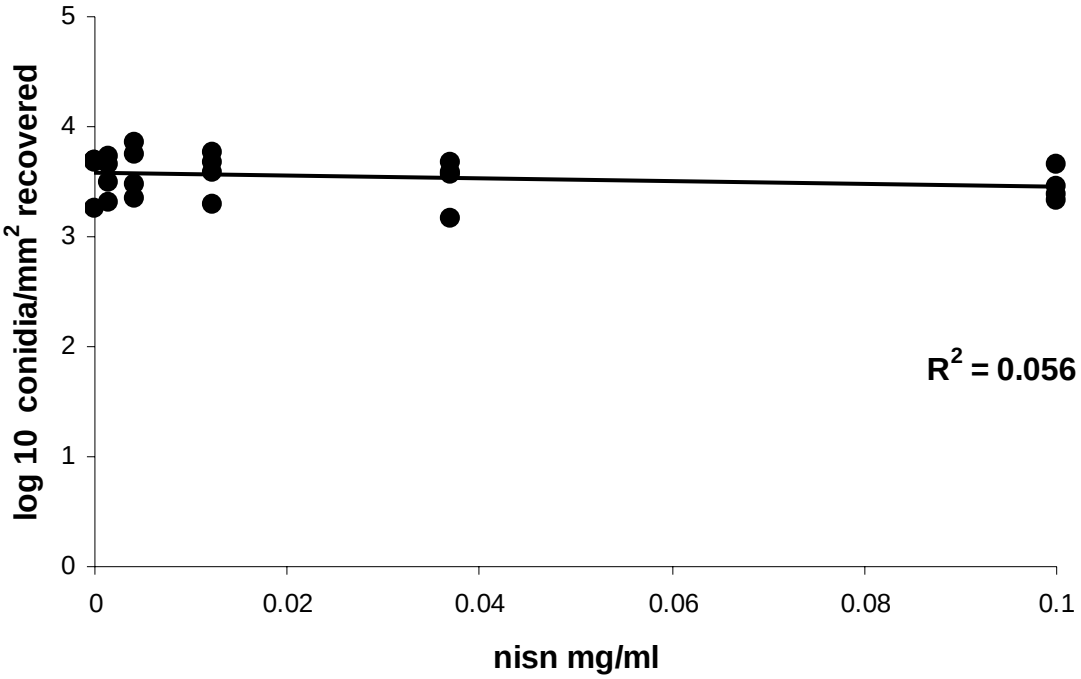

Figure 6. The effect of nisin on the production of *L. muscarium* conidia on Petri dish lids treated with 10% sweet whey plus 1 x10^6 conidia/ml and varying concentrations of nisin. There was no significant fit of the data to the linear model indicating a lack of effect ($P > 0.05$) on conidia production.

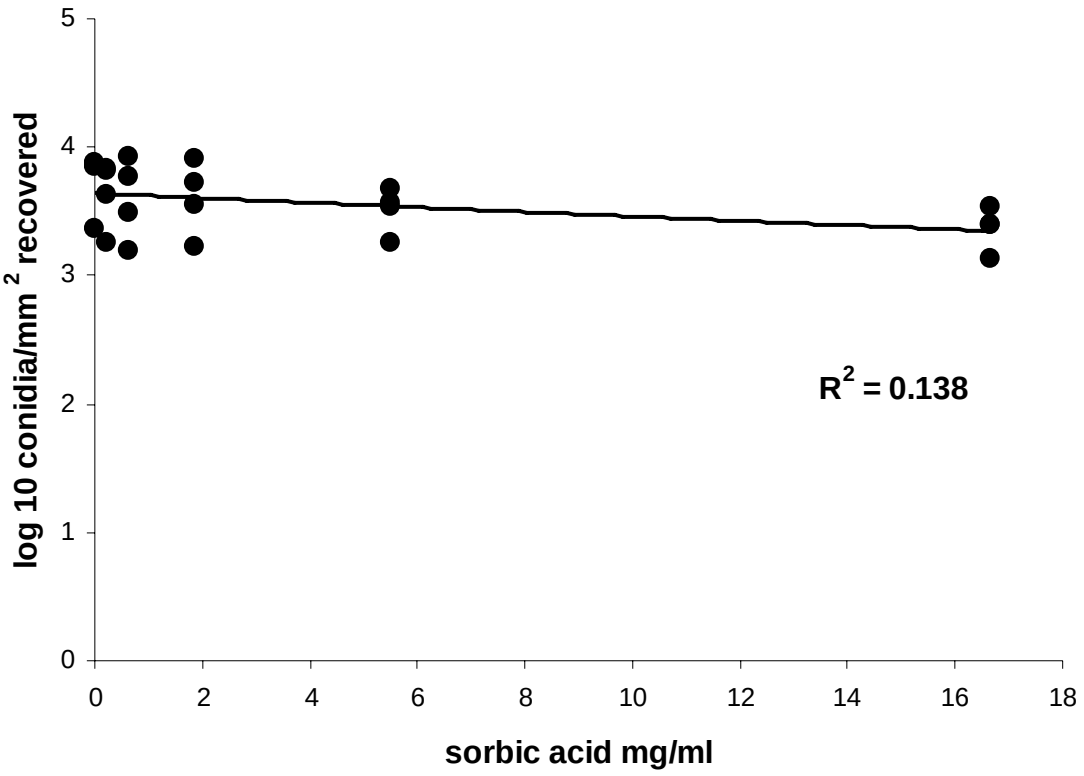

Figure 7. The effect of sorbic acid on the production of *L. muscarium* conidia on Petri dish lids treated with 10% sweet whey plus 1 x10^6 conidia/ml and varying concentrations of sorbic acid. There was no significant fit of the data to the linear model indicating a lack of effect ($P > 0.05$) on conidia production.

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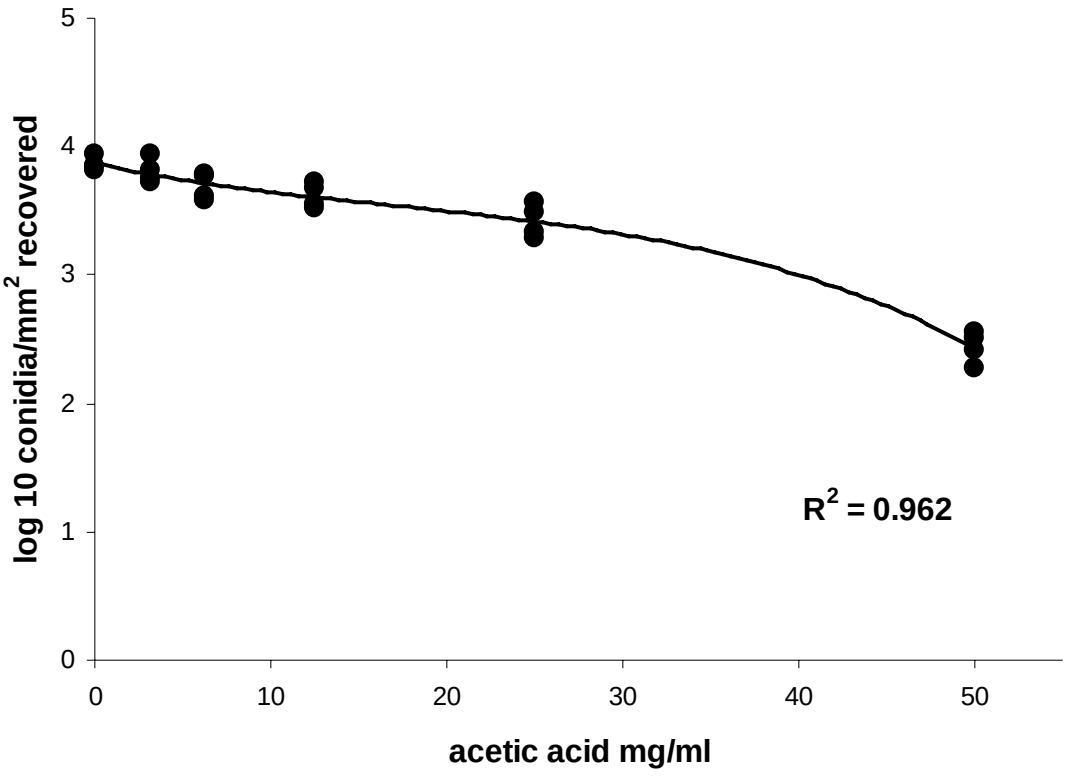

Figure 8. The effect of acetic acid on the production of *L. muscarium* conidia on Petri dish lids treated with 10% sweet whey plus 1 x10^6 conidia/ml and varying concentrations of acetic acid. Decreased production of conidia is characterized by the quadratic effect ($P \leq 0.05$) of increasing concentration.

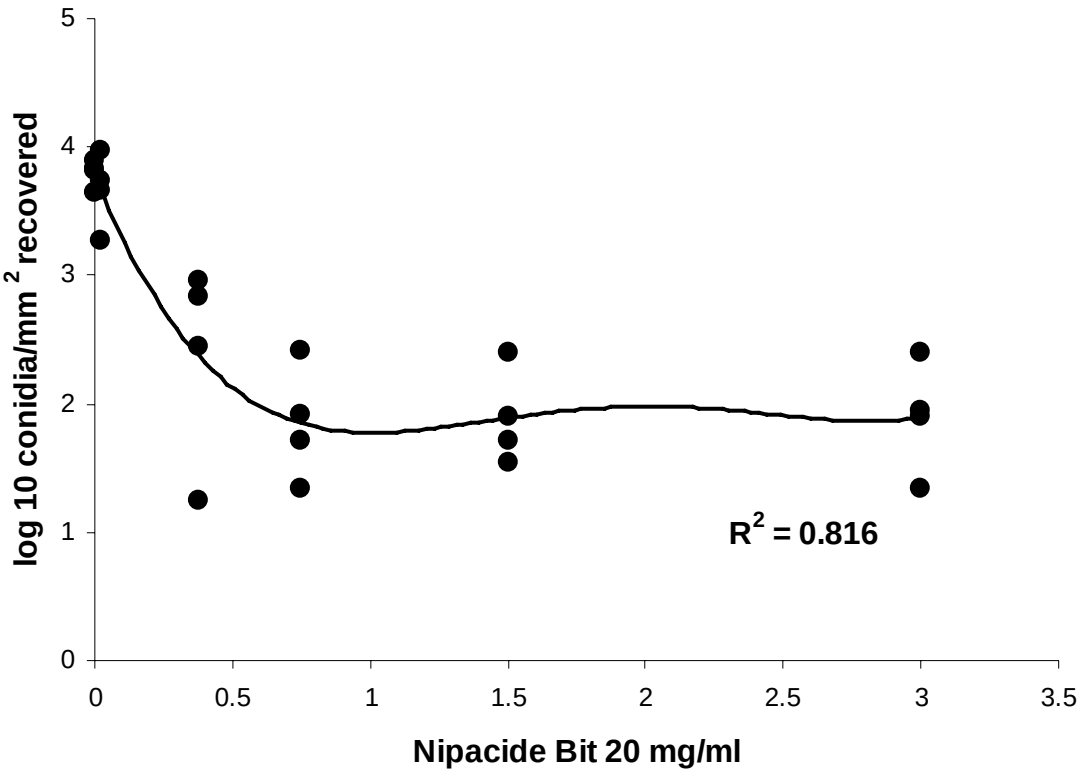

Figure 9. Effect of Nipacide Bit 20 on the production of *L. muscarium* conidia on Petri dish lids sprayed with 10% sweet whey plus 1 x10^6 conidia/ml and varying concentrations of Nipacide Bit 20. Cubic effects characterize the dramatic decrease in conidia production ($P \leq 0.05$).

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Appendix

Table 1. Analysis of variance summary for whey-based fungal microfactory production (conidia/mm^2) recovered from Petri dish lids. Lids were treated with varying concentrations of whey and *L. muscarium* conidia/ml.

Source of Variance	Degrees of Freedom	Type III SS	Mean Square	F test	Pr > F
whey	3	18.66	6.22	81.57	< 0.0001
conidia	2	1.32	0.66	8.65	0.0006
whey*conidia	6	1.95	0.32	4.26	0.0016
error	48	3.66	0.08		

Table 2. Analysis of variance summary for conidia recovered from October whey-based fungal microfactory production on hemlock branches. Branches were treated with varying concentrations of *L. muscarium* conidia in formulations with and without whey.

Source of Variance	Degrees of Freedom	Type III SS	Mean Square	F test	Pr > F
whey	1	9.82	9.81	37.99	< 0.0001
conidia	3	9.58	3.19	12.36	0.0002
whey*conidia	3	4.38	1.46	5.65	0.0085
error	15	3.88	0.25		

Table 3. Analysis of variance summary for conidia recovered from January whey-based fungal microfactory production on hemlock branches. Branches were treated with varying concentrations of *L. muscarium* conidia in formulations with and without whey.

Source of Variance	Degrees of Freedom	Type III SS	Mean Square	F test	Pr > F
whey	1	18.69	18.69	97.14	<0.0001
conidia	3	7.61	2.53	13.19	<0.0001
whey*conidia	3	4.39	1.46	7.61	0.0011
error	22	4.23	0.19		

Table 4. Analysis of variance summary for HWA mortality from October trails. Branches were treated with varying concentrations of *L. muscarium* conidia in formulations with and without whey.

Source of Variance	Degrees of Freedom	Type III SS	Mean Square	F test	Pr > F
whey	1	5.98	5.97	12.53	0.0027
conidia	3	5.10	1.70	3.57	0.04
whey*conidia	3	0.72	0.24	0.50	0.69
error	16	7.63	0.48		

Table 5. Analysis of variance summary for HWA mortality from January trails. Branches were treated with varying concentrations of *L. muscarium* conidia in formulations with and without whey.

Source of Variance	Degrees of Freedom	Type III SS	Mean Square	F test	Pr > F
whey	1	24.36	5.97	33.92	< 0.0001
conidia	3	3.24	1.70	1.87	0.1613
whey*conidia	3	0.161	0.24	0.22	0.8822
error	24	17.05	0.71		

Table 6. Analysis of variance summary for nisin examining the linear effects. Quadratic and cubic effects were insignificant and dropped from the final analysis. Petri dish lids were treated with varying concentrations of nisin in formulations containing 1 x 10^6 conidia/ml *L. muscarium* with and without whey.

Source of Variance	Degrees of Freedom	Type III SS	Mean Square	F test	Pr > F
concentration	1	0.05	0.05	1.30	0.2661
error	22	0.76	0.03		

Table 7. Analysis of variance summary for sorbic acid examining the linear effects. Quadratic and cubic effects were insignificant and dropped from the final analysis. Petri dish lids were treated with varying concentrations of sorbic acid in formulations containing 1 x 10^6 conidia/ml *L. muscarium* with and without whey.

Source of Variance	Degrees of Freedom	Type III SS	Mean Square	F test	Pr > F
concentration	1	0.20	0.20	3.53	0.0735
error	22	1.28	0.06		

Table 8. Analysis of variance summary for acetic acid examining for linear and quadratic effects. Cubic effects were insignificant and dropped from the final analysis. Petri dish lids were treated with varying concentrations of acetic acid in formulations containing 1 x 10^6 conidia/ml *L. muscarium* with and without whey.

Source of Variance	Degrees of Freedom	Type III SS	Mean Square	F test	Pr > F
concentration	1	0.03	0.03	2.99	< 0.0985
concentration*concentration	1	0.19	0.19	18.46	0.0003
error	21	0.22	0.01		

Table 9. Analysis of variance summary for Nipacide Bit 20 examining for cubic effects. Petri dish lids were treated with varying concentrations of Nipacide Bit 20 in formulations containing 1 x 10^6 conidia/ml *L. muscarium* with and without whey.

Source of Variance	Degrees of Freedom	Type III SS	Mean Square	F test	Pr > F
concentration (conc.)	1	6.86	6.86	36.76	< 0.0001
conc.*conc.	1	3.18	3.18	17.10	0.0005
conc.*conc.*conc.	1	2.19	2.19	11.76	0.0027
error	20	3.73	0.19		