Bacillus thuringiensis Subspecies Kurstaki Reduces Competition by Parapoynx diminutalis (Lepidoptera: Crambidae) in Colonies of the Hydrilla Biological Control Agent Cricotopus lebetis (Diptera: Chironomidae)

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Bacillus thuringiensis subspecies *kurstaki* reduces competition by *Parapoynx diminutalis* (Lepidoptera: Crambidae) in colonies of the hydrilla biological control agent *Cricotopus lebetis* (Diptera: Chironomidae)

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Abstract

The hydrilla tip mining midge, *Cricotopus lebetis* Sublette (Diptera: Chironomidae), is a biological control agent of the invasive aquatic weed hydrilla, *Hydrilla verticillata* (L.f.) Royle (Hydrocharitaceae). Mass rearing of the midge for augmentative releases can be compromised by competition from the adventive hydrilla leafcutter moth, *Parapoynx diminutalis* Snellen (Lepidoptera: Crambidae). The objective of this study was to evaluate the biorational pesticide *Bacillus thuringiensis* (subspecies *kurstaki*) (Btk) for reducing competition by the moth in laboratory colonies of the hydrilla tip mining midge. Dose response bioassays with Btk were completed with both species to determine the minimum concentration that kills the moth without affecting midge production. Adult moth emergence was significantly reduced at concentrations of 2.0 mL Btk per 3.8 L of well water. A concentration of 0.2 mL Btk per 3.8 L of well water also reduced the number of emerging moths by 50%. However, midge development declined, although not significantly at the higher 2.0 mL Btk concentration compared with the control. Therefore, concentrations of 0.2 or 2.0 mL Btk per 3.8 L of well water would be appropriate for controlling competition by *P. diminutalis* in *C. lebetis* colonies. The lower concentration (0.2 mL Btk) would be appropriate for maintenance control due to its minimal impact on the midge. However, a severe infestation of *P. diminutalis* might justify the higher concentration of 2.0 mL Btk for maximum impact on the moth. Implementing selective pest management strategies in a biological control agent colony can help increase production and improve the quality of the insects.

Key Words: herbivorous midge; *Hydrilla verticillata*; mass rearing; quality control

Resumen

El jején minador de la punta de hidrilla, *Cricotopus lebetis* Sublette (Diptera: Chironomidae), es un agente de control biológico de la maleza hidrilla acuática invasora, *Hydrilla verticillata* (L.f.) Royle (Hydrocharitaceae). La cría en masa del jején para la liberación aumentativa puede estar comprometida por la competencia con la polilla cortadora de hoja de hidrilla adventiva, *Parapoynx diminutalis* Snellen (Lepidoptera: Crambidae). El objetivo de este estudio fue evaluar el pesticida biorracional *Bacillus thuringiensis* (subespecie *kurstaki*) (Btk) para reducir la competencia por la polilla en las colonias de laboratorio del jején minador de la punta de hidrilla. Se hicieron bioensayos de la respuesta a la dosis de Btk con las dos especies para determinar la concentración mínima que mata a la polilla sin afectar la producción del jején. La emergencia de los adultos de la polilla se redujo significativamente a concentraciones de 2,0 ml Btk por 3,8 L de agua de pozo. Una concentración de 0,2 ml Btk por 3,8 L de agua de pozo también redujo el número de polillas emergentes en un 50%. Sin embargo, el desarrollo del jején se redujo, aunque no de forma significativa en la mayor concentración de 2,0 ml de Btk en comparación con el control. Por lo tanto, las concentraciones de 0,2 o 2,0 ml Btk por 3,8 L de agua así serían apropiadas para el manejo de la competencia por *P. diminutalis* en colonias de *C. lebetis.* La concentración más baja (0,2 ml Btk) sería apropiada para el mantenimiento del control debido a su impacto mínimo sobre el jején. Sin embargo, una infestación severa de *P. diminutalis* podría justificar la mayor concentración de 2,0 ml Btk para el máximo impacto sobre la polilla. La aplicación de estrategias de manejo de plagas selectivas en una colonia agente de control biológico puede ayudar a aumentar la producción y mejorar la calidad de los insectos.

Palabras Clave: jején herbívoros; *Hydrilla verticillata*; cría en masa; control de calidad

Hydrilla, *Hydrilla verticillata* (L.f.) Royle (Hydrocharitaceae), impacts the environment and alters ecosystems by competing with native plant species and causing changes in the fauna due to its presence as the dominant vegetation (Glomski & Netherland 2012). Hydrilla also causes severe economic problems in Florida through interference with water body use. For example, recreation, transport, drainage, and irrigation in lakes and canals can be impeded (Haller & Sutton 1975; Puri et al. 2007). Furthermore, management costs are high, especially in areas where herbicide resistance has been detected (Michel et al. 2004; Berger & MacDonald 2011; Giannotti 2013). Consequently, biological control should be considered as part of an integrated pest management strategy. Biological methods for hydrilla management that have been studied include sterile grass carp and insect species of the families Crambidae (moths), Curculionidae (weevils), and Ephydridae (shore flies) (Center et al. 2002; Cuda 2014). These species have either failed to establish in Florida ecosystems, such as the weevils (*Bagous* species), or are generalist plant herbivores nonspecific to hydrilla, such as the moth *Parapoynx diminutalis* Snellen (Lepidoptera: Crambidae) and grass carp, *Ctenopharyngodon idella* (Valenciennes) (Cypriniformes: Cyprinidae: Squaliobarbinae).

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Cricotopus lebetis Sublette (Diptera: Chironomidae) is a midge that has a semi-aquatic life cycle. It was discovered attacking hydrilla in Kings Bay, Crystal River, Citrus County, Florida, by United States Department of Agriculture researchers in 1992 (Cuda et al. 2002). The herbivorous larvae are free swimming and mine into the apical meristem of hydrilla, where they feed and develop (Cuda et al. 2002). Tunneling by the larvae kills the apical meristems and changes the architecture of hydrilla by preventing or delaying the formation of the surface mats (Cuda et al. 2011). The midge appears to be host specific; it has been collected only from hydrilla and occurs naturally in Florida ecosystems (Stratman et al. 2013a,b). The midge currently is being mass reared for augmentative biological control of hydrilla as part of an integrated approach for hydrilla management.

Field-collected hydrilla, which is used for midge rearing and experiments, often is contaminated with non-target organisms. Although efforts are made to remove these organisms by careful processing, some species are not amenable to physical removal. One such species is the hydrilla leafcutter moth, *P. diminutalis*, an adventive moth whose larvae feed on hydrilla (Buckingham & Bennett 1996). Larvae of *P. diminutalis* live within tubular cases of plant material as they feed, and they use the hydrilla leaf material to construct cocoons in which they pupate (Buckingham & Bennett 1996; Baniszewski et al. 2014). Because they are generalists and not host specific, larvae of *P. diminutalis* are considered poor hydrilla biological control agents (Buckingham & Bennett 1989). However, in a laboratory setting devoid of predators, the moth impedes midge development via resource competition. Because larvae of *P. diminutalis* and *C. lebetis* consume the same food source, and *C. lebetis* is sensitive to water quality, careful management of *P. diminutalis* is necessary to prevent the midge colony from collapsing. Consequently, a selective control method that targets only Lepidoptera without affecting midge survival or water quality would be beneficial.

Bacillus thuringiensis (subspecies *kurstaki*), or Btk, is a bacterium commonly used to selectively control lepidopteran pests in agriculture (Aronson et al. 1986). This biorational pesticide could potentially be used in mass rearing *C. lebetis* to maximize development of the midge when *P. diminutalis* is a contaminant of the hydrilla, provided there are no adverse effects of Btk on the midge. Btk produces proteins specifically toxic to lepidopteran larvae; the proteins are consumed, bind to the gut, and ultimately kill the immature insect (Bauce et al. 2006; Van Driesche et al. 2008). Btk tested on *P. diminutalis* larvae at 10% of the recommended spray application rate significantly reduced larvae within 4 d and eradicated all larvae within 10 d (Buckingham & Bennett 1996). The use of Btk to control lepidopteran pests has made the active ingredient commercially available in many easy-to-obtain products. Btk is naturally occurring in the environment, and although it has been shown to persist longer after binding to humic acid than as free-state toxins (Crecchio & Stotzky 1998), it persists mostly in soils and less in water or on foliage (Van Cuyk et al. 2011). Because it is specific to one group of insects, it does not have adverse effects on other insects, humans, or other mammals, and it is not an opportunistic pathogen (Auckland Regional Public Health Service 2003). Consequently, Btk may be a viable tool for suppressing the hydrilla midge colony pest *P. diminutalis* because of its selectivity.

The aim of this study was to determine the suitability of Btk in reducing competition from *P. diminutalis* in a *C. lebetis* laboratory colony. Appropriate concentrations of the label rate and dilutions were tested to maximize *P. diminutalis* mortality and minimize any adverse effects on *C. lebetis.*

Materials and Methods

The *B. thuringiensis* subspecies *kurstaki* (Btk) formulation used (Garden Safe® Brand Bt Worm & Caterpillar Killer; Schultz Company, Bridgeton, Missouri) was 98.35% Btk strain SA-12 solids, which according to the label includes spores and lepidopteran-active toxins (>6 million viable spores per mg).

Hydrilla was collected from the University of Florida Institute of Food and Agricultural Sciences Center for Aquatic and Invasive Plants (UF/IFAS CAIP, Gainesville, Florida; 29.72639°N, 82.41778°W) and used as a source for hydrilla tips and *P. diminutalis* adults. Hydrilla tips were harvested as described in Baniszewski et al. (2015). Larvae of *P. diminutalis* were obtained by placing males and females that emerged from the field-collected hydrilla in a mosquito breeder (BioQuip® Products, Rancho Dominguez, California) with the cone removed to allow the females access to hydrilla in well water in the base. These breeders were held in an environmental chamber at a 14:10 h L:D photoperiod and 25 °C. Breeders were monitored for larval activity; once larvae were 4th to 5th instars, they were used in experiments. Five 1.5 L solutions were prepared using the following rates: 20.0 (spray label rate), 10.0, 2.0, and 0.2 mL Btk per 3.8 L of well water and a control with no Btk (well water only). Btk solutions were transferred to separate 2 L plastic trays with 20 to 25 *P. diminutalis* larvae and 100 hydrilla tips, both collected from the UF/IFAS CAIP (Gainesville, Florida). Plastic trays were placed inside 30 cm^3 insect cages, aerated, and monitored daily for moth emergence. The trays were maintained in a greenhouse under ambient conditions (approximately 21 to 38 °C and a 14:10 h L:D photoperiod). The numbers of adult moths emerging from Btk-treated trays were recorded and compared between treatments. The experiment was repeated twice.

Cricotopus lebetis was reared according to procedures described by Cuda et al. (2002). After collecting eggs from the midge oviposition chamber, egg masses were evaluated for fecundity and fertility as described by Baniszewski et al. (2015). Btk solutions were prepared as described for the aforementioned moth experiment (20.0, 10.0, 2.0, 0.2 mL Btk per 3.8 L of well water and a control), but the assay was set up in 35 mL test tubes (n = 5) with a single tip of hydrilla and 1 midge larva in each tube. Solutions (20 mL) were added to the test tubes with hydrilla. Larvae were added using a glass pipette under a compound microscope within 6 h of hatching. These tubes were held in an environmental chamber and monitored for 22 d (14:10 h L:D photoperiod; 25 °C). Midge development was recorded as either pupae or final adult emergence because it is larval development and feeding that impedes hydrilla growth. Midge development was compared among treatments to determine the effects of Btk on the midge. The experiment was repeated 4 times.

Moth emergence as a proportion of initial larvae was tested for normality by Shapiro–Wilk W test, and the data were found to be normally distributed. Moth emergence was analyzed with ANOVA using JMP (Pro 9 software; SAS 2010). A Dunnett's test was used to show statistical differences between each Btk concentration and the control. Midge development, recorded as number of pupae and number of adults combined, was found to not be normally distributed by Shapiro–Wilk W test so that data were analyzed by the non-parametric Kruskal–Wallis test. Non-parametric means comparisons using the Wilcoxon method were completed to show statistical differences between each Btk concentration and the control. For all tests, the significance level was set at α = 0.05. All analyses were completed in JMP (Pro 9 software; SAS 2010).

Results

Moth emergence decreased significantly compared with the control for concentrations of ≥2.0 mL Btk per 3.8 L of well water (P = 0.0442; Fig. 1). Moth emergence from trays containing concentra-

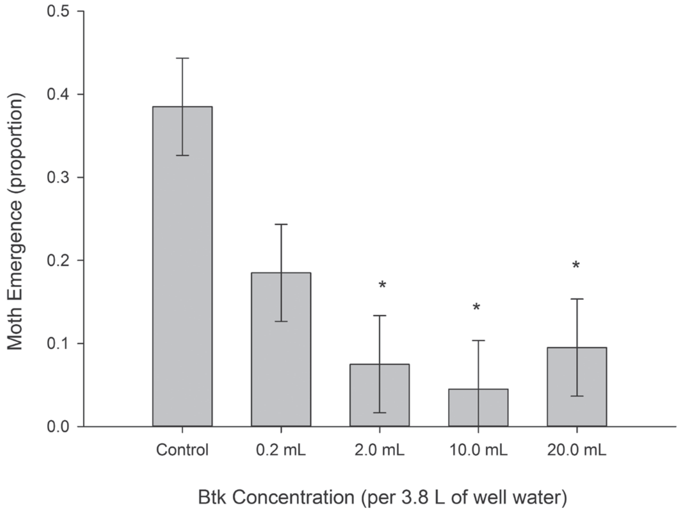

Fig. 1. Emergence of *Parapoynx diminutalis* adults from *Bacillus thuringiensis* (subspecies *kurstaki*; Btk) treated water containing *Hydrilla verticillata*. Moth emergence is shown as a proportion of initial larvae. Bars are means ± SE. Asterisks indicate significance between concentrations of Btk per 3.8 L of well water and the control using Dunnett's test.

tions of Btk at 2.0, 10.0, and 20.0 mL per 3.8 L of well water was less than 10%. The lowest concentration of Btk (i.e., 0.2 mL per 3.8 L of well water) reduced moth emergence to about 20% of the initial larval density ($P = 0.1601$), but did not significantly reduce moth emergence compared with the control, which had almost 40% moth emergence.

Midge development (recorded as number of pupae and adults combined) was found to be significantly affected by the concentration of Btk ($P = 0.0087$; Fig. 2). There was no difference in midge development between the control and the 0.2 mL Btk per 3.8 L well water ($P = 1.000$); these 2 treatments had about 80% larval survival. Additionally, a concentration of 2.0 mL Btk per 3.8 L well water had no significant effect on midge development ($P = 0.1016$). However, concentrations of Btk at 2.0 and 10.0 mL per 3.8 L of well water had an average midge development of less than 40% whereas no development occurred at 20.0 mL per 3.8 L of well water.

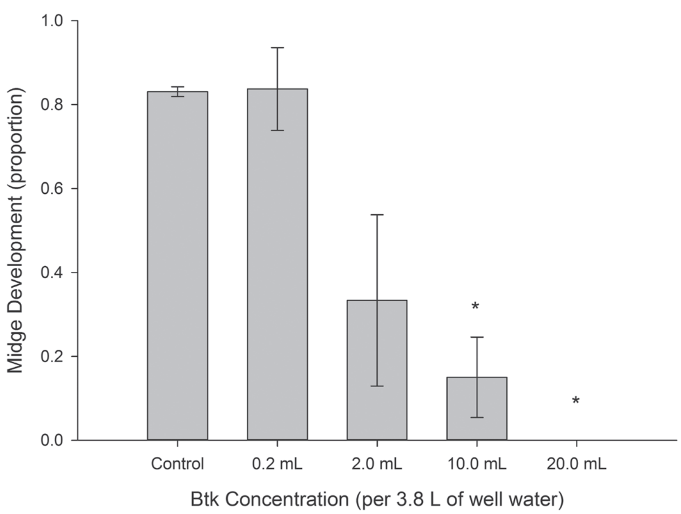

Fig. 2. *Cricotopus lebetis* development in *Bacillus thuringiensis* (subspecies *kurstaki*; Btk) treated water containing *Hydrilla verticillata*. Hydrilla tip mining midge development is recorded as the proportion of pupae and adults that successfully developed from the inoculated larvae. Bars are means ± SE. A Wilcoxon means comparison test indicated significance between the control and Btk treatments as indicated by asterisks.

Discussion

As expected, *B. thuringiensis* subspecies *kurstaki* (Btk) reduced emergence of *P. diminutalis* in the cages treated with this biorational insecticide. Concentrations of at least 2.0 mL Btk per 3.8 L significantly reduced moth emergence in a colony setting to less than 10%. This is a significant reduction of moth larvae and could improve colony rearing of *C. lebetis* by reducing resource competition between the two insect species. Similarly, Buckingham & Bennett (1996) showed significant mortality of up to 80% for *P. diminutalis* larvae in 4 d when 10% of the label rate of Btk was used (label rate not specified). The surviving larvae died after 10 d.

Previous studies have shown that midges were not severely impacted by low concentrations of Btk applied to soils (Crecchio & Stotzky 1998) or water systems (Menon & de Mestral 1984), and that other non-target organisms were not impacted (Auckland Regional Public Health Service 2003). In the current study, the 2.0 mL Btk per 3.8 L concentration not only reduced moth emergence but also reduced the number of midges completing development by approximately 50% albeit not significantly. Btk is a strain that contains 2 toxins, P1 or δ-endotoxin and P2. The P1 toxin is lepidopteran specific, and the P2 toxin is active on Lepidoptera and Diptera (Aronson et al. 1986; Menon & de Mestral 1984). Therefore, although Btk is specific to Lepidoptera, some impact on Diptera, such as chironomid midges, can be expected.

Furthermore, at Btk concentrations greater than 2.0 mL per 3.8 L of well water, there was a significant decrease in midge development and an impact on water clarity, which decreased with increasing Btk concentration. Higher Btk concentrations therefore had lower clarity even before hydrilla decomposition and possibly reduced the ability of *C. lebetis* to survive. It has been shown that Btk can persist for several days in various water systems, persisting longest in freshwater lake systems (Menon & de Mestral 1984). Btk persistence in a small-scale aquatic colony could limit nutrients and reduce water quality for the hydrilla tip mining midge; the reduced water quality throughout the 22 d midge life span may have affected the ability of the midge to pupate or eclose as adults.

Chironomid species often are used as indicators of water quality due to their specific requirements of oxygen concentration, total phosphorous, and chlorophyll in the water (Saether 1979). Consequently, the level of pollution in a water body can be determined from the presence or absence of chironomid indicator communities and their division in different trophic ranges (Saether 1979). Because *C. lebetis* has been discovered inhabiting a diverse range of water bodies in Florida and Louisiana, it is thought that this species is well adapted to a range of water quality conditions (Stratman et al. 2013a).

This study demonstrated the difficulty associated with rearing one beneficial insect at the expense of another that is competing for the same resource. These data indicate that a concentration of 2.0 mL Btk per 3.8 L may have some negative effects on midge development. However, a concentration of 0.2 mL Btk per 3.8 L of well water did not have any adverse effects on *C. lebetis* development compared with the control. Depending on colony rearing situations, either 0.2 or 2.0 mL of Btk per 3.8 L of well water would be recommended. If there is an excess of midge eggs or moth competition is extremely high, the 2.0 mL concentration of Btk per 3.8 L of well water would provide faster control of the moth population. However, if eggs of the hydrilla tip mining midge are scarce or in high demand and moth competition in the midge colony is manageable, the lower concentration of 0.2 mL Btk per 3.8 L of well water would be recommended because the lower Btk concentration would have less impact on midge colony production.

Resistance to Bt in open populations of insects has been documented (Tabashnik 1994). Furthermore, Tabashnik (1994) demonstrated that artificial selection for resistance in the laboratory can increase resistance to Btk 10-fold in multiple families of Lepidoptera. Therefore, it is important to monitor resistance in colony settings, especially when using below-label-rate concentrations of Btk to control colony pests, which could select for resistance to Btk in *P. diminutalis*.

Future bioassays analyzing concentrations of Btk between 0.2 and 2.0 mL Btk per 3.8 L of well water may be beneficial. Also, the data for both the 10.0 and 20.0 mL concentrations of Btk indicate that variation may be high in *P. diminutalis* mortality, and more replicates may be needed to resolve this discrepancy. Additionally, selecting instars of more similar age in future studies would ensure a more accurate response to tested Btk concentrations. Further studies also should evaluate the effect of Btk with *P. diminutalis* and *C. lebetis* in combination. As their life cycles occur over different times, the effects of Btk on water quality, Btk persistence, or the lethality of Btk to one organism may have other effects on the colony than can be anticipated by testing the organisms individually.

In summary, using a low rate of 0.2 mL Btk per 3.8 L of well water can reduce moth emergence by 50% compared with the control and may be incorporated into the midge colony rearing protocol as it imposes little risk to *C. lebetis*. Using 2.0 mL or more Btk per 3.8 L of well water can further reduce moth emergence but poses a higher risk of mortality to *C. lebetis* and should only be used for management of severe *P. diminutalis* infestations. Therefore, Btk at the appropriate concentration can be used to effectively control resource competition in a colony by reducing moth larvae and thereby hydrilla consumption without detrimental effects on the production of hydrilla tip mining midge.

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