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The Efficacy of *Bacillus thuringiensis* spp. *galleriae* Against Rice Water Weevil (Coleoptera: Curculionidae) for Integrated Pest Management in California Rice

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ABSTRACT Rice water weevil (*Lissorhoptrus oryzophilus* Kushel) is the most damaging insect pest of rice in the United States. Larval feeding on the roots stunt growth and reduce yield. Current pest management against the weevil in California relies heavily on pyrethroids that can be damaging to aquatic food webs. Examination of an environmentally friendly alternative biopesticide based on *Bacillus thuringiensis* spp. *galleriae* chemistry against rice water weevil larvae showed moderate levels of activity in pilot studies. We further examined the performance of different formulations of Bt.galleriae against the leading insecticide used in California rice, λ-cyhalothrin. The granular formulation performed as well as the λ-cyhalothrin in use in California in some of our greenhouse and field studies. This is the first reported use of *B. thuringiensis* spp. *galleriae* against rice water weevil.

KEY WORDS *Lissorhoptrus oryzophilus*, *Oryza sativa*, biopesticide, microbial control, crop protection

The rice water weevil (*Lissorhoptrus oryzophilus* Kuschel) is the most destructive insect pest of rice in the United States (Grigarick and Beards 1965, Saito et al. 2005). It is a member of the beetle family Curculionidae and is native to the eastern United States, with a range extending from southern Canada down to the Gulf coast (Way 1990). It was first discovered outside its native range in California in 1957 and from there it has continued to expand across much of northern temperate rice production zones starting from Japan, proceeding southwest through Korea and China, and has most recently established in Italy (Lange and Grigarick 1959, Jiang and Cheng 2003, Lupi et al. 2010). The species reproduces sexually in its native range but reproduces exclusively through parthenogenesis in its expanded range. It feeds primarily on sedges and aquatic grasses in the families Cyperaceae and Poacecae, which includes cultivated rice (*Oryza sativa* L.; Palrang et al. 1994, Lupi et al. 2009). The adult lay eggs into the rice leaf sheath, and the larvae mine into the submerged root zone. The larvae survive in the submerged root zone using modified spiracles that hook into the rice plant's spongy tissue that facilitate gas exchange between the roots and the atmosphere (Zhang et al. 2006). The pupa is attached to the rice root to facilitate gas exchange during metamorphosis. After emergence the adults diapause over the winter on levees and vegetated areas and later return in the spring to infest the rice field after the field is flooded and seeded (Stout et al. 2002, Zou et al. 2004).

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Even though rice has been commercially cultivated in the United States since the 1700s (Adair et al. 1966), rice water weevil was not considered a major pest until Isely and Stewart (1934) definitively demonstrated that it reduced rice yields. The low yields in rice are caused by damage to the roots from larval feeding in the submerged root zone (Stout et al. 2002, Tindall and Stout 2003). This reduces the amount of grain bearing tillers on the individual plant (Tindall and Stout 2001). In contrast to the larvae, the adults feed on rice leaf tissues and leave diagnostic longitudinal feeding scars that do not cause significant yield loss (Zou et al. 2004).

Current pest management strategies in California rely on the use of pyrethroids, which are toxic to aquatic organisms (Soderlund et al. 2002, Godfrey et al. 2007). In the southern United States, growers use sprays and seed treatments with anthralic diamides, neonicotinoids, or pyrethroids (Saichuk 2012, Taillon et al. 2014, Way and Espino 2014). Even though synthetic insecticides with greater insect-specific action such as neonicotinoids have been recently approved for use in California (California Department of Pesticide Regulation [CDPR] 2013), the search for less toxic alternatives is still preferable. Reducing the effect of management on nontarget insects and aquatic food webs is important for maintaining endangered wildlife in rice fields and in wetlands downstream (Lawler and Dritz 2005).

The current alternative strategies include weed control around fields to reduce habitat for rice water weevil adults, drill seeding, delayed flooding or planting, or winter flooding (Bernhardt 2012, Flint et al. 2013). There are also various biopesticides that are available for use in rice. Azadirachtin, which is a common alternative botanical insecticide derived from the insecticidal compounds of the neem tree (*Azadirachta indica)*, is also an option but works as an antifeedant and may not fully prevent oviposition, which would lead to the development of a damaging population of larvae (Schmutterer 1990, Copping and Menn 2000). Pyrethrin (or pyrethrum) is another alternative botanical pesticide that gave rise to the use of pyrethroids, but the original chemistry is not used because it is susceptible to degradation by UV light that shortens residual in the field and reduces efficacy (Buss and Park-Brown 2002, Barcic et al. 2006). Entomopathogenic nematodes were also considered for use in rice pest management after the discovery of a mermithid nematode infecting weevils in Arkansas (Bunyarat et al. 1977). However, the use of nematodes in the field was shown to be ineffective and costly because it required field drainage. (Grigarick and Oraze 1990, Smith 1990).

A more promising tool is the entomopathogenic bacteria, *Bacillus thuringiensis* Berliner, because of the specificity and effectiveness of various subspecies against particular insect species (Lacey and Goettel 1995, Bravo et al. 2011). Research by Godfrey with Novodor (*Bacillus thuringiensis* spp. *tenebrionis*) found it to be as effective as λ-cyhalothrin, but the formulation was discontinued after a company merger (L.D.G., personal communication). Another subspecies that could play a role in rice water weevil management is *Bacillus thuringiensis* spp. *galleriae* that has been used to control different species of white grubs (Coleoptera: Scarabeidae) in turfgrass systems (Matthews, personal communication, Asano et al. 2003). Because of its known efficacy against several species of soil dwelling Coleoptera and moderate activities against rice water weevil immatures in pilot studies, it is a promising candidate for rice water weevil control.

The objective of this study was to evaluate the efficacy of *B. thuringiensis* spp. *galleriae* (henceforth mentioned as Btg) in comparison with λ-cyhalothrin, which is a commonly used pyrethroid in California rice agriculture, and azadirachtin, the active ingredient in neem oil, which is a known botanical insecticide but not commonly used against rice water weevil. We hypothesized that 1) Btg would have a significant effect in reducing the number of weevil immatures, 2) Btg granular formulation would outperform the foliar formulations, 3) the Btg granular formulation would perform as well if not better than λ-cyhalothrin, and 4) all Btg products would have greater control of weevils if applied preflood compared with postflood.

Materials and Methods

Experimental Design. We tested our hypotheses in a series of experiments in the greenhouse and in the field. Granular and foliar formulations of *B. thuringiensis* spp. *galleriae* products (Phyllom BioProducts Corp., Oakland, CA) were evaluated against rice water weevil at different concentrations based on suggestions from the manufacturer and pilot study results. The granular formulation labeled as Phy-2-12 was tested at 24.6, 28.2, and 31.7 kg/ha. Granular formulations Phy-4-12 and Phy-4-13 were only tested at 31.7 kg/ha. Granular formulation Phy-4-13 was made in 2013 and was produced at a different facility from Phy-2-12 and Phy-4-12. The first iteration of foliar formulations, labeled as Phy-3-11, was tested at 100, 300, and 500 mg/liter of water. The second iteration of foliar formulations, labeled as Phy-3-13, was tested at 3.56, 7.12, and 14.24 kg/ha. λ-Cyhalothrin (Syngenta Basel, Switzerland), a commonly used pyrethroid in rice cultivation, and Aza-direct (Gowan Company, 370 South Main Street Yuma, Arizona), a neem oil product, were tested at 22.6 ml/ha and 191 ml/ha, respectively. No products were added for the negative control. The effect of application timing against rice water weevil was evaluated by test products in preflood or postflood scenarios. In preflood applications, insecticides were sprayed directly to the soil, but in the postflood applications, insecticides were sprayed directly onto the plant and into the water column. These treatment combinations of insecticide and application timing were tested in studies over two years at the UC Davis campus greenhouses in Davis, CA, and in the field at the Rice Experiment Station in Biggs, CA.

Greenhouse Experimental Setup. The greenhouse treatments were applied in a complete randomized design and were replicated four times in the first trial and five times in the second trial to increase power. Five rice seeds of M-202, a medium grain rice variety grown in California, were planted in 18- by 10-cm plastic cylindrical pots filled to a 6 cm depth. The rice was planted in Esquon-Neerdobe complex rice paddy soils from Biggs, CA, homogenized for uniformity of soil texture and mineral content. No fertilizer was applied to pots because plants were not grown out to maturity. Pots were flooded to a 10 cm depth of deionized water. Rice water weevil adults were collected from untreated grower rice fields at the Rice Experiment Station in Biggs, CA, and held in the laboratory for 24 h to acclimate to indoor conditions and to eliminate weakened weevils. Preflood insecticide applications were made 2 d before planting. Postflood insecticide applications were applied 10–14 d after planting depending on when the rice plants reached the 3–4 leaf stage of development. All insecticides with the exception of the granular were applied by bottle spray to the pots. The granular formulations were measured using laboratory scale and sprinkled onto the soil preflood or into the water column postflood.

Plants were infested with three field-collected adult female weevils at the 3–4 leaf stage in rice plant development when rice is most vulnerable and likely to be attacked by rice water weevil (Flint et al. 2013). Weevils were placed in 60-cm-tall cylindrical mylar plastic cages with a fabric mesh on the top opening to prevent escape. The weevils were applied 3 d before post-insecticide applications to allow enough time for oviposition on rice plants in both preflood and postflood insecticide treatments. Weevils were removed from all cages after 24 h of postflood treatment application to reduce the confounding factor of death by starvation caused by overpopulation. After 2.5 wk, pots were destructively sampled by removing rice plants with the intact rhizosphere and washing them through a 2 mm sieve to retrieve weevil larvae and pupae (collectively termed immatures) that were still alive for counting.

In 2012, two greenhouse experiments were conducted, with adults collected in April and June. Granular and foliar formulations of Btg products were evaluated, based on recommendations from the manufacturer, at different concentrations to evaluate efficacy against rice water weevil in both 2012 studies (Table 1).

The third greenhouse experiment was conducted in June 2013 with reformulations of the granular and foliar Btg products. The granular Btg product was tested at the highest recommended rate of 31.7 kg/ha, and the foliar was tested at rates as recommended by product scientists at Phyllom BioProducts Corp. (Matthews, personal communication, Table 1).

Field Experiment Setup. Field experiments were conducted in a randomized complete block design with 13 treatments that were replicated five times. A total of 65 aluminum rings that measured 1 m^2 in area and a height of 46 cm were used as the experimental units with approximately 100 rice plants established in each ring on May 27. Pre-germinated rice seeds of variety M-202 were distributed in each ring in a continuously flooded production system. Each ring was infested with 10 adult rice water weevils at the 3–4 leaf stages by June 8. Each ring was subsampled five times. Weevil immatures were sampled with a cylindrical soil corer that measured 12 cm in height and 10 cm in diameter. Each soil core contained the rice plant with the surrounding rhizosphere and was placed in a plastic bag, then stored at −20°C for later processing. Two separate rounds of sampling were conducted in weeks 5 and 7 after planting. Field cores were thawed and processed between August and December of 2013. Field cores were processed for weevil immatures using the same method as the greenhouse study.

Table 1. List of all greenhouse treatments with application timing, product, and rates test from 2012–2013

Study	Product	Active ingredient	Rates
1	Warrior II	λ-Cyhalothrin	22.67 ml /ha
	Aza-Direct	Azadirachtin	191 ml/ha
	Phy-2-12	*B. thuringiensis* spp. *galleriae*	24.6 kg/ha
	Phy-2-12	*B. thuringiensis* spp. *galleriae*	28.2 kg/ha
	Phy-2-12	*B. thuringiensis* spp. *galleriae*	31.7 kg/ha
	Phy-3-11	*B. thuringiensis* spp. *galleriae*	100 mg/ml
	Phy-3-11	*B. thuringiensis* spp. *galleriae*	300 mg/ml
	Phy-3-11	*B. thuringiensis* spp. *galleriae*	500 mg/ml
2	Warrior II	λ-Cyhalothrin	22.67 ml /ha
	Aza-Direct	Azadirachtin	191 ml/ha
	Phy-4-12	*B. thuringiensis* spp. *galleriae*	31.7 kg/ ha
	Phy-3-11	*B. thuringiensis* spp. *galleriae*	500 mg/ml
3	Warrior II	λ-Cyhalothrin	22.67 ml /ha
	Aza-Direct	Azadirachtin	191 ml/ha
	Phy-4-13	*B. thuringiensis* spp. *galleriae*	31.7 kg/ha
	Phy-3-13	*B. thuringiensis* spp. *galleriae*	3.56 kg/ha
	Phy-3-13	*B. thuringiensis* spp. *galleriae*	7.12 kg/ha
	Phy-3-13	*B. thuringiensis* spp. *galleriae*	14.24 kg/ha
Field	Warrior II	λ-Cyhalothrin	22.67 ml /ha
	Aza-Direct	Azadirachtin	191 ml/ha
	Phy-4-13	*B. thuringiensis* spp. *galleriae*	31.7 kg/ha
	Phy-3-13	*B. thuringiensis* spp. *galleriae*	3.56 kg/ha
	Phy-3-13	*B. thuringiensis* spp. *galleriae*	7.12 kg/ha
	Phy-3-13	*B. thuringiensis* spp. *galleriae*	14.24 kg/ha

Statistical Analyses. All data were analyzed using factorial analysis of application timing by insecticide, and means were separated with the Tukey HSD test for comparison between treatments. If the Tukey test showed no differences between any of the insecticides and the negative control, then the Dunnett's test for treatment comparison with the control was used to better understand the effect of the individual insecticide. All analyses were conducted using an alpha of 0.05. A Poisson distribution with Proc Glimmix model in SAS 9.3.1 (SAS Institute 2010, Cary, NC) was used to account for overdispersion with study data that had variances that were greater than the means of treatments. If count data were not overdispersed or random, but still failed to meet assumptions of normality, they were square root transformed. Interactions between application timing and insecticide that were tested with Proc Glimmix were further analyzed by the simple effect of application timing if interactions were significant. Untransformed means are presented graphically in the results.

Results

2012 Greenhouse. All of the insecticides, including the Btg, reduced the mean abundance of rice water weevil immatures relative to untreated controls by >50% ($F = 3.30$, df = 8,54, $P = 0.0039$) in the first greenhouse study (April 2012; Fig. 1). There were no significant effects of application timing ($F = 0.00$, df = 1, 54, $P = 0.9988$) either as an individual factor or in interaction with insecticide ($F = 0.13$, df = 8, 54, $P = 0.9978$). Tukey post hoc tests did not show differences among insecticides or between application rates of Btg.

In the second study (June 2012), the effect of insecticide was significant in reducing the number of immatures ($F = 3.81$, df = 5, 48, $P = 0.0055$), with the exception of the foliar formulation Phy-3-11 that had more immatures than the control (Fig. 2). Application timing did not have a significant effect on reducing immature populations ($F = 0.00$, df = 1, 48, $P = 0.9934$). However, the interaction of application timing by insecticide was significant ($F = 3.51$, df = 5, 48, $P = 0.0205$), so the simple effect of insecticide was analyzed by application timing. In preflood applications, Tukey–Kramer grouping of the least square means showed that the Phy-4-12, azadirachtin, and λ-cyhalothrin were not significantly different in their effect of reducing the number of immatures (Fig. 2). In the postflood applications, the Btg formulations did not have any effect in reducing immatures compared to the control using Dunnett's test ($P < 0.05$). Only the λ-cyhalothrin and azadirachtin had significant effects of reducing immature numbers compared with the control, and the Tukey–Kramer Pairwise test showed no difference between those two treatments ($P < 0.05$)

2013 Greenhouse. In the greenhouse experiments, insecticides significantly decreased immature numbers ($F = 9.26$, df = 6, 63, $P < 0.001$), but application timing

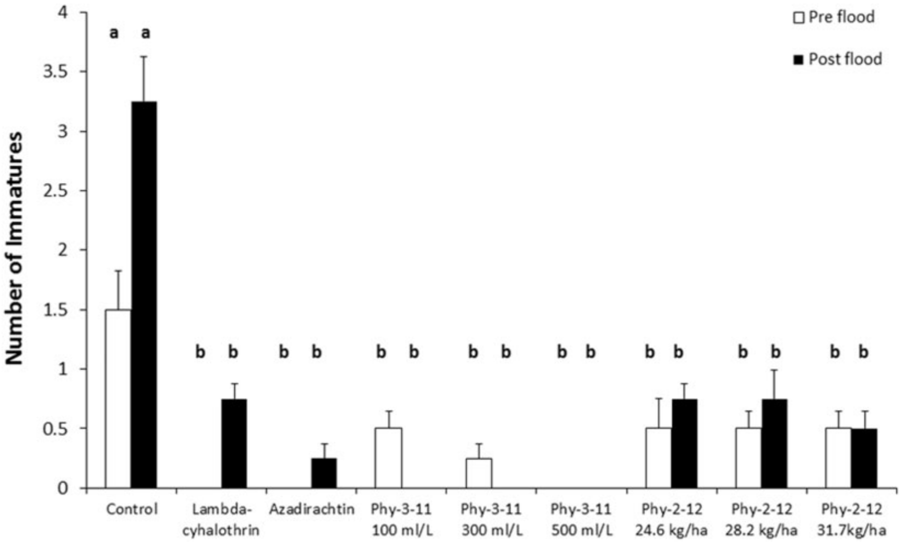

Fig. 1. Summary of first study results from April 2012 with average number of immatures per pot for each insecticide treatment in preflood and postflood. Means with the same letter are not significantly different from each other (Tukey–Kramer test, $P < 0.05$, $n = 36$) and show differences between insecticides across application timing.

had no significant effect on the number of immatures ($F = 0.01$, df = 1, 63, $P = 0.9053$). There was no significant interactive effect of application timing by insecticide ($F = 1.27$, df = 6, 63, $P = 0.2854$). None of the Btg treatments were significantly different from the control in this study. Only λ-cyhalothrin showed a significant decrease in the number of weevils according to the Tukey HSD test ($P < 0.05$) (Fig. 3).

2013 Field. In the field study, data from the negative controls in the postflood were lost from a few plots and this reduced the reliability of the analysis using the unbalanced design option in SAS. Analysis of the data from the first sampling of immatures (5 wk after planting) revealed that the effect of insecticide was significant in reducing the number of immatures ($F = 7.49$, df = 6, 306, $P < 0.0001$). Application timing did not have a significant effect on the number of larvae in the plots ($F = 0.07$, df = 1, 306, $P = 0.7898$) and neither did the application timing by insecticide interaction ($F = 0.78$, df = 6, 306, $P = 0.5824$). Analyzing across application timing, the Btg granular (Phy-4-13), azadirachtin, and λ-cyhalothrin formed a group with the lowest means for larval population and were not significantly different from each other using the Tukey post hoc test (Fig. 4). The analysis of the data for the second immature sampling (7 wk after seeding) revealed no significant effect of treatments ($F = 1.50$, df = 6, 301, $P = 0.177$) or of application timing ($F = 0$, df = 1, 301, $P = 0.9887$) on larval populations.

Discussion

Our studies provide moderate support for our hypotheses that Btg reduces the number of immatures when compared with a negative control and that the Btg granular performs as well as the λ-cyhalothrin insecticide. Some of the studies showed that the Btg granular formulations were more effective than the Btg foliar formulations. However, an effect of application timing on the efficacy of Btg products was not supported by the data.

We speculate that the Btg granular formulations were the most effective Bt formulations tested because they reach and are ingested by the immatures after being applied directly to the soil in the preflood applications. In our observations of the granular application in the postflood situation, we saw that the Btg granules did not penetrate the soil layer. However, they may have been mixed into the soil when plants were being watered to maintain flood conditions. In the field this probably could happen as a result of wind action and tadpole shrimp that disturb the soil layer, allowing the Btg granules to mix into the top layer before rice plants take root and rice water weevils infest.

The Btg granular formulation did not completely outperform the foliar formulations as we hypothesized because it was statistically grouped with the foliar formulation Phy-3-11 in the second study. However, the significant interaction between application timing and insecticide effects made it necessary to analyze the treatment means within application time. This interaction may have been an artefact resulting from large differences in the number of immatures between preflood and postflood controls and foliar (Phy-3-11) treatments (Fig. 2). The interaction made it more difficult to detect a treatment effect of the formulations.

Although Btg granular had a similar effect as λ-cyhalothrin in the third study, we found that there was no statistical separation between the untreated control and Btg (Phy-4-13). The underperformance of the granular formulation may have resulted from its production at a different facility from the granular formulation that we had tested in 2012, with slight modifications according to our discussions with the manufacturer. The granules

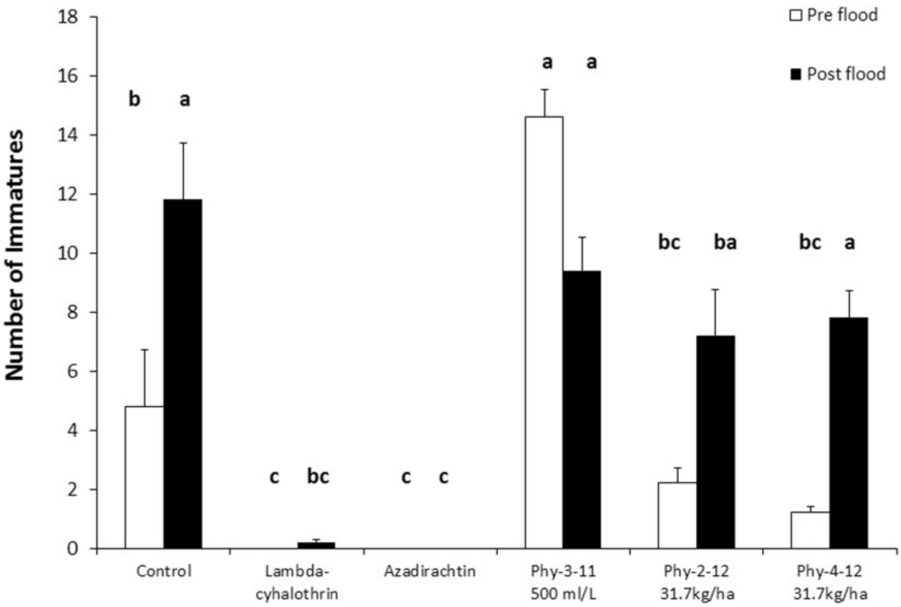

Fig. 2. Summary of second study results from June 2012 with average number of immatures per pot for each insecticide treatment in preflood and postflood. Means with the same letter are not significantly different from each other (Tukey–Kramer test, $P < 0.05$, $n = 48$) and show differences between insecticides across application timing.

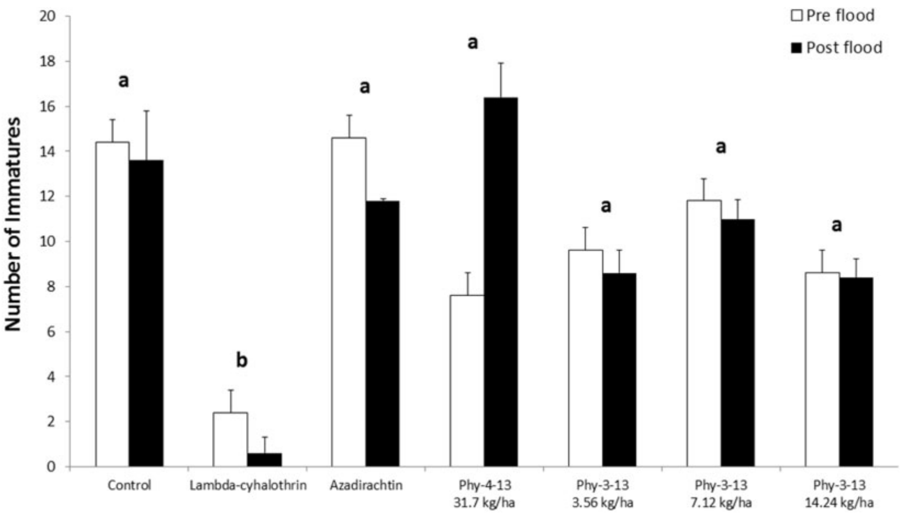

Fig. 3. Summary of third study results from June 2013 with average number of immatures per pot for each insecticide treatment in preflood and postflood. Means with the same letter are not significantly different from each other (Tukey–Kramer test, $P < 0.05$, $n = 70$) and show differences between insecticides across application timing.

from the 2013 formulation were visibly and olfactorily different from previous formulations. The higher immature count in the azadirachtin treatment in that same study could have been due to a loss in efficacy after a year in storage or excessive dilution prior to application in the greenhouse.

The field studies corroborated the second and third greenhouse experimental results that showed similar performances for Btg granular (Phy-4-13) and λ-cyhalothrin when analyzed across application times. Within application times there were no statistically significant differences between treatments using the Tukey HSD test, which is unexpected because at the very least the pyrethroid should have been effective. It could be that the loss of a few experimental units masks differences when analyzing the data within application times.

The first study also corroborated our conclusions with regard to Btg in general, but it also presented some analytical challenges because of the lack of

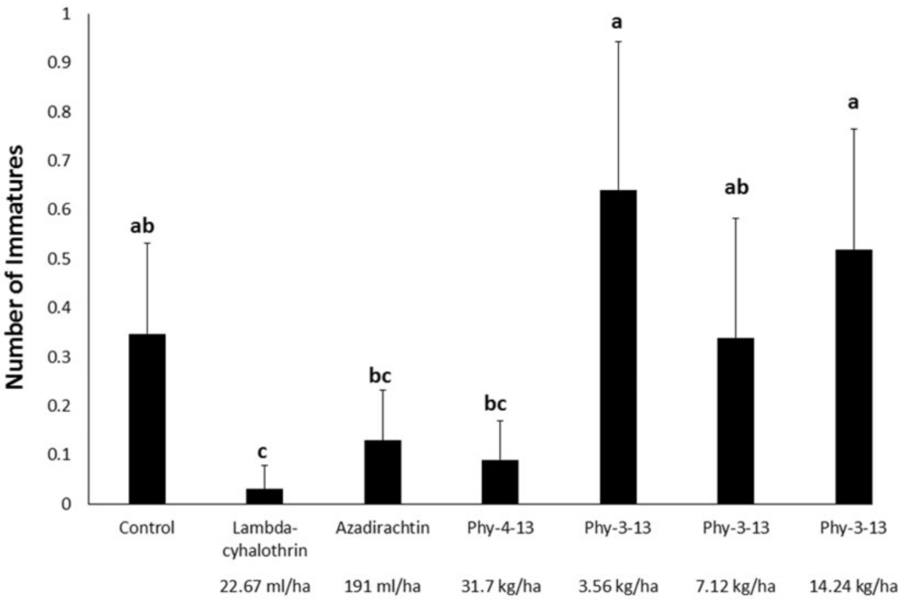

Fig. 4. Summary of field results from the first coring in July 2013 with average number of immatures for each insecticide tested in across application timing. Means with the same letter are not significantly different from each other (Tukey–Kramer test, $P < 0.05$, $n = 65$) and show differences between insecticides across application timing.

statistical separation between the insecticide treatments. This may have been caused by the presence of many zero counts among the pots. The most plausible explanation for what is observed in the first study (April 2012) is that the weevil adults used may not have had fully developed ovaries. The adults from that study were captured in early April when most weevils have emerged from diapause. Many of these weevils have depleted the stores of fat from their winter hibernation and need to feed on weeds to help regenerate flight muscles and develop their ovaries (Shi et al. 2007). The only conclusion that we drew from that study was that the Btg had an effect relative to the negative control.

Data trends from the foliar formulations were not clear, and there were no linear dose responses in any of the studies testing Phy-3-11 and Phy-3-13 at incrementally increasing rates. The foliar formulations from 2012 (Phy-3-11) were very difficult to mix in water and would occasionally clog the bottle sprayer used in the greenhouse. The foliar formulation from 2013 (Phy-3-13) was an easily mixable powder. In the field, the Btg foliar formulations did not perform well and had the highest mean number of immatures (Fig. 4). We did not find a linear relationship between immature mortality and increasing concentrations of the foliar formulation Phy-3-13. It is unclear why the Phy-3-13 at 7.12 kg/ha rate had a higher count of immatures compared to the formulations at the 3.56 and 14.24 kg/ha rates, although it may be due to random variation in oviposition rates between the weevils in the treatments. The foliar formulation rates were based on producer recommendations that may ultimately have been too low to be effective against rice water weevil. The field performance could be explained by inactivation of the Bt endotoxin from exposure to ultraviolet light. However, this was not evident with the granular Btg, which outperformed the foliar formulations in both preflood and postflood applications. It is likely that one or more of the ingredients for the granular formulation of the product tested provides more UV protection and environmental persistence compared with the foliar products that we tested. This increased persistence in the field increases the chances for the immatures to come into contact and ingest the Bt (Nicholson 2002, Sanahuja et al. 2011).

There was very little support for our hypothesis that the insecticides would have been effective when applied prior to rice planting and flooding. Time-dependent application effects were only observed in the second study (June 2012). Our hypothesis was based on the assumption that application of insecticides directly to the soil matrix prior to flooding and planting would allow the Btg to persist within the soil space away from UV damage. We expected some level of time-dependent application effects because some insecticides have been reported to have better efficacy before or after flooding in rice fields. Field tests with azadirachtin found the product to only be effective when sprayed at the time of seeding or 19 d after seeding in the postflood period (Godfrey 2004). Tests with neonicotinoids such as the Belay showed that the insecticide was more effective when sprayed at the 3 leaf stage postflood compared with a preflood spray (Godfrey 2013). The design of the study may have obscured these effects because the preflood and postflood treatments were only 2 and 3 wk apart in the greenhouse and field studies, respectively. In addition, *B. thuringiensis* products have a short window of effectiveness in the field compared with λ-cyhalothrin, which can persist up to 3 wk (Choo and Rice 2007, Barbee and Stout 2009).

At this point in our research the application of Btg granules (Phy-4-12 and Phy-4-13) at the 31.7 kg/ha is the most effective rate for controlling rice water weevil compared with other Btg products and rates that were tested. Our greenhouse studies showed that products based on *B. thuringiensis* spp. *galleriae* can have a significant effect on immature mortality regardless of the timing of application. However, direct comparison with the leading synthetic chemical (λ-cyhalothrin) showed that the Btg granular does not work consistently well. They are most likely to have similar effects on the weevils when applied preflood.

It is very desirable for these alternative biopesticides to have similar efficacy as the leading synthetic active ingredients in the field, without the secondary target effects. Nontarget studies are required to confirm that the Btg has no effect on nonpest aquatic beetles such as Dytiscids and Hydrophilids (Lawler et al. 2007) that inhabit the rice fields. The next step is to continue greenhouse and field testing to track possible changes in the formulation mixture as the product continues to be developed for large scale commercial field applications. Our experimental results show that while the product cannot yet be relied upon for consistent performance, it has promising potential as an effective new tool for rice water weevil control.

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