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SUSCEPTIBILITY OF *DIAPREPES ABBREVIATUS* (COLEOPTERA: CURCULIONIDAE) TO A COMMERCIAL PREPARATION OF *BACILLUS THURINGIENSIS* SUBSP. *TENEBRIONIS*

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ABSTRACT

A commercial preparation of the microbial entomopathogen, *Bacillus thuringiensis* subsp. *tenebrionis* (*Btt*) was evaluated for biological activity against the Diaprepes root weevil, *Diaprepes abbreviatus* (L.). A reduction in survival was observed for neonatal larvae exposed to insect diet incorporated with *Btt* and in potted citrus treated with a *Btt* soil application. A treatment-induced, weight gain reduction for neonates was indicated only in the diet assay. Larvae exposed at 5 weeks old to diet treated with *Btt* demonstrated a dose-dependent mortality response. The mean ages for larval death ranged from 111 to 128 days among treatments. The LC_{50} for larvae in this age group was 6.2 ppm [AI] and the slope of the probit line was 2.29. The mortality response of larvae exposed at 12 weeks old also was dose dependent and the mean ages for larval death ranged from 130 to 141 days among treatments. The LC_{50} for larvae in this age group was 25.4 ppm [AI] and the slope of the probit line was 2.75. The delayed patterns of mortality that we observed among larvae treated at 5 and 12 weeks old indicates that disease is slow to develop in older larvae but that death occurs before maturation is completed.

Key Words: *Diaprepes abbreviatus*, *Bacillus thuringiensis*, entomopathogen, citrus, Diaprepes root weevil

RESUMEN

Se evaluó una formulación comercial del entomopatógeno microbial, *Bacillus thuringiensis* subsp. *tenebrionis (Btt)* por su actividad biológica en contra del gorgójo *Diaprepes abbreviatus* (L.). Se observó una reducción en la sobrevivencia de larvas neonatas expuestas a una dieta artificial que contenia *Btt*, y de larvas en potes con arboles tratados con *Btt*. Una reducción del peso ganado por las larvas neonatas fue inducida por el tratamiento solamente en el caso del ensayo con dieta artificial. Larvas expuestas a dieta tratada a las 5 semanas de edad manifestaron una mortalidad proporcional a la dosis. El rango del promedio del número de dias hasta la muerte de las larvas fue de 111 a 128 dias entre tratamientos. La DL_{50} para las larvas de este grupo fue de 6.2 ppm (IA) y el pendiente de la linea probit fue de 2.29. La mortalidad de larvas expuestas a las 12 semanas de edad fue dependiente de la dosis y la edad promedia de la muerte varió entre 130 y 141 dias entre tratamientos. La DL_{50} para las larvas de este grupo fue de 25.4 ppm (IA) y el pendiente de la linea probit fue de 2.75. El patron de mortalidad que observamos entre larvas tratadas a las 5 y a las 12 semanas de edad indica que la enfermedad se desarrolló lentamente en las larvas mayores pero la muerte ocurrió antes de la maduración.

The most economically important insect pest of citrus in Florida is the Diaprepes root weevil, *Diaprepes abbreviatus* (L.). Losses to citrus growers are estimated in excess of $75 million yearly (Anonymous 1997). This root weevil also attacks sugar cane, vegetable crops, and ornamental plantings in areas that are infested. The subterranean larvae extensively damage host plant root systems on which they feed. Larval feeding in citrus predisposes root tissues to infections by pathogens such as *Phytophthora* spp. (Rogers et al. 1996), induces a decline in tree health and fruit production, and may eventually kill the tree (Schroeder & Sutton 1977). In severe cases of uncontrolled weevil populations, entire citrus groves are destroyed (pers. obs.).

Entomopathogens have received considerable attention as potential biocontrol agents of root weevil larvae. Beavers et al. (1983) and Román

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and Beavers (1983) initially demonstrated the pathogenicity and seasonal prevalence of several naturally occurring fungi and nematodes attacking *D. abbreviatus.* Since then, many others have demonstrated the potential for biological control of root weevil larvae by entomopathogens (Duncan et al. 1996; Figueroa & Román 1990; Quintella & McCoy 1997, 1998; Schroeder 1987, 1994). Nematode applications were recommended for control as recently as 1999, but the insecticidal compound, bifenthrin, is currently the only material listed in the Florida Citrus Pest Management Guide for control of larval stages (Futch et al. 2001).

Bacterial entomopathogens have not been previously investigated as potential biocontrol agents for Diaprepes root weevil. The microbial pathogen, *Bacillus thuringiensis* subsp. *tenebrionis* (*Btt*), was shown to have activity against representatives of the order Coleoptera including the families Chrysomelidae, Bostrichidae, and Curculionidae (Herrnstadt et al. 1986; Krieg et al. 1983, 1987; Beegle 1996; Saade et al. 1996). The bacterium produces a crystalline δ-endotoxin during the process of sporulation (Hofte & Whiteley 1989). Upon ingestion by a susceptible insect host, the endotoxin is proteolytically activated in the midgut and causes feeding inhibition, septicaemia, and eventual death (Knowles 1994). We investigated a commercially available *Btt* product, Novodor® (Novo Nordisk, North Chicago, IL), to determine if it had biological activity against *D. abbreviatus*, envisioning that a demonstration of activity may provide the impetus to develop this and possibly other *B. thuringiensis* subsp. for field application.

Materials and Methods

Insect Rearing

Neonatal, 5-week-old, and 12-week-old larvae of *D. abbreviatus* were obtained from a laboratory colony maintained by the U.S. Horticultural Research Laboratory, Fort Pierce, FL. Larvae were reared on a commercially-prepared insect diet (Product No. F1675, Bio-Serv, Frenchtown, NJ) placed within sealed diet cups (PC100, 30 ml cups and lids, Jet Plastica, Harrisburg, PA). Preparation of insect diet and rearing of larvae were similar to the methods described by Beavers (1982) with temperature and moisture content of diet optimized for larval development (Lapointe 2000, Lapointe & Shapiro 1999). Weevil larvae used in the bioassays were maintained in diet cups stored in plastic trays in a growth chamber at 26°C and a 24-h dark cycle.

Diet Incorporation of *Btt*

Prepared insect diet was heated to 90°C for 15 min., covered with foil, and allowed to cool to 56°C in a heated water bath before incorporating treatments. A commercial preparation of *B. thuringiensis* subsp. *tenebrionis* (Novodor 3% [AI], [30 mg spores and δ-endotoxin crystals per ml product]) was incorporated into the diet at rates of 0, 0.3, 3.0, 30, and 300 ppm (µg AI/ml diet). Treatments were incorporated into the agar-laden diet with the aid of a heated, stirrer plate. The resulting mixtures were pipetted into diet cups (15 ml diet per cup), allowed to solidify and cool to room temperature, and then were closed with a lid. All steps after heating of the diet were performed in a laminar flow, clean bench to avoid contamination.

Evaluation of *Btt* Activity

The biological activity of Novodor was evaluated initially against neonatal larvae exposed to treated insect diet. Neonates were briefly surface sterilized in a 5% bleach solution and rinsed with sterile, deionized water prior to being placed in diet cups. The treatments included the 5 rates of treatment-incorporated diet described above and there were 3 replications of each treatment. A treatment comprised 18 diet cups, each infested with 5 neonatal larva. The numbers and weights of surviving larvae in each diet cup were assessed after 6 weeks of exposure to treated diet.

The activity of Novodor also was evaluated against 5-week old weevil larvae. Larvae used in the experiment weighed ~110 mg before exposure to treatments. Treatments were prepared as above and replicated 6 times. Each treatment comprised 18 diet cups, each containing 1 larva. Mortality, age at death, and weights of eclosed adults were recorded twice weekly. The experiment was terminated after 5 months when most larvae had either died or matured to adults.

Novodor activity also was evaluated against 12-week old larvae that weighed ~510 mg before exposure to treatments. The treatments were incorporated in diet at the rates described above. There were 3 replications of treatments, each comprising 30 larvae confined individually to diet cups. Mortality, age at death, and weights of eclosed adults were recorded twice weekly. The experiment was terminated after 3 months when most larvae had either died or matured to adults.

Another experiment was conducted to determine the effect of Novodor suspensions applied as soil treatments against neonatal larvae feeding on potted citrus roots. The citrus plants used in the study were 1 year old Cleopatra Mandarin (*Citrus reshni* Hort., ex Tan.) rootstock potted in 473 cm^3 containers of potting soil (Metromix 500, Scotts, Marysville, OH). The treatments included 4 rates of Novodore (0, 3.0, 30, and 300 ppm [(g AI/ml DI water]) applied as 50 ml suspensions to the soil of each container. Two drench applications were made 14 days apart. There were 6 replications of each treatment. A treatment comprised 1

potted citrus plant infested with 20 neonates immediately after the first drench application. All treatments were maintained in a growth chamber at 26°C with a photoperiod of 14:10 (L:D) h. The numbers and weights of surviving larvae were assessed after 6 weeks.

Data Analyses and Statistics

Percentage data were adjusted for control mortality using the Abbott (1925) formula and transformed (arcsine) before analyses. Data were analyzed by the General Linear Models Procedure, and differences among treatment means were determined by Tukey's studentized range test (SAS Institute 1990). Differences among means were considered significant at a probability level of 5 percent ($P \leq 0.05$). Probit analyses were conducted using the Probit Procedure (SAS Institute 1990) to generate LC_{50} values and slopes of probit lines for larval mortality due to treatments. Untransformed means were presented in the data tables.

Results

Neonatal Larvae

The survival of root weevil larvae exposed as neonates to diet treated with Novodor was significantly reduced ($F = 9.36$; df = 4, 262; $P < 0.0001$) after 6 weeks. While 2 of 5 larvae survived after 6 weeks in the control group, only 1 larvae survived on average in the 300 ppm treatment (Table 1). The low survival rate observed for control larvae is addressed in the discussion section. The fresh weights of surviving larvae were significantly reduced ($F = 8.46$; df = 4, 262; $P < 0.0001$) by treatments indicating that larval feeding or nutrient assimilation may have been inhibited. Surviving larvae in the 300 ppm treatment weighed 53% less than those in the control group (Table 1).

5-Week Old Larvae

Insect diet treated with Novodor caused a significant increase ($F = 76.57$; df = 4, 20; $P < 0.0001$) in mortality of larvae exposed at the age of 5 weeks. Mortality of larvae exposed to the 3 ppm treatment (44%) was significantly ($P \leq 0.05$) greater than that of the controls (15%). Larval mortality in the 30 ppm treatment exceeded 50% (Table 2). The calculated LC_{50} for larvae in this age group was 6.2 (95% FL = 2.7-13.2) ppm [AI]. The slope of the probit line was 2.29 (SE = 0.31) ($\chi^2 = 43.07$; df = 1; $P < 0.0001$). Treatments also caused a significant reduction ($F = 6.25$; df = 4, 20; $P = 0.0020$) in the mean death ages of larvae. The death ages for larvae maintained on treated diets ranged from 111 to 128 d while that of control larvae was 154 d. Larvae in the 30 and 300 ppm treatments died significantly ($P \leq 0.05$) earlier than the control larvae (Table 2). The mortality response we observed occurred later than is typical for insects susceptible to *B. thuringiensis*; nevertheless, the response was consistent, dose-dependent, and occurred earlier than that observed for the controls. We observed that disease and death occurred in treated insects during the later stages of larval development and early phases of pupation (Fig. 1). The presence of *Btt* was confirmed by isolating the bacteria from internal tissues of dead larvae. Bacterial isolates were cultured in 40 ml LB broth in a 125 ml baffled flask maintained in an incubator shaker at 28°C and 200 rpm until sporulation phase. Cultures were examined using Hoffman contrast microscopy at 1000× magnification to identify *Btt* endospores and δ-endotoxin crystals. The mean fresh weights of adults that eclosed on treated diets also were significantly reduced ($F = 3.39$; df = 4, 20; $P = 0.0286$). Adult weevils that survived the 300 ppm treatment weighed 15% less than control adults, indicating that inhibition of feeding or disruption of nutrient assimilation may have occurred among survivors of treatments (Table 2).

12-Week Old Larvae

A significant increase ($F = 8.82$; df = 4, 8; $P < 0.0050$) in mortality was observed for 12-week old larvae fed Novodor treated diet. Mortality in the 30 ppm treatment (53%) was significantly ($P \leq$

TABLE 1. SURVIVAL AND AVERAGE FRESH WEIGHT OF *D. ABBREVIATUS* LARVAE EXPOSED AS NEONATES (5 PER DIET CUP) FOR 6 WEEKS TO INSECT DIET INCORPORATED WITH NOVODOR.

Treatment (ppm AI)[a]	Number of surviving larvae ± SE (n = 3)[b]	Weight (mg) of surviving larvae ± SE (n = 3)[b]
0.0	2.0 ± 0.1 a	306.3 ± 22.4 a
0.3	1.9 ± 0.1 a	251.8 ± 16.7 a
3.0	1.8 ± 0.1 a	236.1 ± 21.5 a
30.0	1.6 ± 0.1 a	231.1 ± 20.5 a
300.0	1.0 ± 0.1 b	144.0 ± 20.9 b

[a]AI refers to the concentration (μg/ml) of active ingredient, comprising spores and δ-endotoxin of *B. thuringiensis* var. *tenebrionis*, in prepared diet.
[b]Means within a column sharing the same letter were not significantly different ($P > 0.05$, Tukey's studentized range test [SAS Institute 1990]).

TABLE 2. MORTALITY AND AGE AT DEATH OF LARVAE, AND FRESH WEIGHT OF ECLOSED ADULTS, FOR *D. ABBREVIATUS* EXPOSED AS 5-WEEK-OLD LARVAE TO INSECT DIET INCORPORATED WITH NOVODOR.

Treatment (ppm AI)[a]	% Mortality ± SE (n = 6)[b,c]	Death age (d) ± SE (n = 6)[b,c]	Adult wt. (mg) ± SE (n = 6)[b,c]
0.0	15.3 ± 4.4 a	154.3 ± 14.5 a	315.0 ± 12.0 a
0.3	30.3 ± 4.3 a	127.9 ± 10.0 ab	329.2 ± 23.8 ab
3.0	43.7 ± 5.8 b	126.9 ± 8.6 ab	323.2 ± 15.2 ab
30.0	58.1 ± 4.9 c	114.4 ± 8.1 b	294.3 ± 9.0 ab
300.0	71.0 ± 4.4 d	110.6 ± 2.9 b	266.9 ± 19.5 b

[a]AI refers to the concentration ((g/ml) of active ingredient, comprising spores and δ-endotoxin of *B. thuringiensis* var. *tenebrionis*, in prepared diet.
[b]Mortality, age at death, and fresh weights of eclosed adults were assessed twice weekly during the experiment.
[c]Means within a column sharing the same letter were not significantly different ($P > 0.05$, Tukey's studentized range test [SAS Institute 1990]).

0.05) greater than that for the control group (6%) (Table 3). The calculated LC_{50} for larvae in this age group was 25.4 (95% FL = 12.5-60.0) ppm [AI]. The slope of the probit line was 2.75 (SE = 0.44) ($\chi^2 = 47.37$; df = 1; $P < 0.0001$). The mean age at which mortality occurred also was significantly reduced by treatments ($F = 4.54$; df = 4, 8; $P = 0.0330$). The mean death age for larvae in the 300 ppm treatment (130 d) was significantly ($P \leq 0.05$) less than that for control larvae (153 d) (Table 3). Treatments did not significantly affect the fresh weights of eclosed adults in this age group ($F = 1.34$; df = 4, 8; $P = 0.3341$), likely because larvae used in the experiment had attained near maximum weights before exposure to treatments.

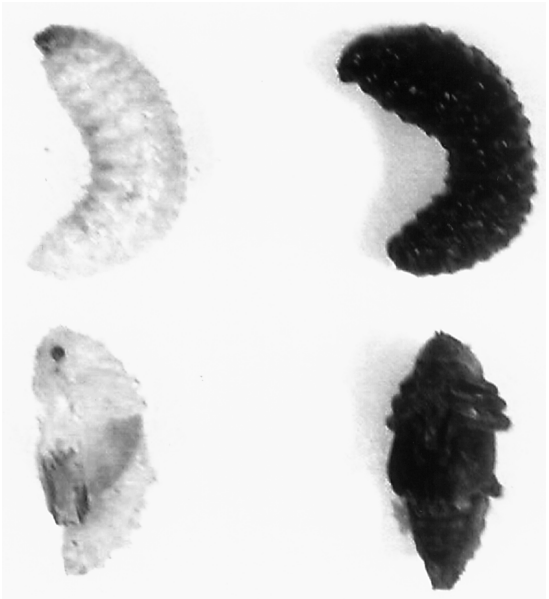

Fig. 1. Healthy (left) and diseased (right) larvae and pupae of *D. abbreviatus* due to feeding on diet incorporated with Novodor.

Soil Treatments

Survival of neonates on potted citrus was significantly reduced ($F = 4.87$; df = 3, 15; $P < 0.0146$) by soil treatments with Novodor. Larval survival was significantly ($P \leq 0.05$) less in all treatments as compared to the controls after 6 weeks exposure to treated soils and citrus roots (Table 4). The low rate of survival for control larvae is addressed in the discussion section. In contrast to the results of the diet bioassay against neonates, the fresh weights of surviving larvae were not significantly reduced ($F = 1.02$; df = 3, 15; $P = 0.4098$) in the potted citrus bioassay (Table 4). Some larvae may have been able to avoid exposure to *Btt* where soil drenches resulted in unequal dispersion of treatments.

DISCUSSION

The results of our experiments indicate that neonatal, 5-week-old, and 12-week-old larvae of *D. abbreviatus* were susceptible to the biological effects of Novodor. Activity for *B. thuringiensis* against older larval stages of insects is not normally expected; nevertheless, the dose-dependent mortalities we observed for larvae exposed at 5 and 12 weeks old were reproducible.

The low survival rates we observed for neonates in our controls are typical in experiments such as this, where multiple larvae are collectively grouped to challenge plants or generate data for bioassays of diet-incorporated materials. Low survival rates for neonatal *D. abbreviatus* have been reported by others (Schroeder & Sieburth 1997, Quintella & McCoy 1997), and are due to natural mortality factors including aggressive interactions among larvae confined together (Lapointe & Shapiro 1999).

The response we observed for older larvae to Novodor may be attributed to the finding that midgut trypsin activity increases to a maximum in *D. abbreviatus* larvae at the age of approximately 7 weeks (Yan et al. 1999). Yan et al. also inferred that the midgut of *D. abbreviatus* larvae

TABLE 3. MORTALITY AND AGE AT DEATH OF LARVAE, AND FRESH WEIGHT OF ECLOSED ADULTS, FOR *D. ABBREVIATUS* EXPOSED AS 12-WEEK-OLD LARVAE TO INSECT DIET INCORPORATED WITH NOVODOR.

Treatment (ppm AI)[a]	% Mortality ± SE (n = 3)[b,c]	Death Age (d) ± SE (n = 3)[b,c]	Adult Wt. (mg) ± SE (n = 3)[b,c]
0.0	5.6 ± 5.6 a	153.1 ± 7.4 a	353.0 ± 6.5 a
0.3	21.1 ± 13.1 ab	132.0 ± 14.9 ab	331.2 ± 22.2 a
3.0	31.1 ± 21.5 abc	141.3 ± 7.8 ab	317.6 ± 32.3 a
30.0	53.3 ± 15.4 bc	134.8 ± 10.8 ab	338.5 ± 1.3a
300.0	67.8 ± 12.5 c	130.1 ± 9.4 b	374.6 ± 13.5 a

[a]AI refers to the concentration (μg/ml) of active ingredient, comprising spores and δ-endotoxin of *B. thuringiensis* var. *tenebrionis*, in prepared diet.
[b]Mortality, age at death, and fresh weights of eclosed adults were assessed twice weekly during the experiment.
[c]Means within a column sharing the same letter were not significantly different ($P > 0.05$, Tukey's studentized range test [SAS Institute 1990]).

is probably alkaline, as shown for many Lepidoptera and Diptera, since the enzyme activity observed was most active at a pH of 10.4. Alkaline solubilization and proteolytic cleavage by serine proteases, such as trypsin, are required for activation of many *B. thuringiensis* δ-endotoxins (Pietrantonio et al. 1993) including those produced by *Btt*. The Cry3a toxin of *Btt* may undergo substantial cleavage at the N-terminus without loss of biological activity (Carroll et al. 1989). It has been suggested that these additional protease cleavage sites may facilitate insertion of a portion of the protein into the target membrane of the midgut epithelium (Knowles 1994) and subsequent pore formation. The mortality responses we observed for larvae treated at 5 and 12 weeks old may have been a product of appropriate levels of pH and serine proteases in midgut tissues during those stages of development. The delayed period to larval death may have been a function of the ability of *D. abbreviatus* to survive for long periods without food or water (personal observation) and the slow development of disease. Any future evaluation of *Btt* on larval *D. abbreviatus,* and possibly other insects, should consider the importance of suitable midgut environments for endotoxin activation and the general hardiness of the insect.

We observed dose-dependent responses for mortality, age at death, and fresh weights of adult survivors from larvae treated at the age of 5 weeks. Although the mortality response developed slowly, the mean ages at death were consistently earlier among treated as compared to control larvae. Also, the fresh weights of adults that survived treatments were reduced compared to those of the control, indicating that midgut damage or feeding inhibition may have occurred among larvae that survived treatments.

A dose-dependent mortality response also was observed for larvae treated at the age of 12 weeks. Larvae treated at this stage of development often died as pupae, further supporting the contention that disease was slow to develop. The fresh weights of the adults that developed from larvae treated at 12 weeks old were not affected by treatments. Lapointe (2000) reported that larval weights of *D. abbreviatus* were maximum at 12 weeks old, and then declined during further development, thus explaining the lack of a weight response for larvae in this age group.

There is much yet to be learned about the activation and binding patterns of *B. thuringiensis* endotoxins, and the subsequent onset of disease in susceptible insects. We observed that disease was slow to develop in *D. abbreviatus*. The results of our experiment indicate that there may be age-related factors that influence larval *D. abbreviatus* response to *Btt* endotoxins. There may also be other endotoxins awaiting discovery that are better adapted to activation and binding by larval *D. abbreviatus*. This report provides the impetus to further explore these processes in the Dia-

TABLE 4. SURVIVAL AND MEAN FRESH WEIGHT OF *D. ABBREVIATUS* LARVAE EXPOSED AS NEONATES (20 PER PLANT) FOR 6 WEEKS TO POTTED CITRUS TREATED WITH NOVODOR.

Treatment (ppm AI)[a]	Number of surviving larvae ± SE (n = 6)[b]	Weight (mg) of surviving larvae ± SE (n = 6)[b]
0.0	4.2 ± 1.1 a	52.0 ± 22.5 a
3.0	1.7 ± 0.3 b	40.7 ± 9.9 a
30.0	1.5 ± 0.4 b	19.8 ± 6.6 a
300.0	1.3 ± 0.3 b	41.2 ± 12.1 a

[a]AI refers to the concentration (μg/ml) of active ingredient, comprising spores and δ-endotoxin of *B. thuringiensis* var. *tenebrionis*, in prepared diet.
[b]Means within a column sharing the same letter were not significantly different ($P > 0.05$, Tukey's studentized range test [SAS Institute 1990]).

prepes root weevil and other insects. Our demonstration of Novodore activity against different larval stages of *D. abbreviatus* is encouraging enough to warrant additional testing.

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