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Source: Florida Entomologist, 89(4) : 441-448

Published By: Florida Entomological Society

URL: https://doi.org/10.1653/0015-4040(2006)89[441:AOBTIA]2.0.CO;2

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ACTIVITY OF *BACILLUS THURINGIENSIS* ISOLATES AGAINST *DIAPREPES ABBREVIATUS* (COLEOPTERA: CURCULIONIDAE)

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ABSTRACT

A collection of *Bacillus thuringiensis* isolates plausibly active against coleopteran insects was obtained from the Agricultural Research Service Culture Collection. Each isolate was cultured, spores and δ-endotoxin *cry*stals were pelleted by centrifugation and lyophilized, and the resulting product was incorporated in insect diet for testing against *Diaprepes abbreviatus* neonates. A bioassay method was developed that utilized small amounts of insect diet and *B. thuringiensis* spores and δ-endotoxin to treat single neonates confined to 0.2-mL clear polymerase chain reaction (PCR) tubes. The method was less expensive in terms of labor and materials as compared to previous methods and reduced control losses due to burrowing and aggressive behaviors of *D. abbreviatus* larvae confined together. Of 19 *B. thuringiensis* isolates screened for activity against *D. abbreviatus* with a discriminating dose of 250 ppm spores and δ-endotoxin on diet, 5 were selected for further evaluation in dose-response experiments. *Diaprepes abbreviatus* larvae demonstrated a significant dose response to 4 of the 5 isolates tested. The most active isolates were those that expressed *Cry*ET33 and *Cry*ET34, or *Cyt*2Ca1 proteins. A wild-type *B. thuringiensis* strain that expressed *Cyt*2Ca1 generated the lowest LC_{50} value (50.7 µg/ml) and steepest slope (1.11) based on log10 probit analysis of the data. These *B. thuringiensis* δ-endotoxins may have utility in transgenic approaches to citrus rootstock protection from *D. abbreviatus*.

Key Words: *Diaprepes abbreviatus*, *Bacillus thuringiensis*, *Cry*, *Cyt*, endotoxin, citrus

RESUMEN

Una colección de aislamientos de *Bacillus thuringiensis* posiblemente activos contra insectos del orden Coleóptera fue obtenido de la Colección de Cultivos del Servicio de Investigación Agrícola (USDA, ARS). Cada aislamiento fue criado, las esporas y cristales de endotoxina-δ fueron sedimentados por una centrifugadora y liofilizado (congelado y secado), y el producto resultante fue incorporado en una dieta de insectos para probarlo contra los neonatos (larvas recién nacidas) del *Diaprepes abbreviatus*. Un método de bioensayo fue desarrollado para utilizar cantidades pequeñas de la dieta de insectos, esporas de *B. thuringiensis* y la endotoxina-δ para tratar individualmente los neonatos confinados en frascos claros de 0.2 ml para la reacción en cadena por la polimerasa (RCP). Este metodo fue menos costoso en terminos de mano de obra y materiales comparado con los metodos de control usados anteriormente para reducir las perdidas asociadas al comportamiento minador y agresivo de las larvas juntamente confinadas de *D. abbreviatus*. De los 19 aislamientos de *B. thuringiensis* evaluados con actividad contra *D. abbreviatus* con una dosis de 250 ppm de esporas y endotoxin-δ sobre la dieta, 5 fueron seleccionados para evaluación adicional en experimentos de respuesta de dosis. Las larvas de *Diaprepes abbreviatus* demonstraron una respuesta de dosis significativa en 4 de los 5 aislamientos probados. Los aislados mas activos fueron los que expresaron las proteinas *Cry*ET33 y *Cry*ET34, o *Cyt*2Ca1. Una raza de tipo-silvestre de *B. thuringiensis* que expreso *Cyt*2Ca1 produjó el valor menor de CL_{50} (50.7 µg/ml) y el pendiente empinado (1.11) basado en el análisis de datos usando el probit de log10. Las endotoxinas-δ de *B. thuringiensis* puede ser útiles en un enfoque transgénico para la protección de rizomas citricas contra el *D. abbreviatus*.

The invasive weevil species, *Diaprepes abbreviatus* (L.), has become one of the most damaging insect pests of citrus and nursery crops in Florida since it was first reported in 1964 (Woodruff 1964). Prior to its introduction into the continental United States, *D. abbreviatus* was known to be a serious pest of sugarcane in the Lesser Antilles and is considered the most important pest of agriculture, horticulture, and silviculture in Puerto Rico (Hantula et al. 1987). Efforts of researchers and pest managers to develop an effective long-term management strategy for this pest have been unsuccessful. Apparently, *D. abbreviatus* is not under effective biological control within its putative native range of Puerto Rico and the Lesser Antilles (Lapointe 2004). The lack of natural enemies, combined with the wide host range of this highly polyphagous weevil (Simpson et al. 1996) and its slow subterranean larval development (Lapointe 2000), makes *D. ab-*

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breviatus particularly well adapted to semi-permanent, tropical, and subtropical agroecosystems such as citrus groves. Efforts to identify plant resistance to *D. abbreviatus* within sexually compatible citrus germplasm have been only marginally successful (Bowman et al. 2001; Lapointe & Bowman 2002; Shapiro et al. 2000).

The bacterial entomopathogen, *Bacillus thuringiensis* (Berliner), has been recommended for the control of other insect pests of citrus, particularly those in the order Lepidoptera (Shapiro et al. 1998; Stansly et al. 2006). Although use of *B. thuringiensis* as an applied biopesticide can be an effective control method for some insects, the subterranean feeding habits of *D. abbreviatus* larvae make them difficult to target with a biocontrol agent, such as *B. thuringiensis*, that must be ingested to be effective. It has been suggested that the most appropriate and economically viable method for control of *D. abbreviatus* will be the production of transgenic rootstocks engineered to express exogenous toxins (Lapointe 2004). Al-Deeb & Wilde (2005) reported that transgenic corn, expressing the *Cry*3bb1 toxin from *B. thuringiensis*, was protected from another root-feeding coleopteran, the western corn rootworm, *Diabrotica virgifera virgifera* LeConte. Transgenic crops that express *B. thuringiensis* proteins display resistance to some of the most devastating pests of agriculture, yet are virtually safe to nontarget organisms (Betz et al. 2000). A transgenic approach that uses a genetically-engineered citrus rootstock to express a δ-endotoxin active against *D. abbreviatus* is a plausible solution.

Currently, the few *B. thuringiensis* δ-endotoxins known to be active against coleopterans are far outnumbered by known lepidopteran-active toxins. In order to pursue this paradigm toward the development of a transgenic citrus rootstock, *B. thuringiensis* toxins that are active against *D. abbreviatus* larvae must first be identified. One strain of *B. thuringiensis* has been reported to cause mortality of *D. abbreviatus* larvae (Weathersbee et al. 2002), but otherwise *B. thuringiensis* has received minimal attention as a potential biocontrol agent for this pest. We assembled a collection of *B. thuringiensis* isolates that expressed novel δ-endotoxins putatively active against one or more representatives of Coleoptera. This paper presents the results of experiments that determined if any of these toxins were active against *D. abbreviatus* larvae.

Materials and Methods

Source and Culture of *B. thuringiensis* Isolates

Patent databases at the United States Patent and Trademark Office were searched to locate *B. thuringiensis* isolates potentially active against species of Coleoptera. Representative samples of 19 isolates, for which patents had issued (Bradfisch et al. 2005; Donovan et al. 2005; Narva et al. 2005; Rupar et al. 2003; Rupar et al. 2004; Soares et al. 1989), were obtained by request from the curator of the Agricultural Research Service Culture Collection, National Center for Agricultural Utilization Research, Peoria, Illinois (Table 1). Samples were received as dried pellets sealed in glass ampoules. Growing cultures of each isolate were established following instructions provided with the samples. Briefly, an ampoule was scored with a file and broken. The broken end of the ampoule was flame sterilized, the pellet was removed and then cultured in Luria-Bertani (LB) broth in an incubator shaker. Cultures were stored on LB agar slants at 4°C and in LB broth/glycerol stock solutions at -80°C until needed.

Starter cultures of each isolate were prepared by inoculating 10 mL of LB broth with a loopful of bacterial cells removed from storage. Cultures were grown overnight in 125-mL baffled Erlenmeyer flasks at 27ºC and 150 rpm in an incubator shaker. Then 80 µl of starter culture were added to 100 mL of LB broth in a 500-mL baffled Erlenmeyer flask and grown in the incubator shaker until approximately 90% of the cultured cells had sporulated and autolyzed. Cultures were pelleted by centrifugation (15,000 G) for 15 min at 4°C, washed 3 times with phosphate buffered saline (PBS) containing 0.005% Triton X-100, lyophilized, and weighed. Lyophilized pellets, containing *B. thuringiensis* spores and δ-endotoxin *cry*stals, were stored in 1.5-mL microcentrifuge tubes at -80°C until they were used in the experiments.

Insect Source and Rearing

Neonatal larvae of *D. abbreviatus* were obtained from a laboratory colony maintained at 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). Temperature and moisture content of the diet were optimized for larval development according to Lapointe (2000) and Lapointe & Shapiro (1999). Neonate larvae were surface sterilized with a solution of 0.31% sodium hypochlorite and individually inspected to insure only healthy and active larvae were used in the experiments.

Bioassay Method

A bioassay method was developed to test the effectiveness of *B. thuringiensis* isolates on individual *D. abbreviatus* neonates. A clear polymerase chain reaction (PCR) tube containing a small amount of insect diet in the lid was used to hold a single *D. abbreviatus* neonate that could be visually inspected for response to treatments. Monitoring of larvae had not been possible with

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TABLE 1. SCREENING OF *B. THURINGIENSIS* ISOLATES AGAINST *D. ABBREVIATUS* NEONATES WITH A DISCRIMINATING DOSE (250 µG/ML) OF LYOPHILIZED, SPORULATED CULTURES IN DIET.

Isolate[a] number	Isolate description	Endotoxins[b] present	Genbank accession[c]	% Mortality ± SE (n=90)	
				Treated[d]	Control
B-21367	recombinant	*Cry*ET33	AAF76375	91.3 ± 3.0*	20.0 ± 10.1
		*Cry*ET34	AAF76376		
B-21365	wild-type	*Cry*ET33	AAF76375	90.3 ± 6.7*	20.0 ± 10.1
		*Cry*ET34	AAF76376		
B-21366	recombinant	*Cry*3Bb2	AAA74198	87.7 ± 4.7*	20.0 ± 10.1
		*Cry*ET33	AAF76375		
		*Cry*ET34	AAF76376		
B-21582	wild-type	*Cyt*2Ca1	AAK50455	81.7 ± 7.5*	11.5 ± 5.9
B-21583	recombinant	*Cyt*2Ca1	AAK50455	52.3 ± 2.9*	0.0 ± 0.0
B-21784	wild-type	*Cry*35Aa2	AAK64561	20.3 ± 8.8	3.0 ± 0.0
		*Cry*34Aa2	AAK64560		
		*Cry*38Aa1	AAK64559		
B-21783	wild-type	*Cry*35Aa2	AAK64561	15.7 ± 5.9	2.0 ± 1.0
		*Cry*34Aa2	AAK64560		
		*Cry*38Aa1	AAK64559		
B-21915	wild-type	*Cry*35Ba1	AAK64566	15.7± 7.0	4.3 ± 1.3
		*Cry*34Ba1	AAK64565		
		*Cry*ET84	AAK64564		
B-21554	wild-type	*Cry*35Ac1	AAG50117	14.3 ± 1.3*	3.0 ± 0.0
		*Cry*34Ac1	AAG50118		
B-21787	recombinant	*Cry*36Aa1	AAK64558	12.0 ± 4.9	1.0 ± 1.0
B-21786	wild-type	*Cry*36Aa1	AAK64558	11.3 ± 3.0*	2.3 ± 2.3
B-21788	recombinant	*Cry*35Ab2	AAK64563	10.0 ± 4.0	3.0 ± 0.0
		*Cry*34Ac2	AAK64562		
B-18765	wild-type	*Cry*5Ba1	AAA68598	8.7 ± 3.0	1.0 ± 1.0
		*Cry*5Ac1	P56955		
B-21916	recombinant	*Cry*35Ba1	AAK64566	7.7 ± 2.9	3.0 ± 0.0
		*Cry*34Ba1	AAK64565		
		*Cry*ET84	AAK64564		
B-21785	wild-type	*Cry*35Ab2	AAK64563	6.7 ± 2.0	0.0 ± 0.0
		*Cry*34Ac2	AAK64562		
B-18243	wild-type	*Cry*5Aa1	AAA67694	5.7 ± 1.3	2.0 ± 1.0
		*Cry*5Ab1	AAA67693		
B-21553	wild-type	*Cry*35Ab1	AAG41672	4.3 ± 1.3	1.0 ± 1.0
		*Cry*34Ab1	AAG41671		
B-18244	wild-type	*Cry*12Aa1	AAA22355	1.0 ± 1.0	0.0 ± 0.0
B-18679	wild-type	*Cry*14Aa1	AAA21516	1.0 ± 1.0	1.0 ± 1.0
		*Cry*35Aa1	AAG50342		
		*Cry*34Aa1	AAG50341		

[a]Isolate numbers were assigned by curators of the ARS Culture Collection, National Center for Agricultural Utilization Research (formerly the Northern Regional Research Laboratory), Peoria, Illinois USA.

[b]Endotoxins labeled *Cry*ET## have not yet been assigned names recognized by the *B. thuringiensis* δ–endotoxin nomenclature committee.

[c]Protein accessions can be retrieved from the National Center for Biotechnology Information, Genbank at http://www.ncbi.nlm.nih.gov/.

[d]Means for *D. abbreviatus* percent mortality in the treated group marked by an asterisk (*) were ($P \leq 0.05$, paired t-test) greater than those for the control group.

older bioassay methods that used larger volumes of medium (soil or diet) because *D. abbreviatus* larvae burrow into the medium, complicating visual inspection.

All items used in the bioassay procedure were sterilized by autoclaving, filtering, or treating with 75% ethanol, and the procedure was conducted in a biological safety cabinet. A stock solution was prepared that contained 5% sucrose and 0.005% Triton X-100 in deionized distilled water. The previously prepared lyophilized pellets of each *B. thuringiensis* isolate were resuspended in

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the stock solution and diluted with stock to provide a discriminating dose of spores and δ-endotoxin in diet of 250 ppm (µg/mL) for screening experiments. Dose response assays were conducted with isolates that caused >50% mortality of neonates at the discriminating dose level. Concentrations of 300, 150, 75, and 32.5 ppm of spores and δ-endotoxin in diet were used in the dose-response experiments. Diet treatments for the controls received stock solution only.

Prepared insect diet was liquefied by reheating and 80 µl of diet were pipetted onto the inside surface of the lid of a 0.2-mL clear PCR tube. The diet pellets were dried for 15 min to remove approximately 20 µl of water. *Bacillus thuringiensis* treatments were applied in a volume of 20 µl by pipette to each diet pellet and the pellets were dried for an additional 5 min. Controls were treated equally with stock solution only. A #1 fine camel hair brush was used to place a single *D. abbreviatus* neonate into each PCR tube containing diet and the lid was affixed. The PCR tubes were inverted and placed in a tube rack, covered, placed in a sealed plastic bag with a moist paper towel, and stored in an incubator at 27°C. After 2 weeks, each larva was inspected with the aid of a dissecting microscope and mortality was recorded. There were 3 replications, each containing 30 larvae, for the initial screening of each isolate at 250 ppm. A minimum of 3 replications, each with 30 larvae, was used for each level of treatment in the dose-response experiments.

Data Analyses and Statistics

Data collected from the screening experiments were subjected to the Means Procedure (SAS Institute 1999) to determine means and standard errors for mortality of *D. abbreviatus* neonates exposed to the discriminating dose of each isolate. Paired *t*-tests were conducted using the *T*-test Procedure (SAS Institute 1999) to determine if means for mortalities in treated groups differed from those of control groups. A probability level of 5 percent ($P \leq 0.05$) was considered significant.

Data from the dose-response experiments were adjusted for control mortality by the Abbott (1925) formula and transformed (arcsine) before analyses. Transformed data were analyzed by the General Linear Models Procedure, and differences among treatment level means were determined by Tukey's studentized range test (SAS Institute 1999). Differences among means were considered significant at a probability level of 5 percent ($P \leq 0.05$). Untransformed means are presented in the data tables. Data from isolates that elicited a significant response to treatment were subjected to log10 Probit analyses by the Probit Procedure (SAS Institute 1999) to generate LC_{50} values and slopes of probit lines.

RESULTS

Screening Experiments

Of 19 *B. thuringiensis* isolates screened in diet bioassays against *D. abbreviatus* neonates, 7 caused significantly greater ($P \leq 0.05$, paired *t*-tests) mortality compared with the controls (Table 1). Isolates B-21365, B-21366, and B-21367 containing *Cry*ET33 and *Cry*ET34 toxins caused the highest observed mortalities (90, 88, and 91%, respectively). Isolates B-21582 and B-21583 containing the *Cyt*2Ca1 toxin provided 82 and 52% mortalities, respectively. These five isolates (B-21365, B-21366, B-21367, B-21582, and B-21583) provided meaningful levels of mortality (>50%) and were further evaluated in dose-response experiments.

Dose-Response Experiments

A significant effect of spore and δ-endotoxin dose was observed for 4 of the 5 isolates that were subjected to dose-response experiments against *D. abbreviatus* neonates, including B-21365 (F = 15.52; df = 4, 28; $P < 0.0001$), B-21367 (F = 9.46; df = 4, 28; $P < 0.0001$), B-21582 (F = 33.63; df = 4, 8; $P < 0.0001$), and B-21583 (F = 56.60; df = 4, 16; $P < 0.0001$). The highest corrected mortality observed in the dose-response experiments was 81% provided by the wild-type isolate B-21582 at a dose of 300 µg spores and δ-endotoxin/ml diet (Table 2). Recombinant isolates B-21367 and B-21583 also elicited good dose-responses with greater than 60% mortality of *D. abbreviatus* larvae observed at the 300 ppm dose. The effect of spore and δ-endotoxin dose on larval mortality was not significant for isolate B-21366 (F = 1.71; df = 4, 8; P = 0.2406). The dose-response obtained with isolate B-21366 was inconsistent, the response data were variable compared to those of the other isolates, and larval mortality obtained at the highest dose remained below 50%. Consequently, isolate B-21366 was not included in subsequent probit analyses.

Results obtained for isolates B-21365, B-21367, B-21582, and B-21583 were examined further by log10 probit analyses to model the effects of spore and δ-endotoxin dose on mortality of *D. abbreviatus* larvae (Fig. 1). The calculated LC_{50} for larvae exposed to B-21365 in diet was 258.3 (95% FL = 130.5-2779) ppm [AI]. The slope of the probit line was 0.65 (SE = 0.23) (χ_2= 8.11; df = 1; P = 0.0044) (Fig. 1A). The LC_{50} for larvae exposed to B-21367 was 115.3 (95% FL = 40.4-269.5) ppm [AI] and the slope of the probit line was 0.93 (SE = 0.31) (χ^2= 8.79; df = 1; P = 0.0030) (Fig. 1B). The LC_{50} for larvae exposed to B-21582 was 50.7 (95% FL = 28.3-72.1) ppm [AI] and the slope was 1.11 (SE = 0.21) (χ^2= 27.10; df = 1; $P \leq 0.0001$) (Fig. 1C). The calculated LC_{50} for larvae exposed to B-21583 was 174.1 (95% FL = 114.6-361.2) ppm [AI] and

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TABLE 2. MORTALITY OF *D. ABBREVIATUS* NEONATES EXPOSED TO DIET TREATED WITH DIFFERING RATES OF LYOPHILIZED, SPORULATED CULTURES OF *B. THURINGIENSIS* ISOLATES.

	% Mortality ± SE [b] by isolate[c]				
Dose (µg/ml AI)[a]	B-21365	B-21366	B-21367	B-21582	B-21583
0.0	6.7 ± 2.2 a	11.1 ± 2.2 a	17.5 ± 3.1 a	7.8 ± 2.9 a	1.3 ± 1.3a
32.5	26.3 ± 6.7 b	21.1 ± 12.8 a	31.3 ± 7.4 ab	40.6 ± 7.0 b	31.9 ± 3.0 b
75.0	39.4 ± 6.8 bc	29.9 ± 3.5 a	40.9 ± 8.7 ab	58.7 ± 6.5 bc	35.0 ± 6.9 b
150.0	40.2 ± 6.3 bc	28.6 ± 6.6 a	50.3 ± 10.0 bc	68.2 ± 7.2 c	44.1 ± 3.3 bc
300.0	52.9 ± 6.8 c	41.8 ± 16.4 a	67.8 ± 7.9 c	80.7 ± 4.3 c	61.6 ± 4.4 c

[a]AI refers to the active ingredient comprising lyophilized spores and δ-endotoxin of *B. thuringiensis* in diet.

[b]Means within a column sharing the same letter were not different ($P > 0.05$, Tukey's studentized range test [SAS Institute 1999]).

[c]Isolate numbers were assigned by curators of the ARS Culture Collection, National Center for Agricultural Utilization Research, Peoria, Illinois.

the slope was 0.79 (SE = 0.0.19) (χ^2= 17.96; df = 1; $P \leq 0.0001$) (Fig. 1D).

DISCUSSION

The subterranean habit, and aggressive behavior when confined together, complicate the evaluation of control measures for *D. abbreviatus* larvae (Lapointe & Shapiro 1999) and result in high levels of control mortality (Schroeder & Sieburth 1997; Quintella & McCoy 1997; Weathersbee et al. 2002). The bioassay method used here provided an efficient means for screening *B. thuringiensis* isolates against *D. abbreviatus* neonates. It was less expensive in terms of labor and materials than methods used in the past. The current method avoids these problems by confining larvae singly on a nominal amount of diet, thereby reducing losses in the control group. Control mortality in these experiments did not exceed 20% and was most often maintained below 10%.

The highest levels of mortality of *D. abbreviatus* in the screening experiments (90, 88, and 91%) occurred when neonates were fed diet containing spores and δ-endotoxin of isolates B-21365, B-21366, and B-21367, respectively. Donovan et al. (2005) has shown these isolates to be active against the larvae of other species of Coleoptera, including the red flour beetle, *Tribolium castaneum* (Herbst), and the Japanese beetle, *Popillia japonica* Newman. Isolate B-21365 is a wild-type *B. thuringiensis* strain that contains genes for *Cry*ET33 and *Cry*ET34 toxins. Isolate B-21367 is a recombinant strain that was engineered to express *Cry*ET33 and *Cry*ET34 toxins. This isolate was derived from a parent *B. thuringiensis* strain that was *cry*stal negative (*Cry*$^-$). Isolate B-21366 also is a recombinant strain engineered to express *Cry*ET33 and *Cry*ET34 toxins, but the parent strain was wild-type and naturally expressed the *Cry*3Bb2 toxin. Although B-21366 expressed an additional δ-endotoxin compared to B-21365 and B-21367, it performed no better against *D. abbreviatus* than those isolates in the screening experiments, and its performance was inconsistent in the dose-response experiments. Perhaps expressing the *Cry*ET33 and *Cry*ET34 toxins in addition to *Cry*3Bb2 added a burden that affected overall toxin expression and the virulence of this strain. Because this isolate produced erratic results it was not further evaluated.

The wild-type isolate B-21365, expressing the *Cry*ET33 and *Cry*ET34 toxins, exhibited the shallowest dose-response curve and highest LC_{50} value of those tested, probably because the highest concentration of B-21365 tested provided 53% larval mortality. The recombinant isolate B-21367 expressing the same toxins displayed a steeper response curve, a lower LC_{50} value, and provided better predictions in the 50% response range. It is unclear why recombinant strain B-21367 apparently produced a more virulent product than the wild-type B-21365 since both expressed the same toxins. Perhaps B-21367 invests less energy into other processes such as spore formation and more into toxin production. Nonetheless, the dose responses displayed by the recombinant strain B-21367 and the wild type B-21365 against *D. abbreviatus* were not statistically different based on overlap of standard errors.

Isolates B-21582 and B-21583 provided 82 and 52% mortalities, respectively, in the screening experiments. Rupar et al. (2004) demonstrated these isolates were active against the larvae of representative species of Siphonaptera: including the cat flea, *Ctenocephalides felis* (Bouché); and Coleoptera: including the southern corn rootworm, *Diabrotica undecimpunctata howardi* Barber; the western corn rootworm, *Diabrotica virgifera virgifera* LeConte; the Colorado potato beetle, *Leptinotarsa decemlineata* (Say); the red flour beetle; and the Japanese beetle. Isolate B-21582 is a wild-type *B. thuringiensis* strain that contains the gene for the *Cyt*2Ca1 toxin, while recom-

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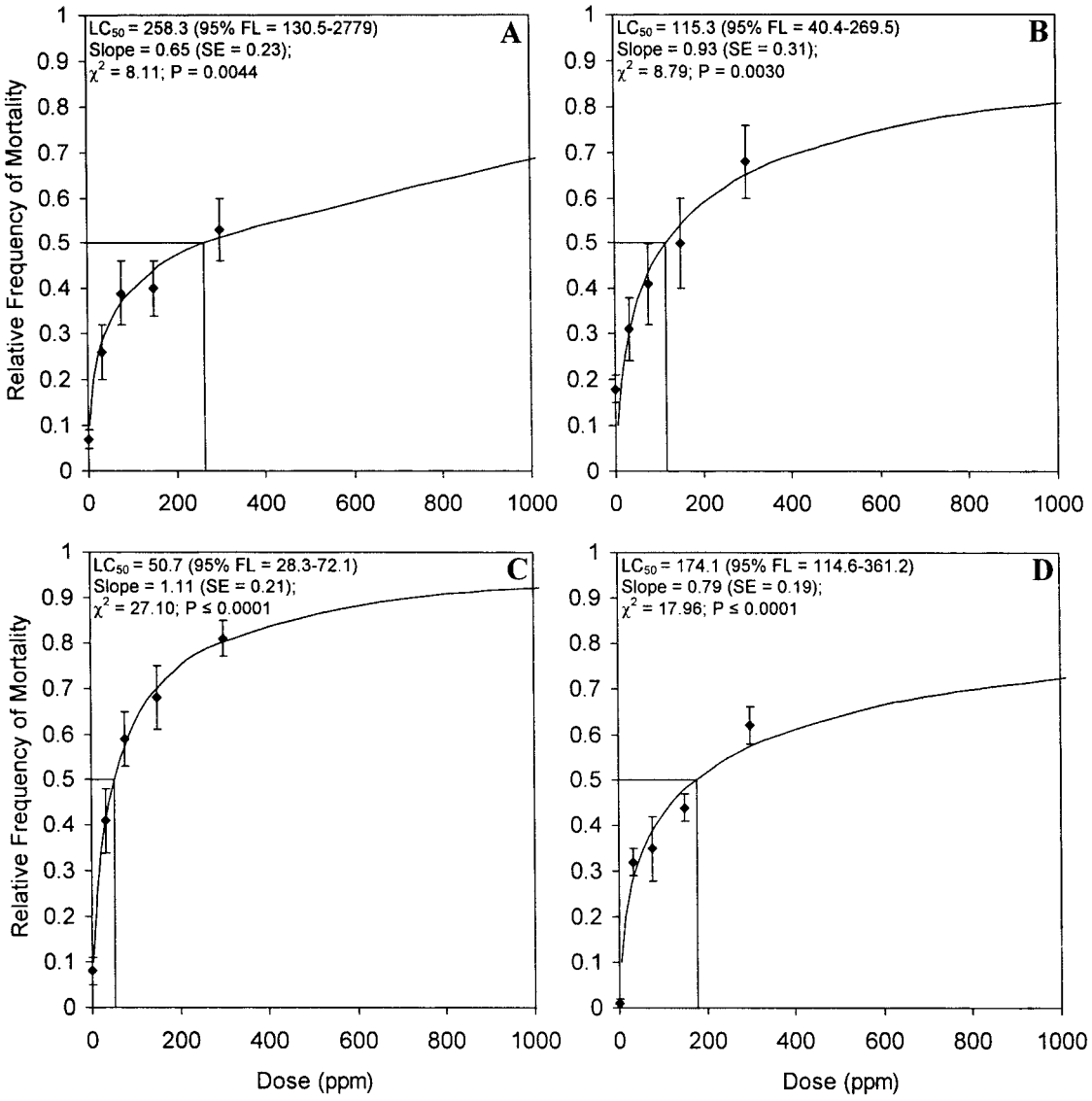

Fig. 1. Observed (♦ ± SE bars) values and predicted (line) dose response curves for *D. abbreviatus* neonates exposed to *B. thuringiensis* spores and δ-endotoxin in diet. LC_{50} values, slopes of log10 probit lines, and χ^2 values are shown for isolates B-21365 (A), B-21367 (B), B-21582 (C), and B-21583 (D).

binant strain B-21583 originally was a *Cry*⁻ *B. thuringiensis* that was engineered to express the *Cyt*2Ca1 toxin. The *Cyt*2Ca1 protein is a δ-endotoxin that fits into a second category, aside from the *Cry* toxins, known as *cyt*olitic (*Cyt*) toxins. The *Cyt* proteins, also known as hemolytic toxins, cause damage to the insect midgut through pore formation and cell lysis much like the *Cry* toxins. Guerchicoff et al. (2001) provides a discussion of the *Cyt* gene family, including similarities and differences with *Cry* genes.

The wild-type isolate B-21582, expressing the *Cyt*2Ca1 toxin, displayed the steepest dose-response curve and lowest LC_{50} value of those tested with the highest confidence in predictions. Though recombinant strain B-21583 also expressed *Cyt*2Ca1, the probit response was not as steep as that for B-21582, the LC_{50} value was greater, and the confidence in predicted values was lower. It appeared in this case that the wild-type isolate B-21582 produced a more virulent product against *D. abbreviatus* than did the recombinant strain B-21583 expressing the same toxin. Perhaps isolate B-21582 produces another, yet undetected product that works in conjunction with the *Cyt*2Ca1 protein to induce mortality in *D. abbreviatus* larvae.

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Bacillus thuringiesis products have been widely accepted in agriculture for the control of many insect pests, but only in recent years have strains been discovered that control coleopteran pests. Control strategies that rely on formulated *B. thuringiensis* applications are used world-wide in many crops. Transgenic plant varieties that express *B. thuringiensis* δ-endotoxins have been used in the U.S. for several years and are now gaining international acceptance (Betz et al. 2000). Unfortunately, transgenic approaches to plant improvement are not currently being exploited in some crops that could benefit most from this technology, such as citrus, where a genetically engineered rootstock could be used to alleviate damage caused by *D. abbreviatus*.

These experiments demonstrated that there are *B. thuringiensis* δ-endotoxin genes currently available that could be used to transform citrus for protection against *D. abbreviatus*. The *Cry*ET33, *Cry*ET34, and *Cyt*2Ca1 genes could be expressed together or separately in a citrus rootstock. An appropriately engineered citrus rootstock, if properly managed, has the potential to offer resistance to *D. abbreviatus* throughout the life of the crop.

Because *D. abbreviatus* has a broad host range and is known to feed on other plants within and around citrus groves (Lapointe 2003), the presence of toxin in citrus roots within a grove may be expected to result in increased feeding by larvae on alternative food sources (either wild or intentionally planted) and thus reduce the likelihood of rapid resistance development to the toxin. The use of transformed citrus rootstocks would also avoid concerns associated with the possible effect of pollen from transformed plants, particularly if the gene inserted into the rootstock is constructed to be expressed only in root tissue.

Development of this technology for citrus should not be delayed since introduction of genetically modified crops requires investments of time and money, and years to complete, particularly for slow-maturing crops like citrus. Moreover, an effective control strategy for *D. abbreviatus* is long overdue and alternatives to the proposed genetically-modified citrus rootstock have not been presented.

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