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Full Length Research Paper

Determination of insecticidal toxicity of three species of entomopathogenic spore-forming bacterial isolates against *Tenebrio molitor* L. (Coleoptera: Tenebrionidae)

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Bioassays were conducted using larvae of mealworms, *Tenebrio molitor,* to determine lethal concentration for five entomopathogenic strains of spore-forming bacteria. Lethal concentration was determined by feeding *T. molitor* larvae cabbage discs dipped in whole cell cultures of these five strains of bacteria. The strains of bacteria were isolates of *Bacillus cereus, Bacillus thuringiensis* and *Brevibacillus laterosporus.* An isolate of *B. cereus* required the highest concentration of viable spores (8.531×10^7 spores ml^{-1}) to achieve its LC_{50}, whereas an isolate of *B. laterosporus* required the lowest concentration of viable spores (3.388×10^6 spores ml^{-1}) to achieve LC_{50}.

Key words: Mealworms, spore-forming bacteria, bioassays.

INTRODUCTION

The first isolates of *B. thuringiensis* Berliner subspecies effective against coleopterans were isolated from a mealworm larva, *Tenebrio molitor* L. (Coleoptera: Tenebrionidae). This subspecies was subsequently named *B. thuringiensis* subsp. *tenebrionis* (Kriege et al., 1983). Another coleopteran specific strain was later isolated, *B. thuringiensis* subsp. *san diego* (Herrnstadt et al., 1986). These subsp. of *B. thuringiensis* are effective against one of the USA's most important potato pests, the Colorado potato beetle, *Leptinotarsa decemlineata* Say (Coleoptera: Chrysomelidae) (Nault and Kennedy, 1999). Colorado potato beetle has since been reported to have developed resistance to the Cry3A toxin (Whaldon et al., 1993). The development of resistance to pesticides is not uncommon in insects repeatedly subjected to a single pesticide, especially when only one toxin is involved (Ferré and Van Rie, 1992; Tabashnik, 1994).

Various other coleopteran specific *B. thuringiensis* strains have been isolated and are effective against scarabaeid beetles such as *Anomala cuprea* Hope (Coleoptera: Dynastidae), *A. rufocuprea* Motschulsky (Coleoptera: Dynastidae) and *Popillia japonica* Newman (Coleoptera: Scarabaeidae) (Ohba et al., 1992).

Bioassays on coleopterans are usually conducted on the Colorado potato beetle because these beetles are of economic importance and are a major pest in Asia, Europe and North America (Hare, 1990). These beetles are also the standard beetles used in bioassays to determine the International Units (IU) of toxicity (Navon, 2000). However, it is absent from South Africa, and therefore *T. molitor* was used as the test organism because it is readily available in South Africa and easy to rear (Hinze, 2000).

The aim of this research was to determine and compare the level of toxicity of five endospore-forming, entomopathogenic bacterial isolates, namely NDR1, NDR2, NDR3, NDR5 and NDR11.

MATERIALS AND METHODS

Samples

Bacterial isolates were obtained from diseased insects such as *T. molitor*, Coleoptera adults and larvae of *Schizonycha* spp.

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collected in sugarcane areas in KwaZulu-Natal (KZN), and debris from insect-rich environments such as compost, grain dust from grain storage bins and *T. molitor* cultures.

Sample collection

Mushroom compost was sampled from a mushroom farm (Karkloof, KwaZulu-Natal), compost from an urban garden (Hillcrest, KwaZulu-Natal) and grain dust was sampled from a chicken grain storage facility (Pietermaritzburg, KwaZulu-Natal). Diseased *T. molitor* larvae were collected from the insect rearing facility (University of KwaZulu-Natal). Adult beetles were collected from light traps in sugarcane areas in the KwaZulu-Natal midlands by employees of the South African Sugar Research Institute (SASRI, Mt. Edgcombe, KZN). White grubs were collected from sugarcane areas by staff members of SASRI and were delivered in plastic containers filled with soil. Diseased grubs were removed from these containers and placed into sterile Petri dishes. All samples were stored in a refrigerator kept at 4°C.

Isolation of endospore-forming bacteria

Isolation of *Bacillus* spp. was conducted using a similar pasteurization method to that described by Ohba and Aizawa (1978). Suspensions were shaken vigorously for 30 s at full speed on a vortex shaker. Mixed suspensions were then left to stand for 1 h at room temperature. Suspensions were then vortexed a second time at full speed for 30 s and then subjected to a pasteurization process in a preheated water bath for 10 min at 80°C. After pasteurization 0.1 ml aliquots of the suspensions were plated out onto nutrient agar (Biolab, Biolab Diagnostics, 259 Davidson Road, Wadeville, Johannesburg) (Thiery and Frachon, 1997).

Plates were incubated for 24 h at 30°C in an incubator and examined for colonies with typical *B. cereus* morphology (fast growing, rhizoid, irregular, raised, smooth, and opaque white) or 'colonies with an 'ice crystal' appearance wider than 2 mm (Damgaard et al., 1997; Prescott et al., 1999; Selvakumar et al., 2007). Various other white colored bacterial colonies larger than 2 mm in diameter were selected for microscopic evaluation. The number of colonies isolated from each isolate was estimated and counted using a colony counter. Selected colonies were then purified by sub-culturing onto nutrient agar plates and incubated for 3 days at 30°C in an incubator. Colonies were stored on nutrient agar slants. Not all colonies from each sample that fitted the description above were selected due to the large number of colonies formed.

Selected isolates were subjected to Gram staining as well as Coomassie Blue staining [Coomassie Blue stain 0.133% (w/v) and methanol 50% (v/v)] for 1 min. Slides were then rinsed gently for 30 s with distilled water and blotted dry with tissue paper (Ammons et al., 2002). Isolates were screened for the presence of crystal proteins using light microscopy with a 100 x oil immersion objective lens (Bernhard et al., 1997; Young et al., 1998). Crystal proteins stained as dark blue structures (Ammons et al., 2002). Isolates were screened for size of cells, spore morphology and the presence of crystal proteins. Isolates without crystal proteins, but with cells broader than 0.9 µm, were also selected. This was done in order to include *B. cereus,* which does not produce crystal proteins. Rod-shaped bacterial cells containing oval spores were selected because they fit the description of *B. cereus, B. thuringiensis* and *B. laterosporus* cells. Selected cultures were purified by sub-culturing and were assigned numbers NDR1-NDR14.

Multiple dose bioassay

A pure culture of *T. molitor* was obtained from a pet shop in Pietermaritzburg, KZN, SA. The culture was reared on a diet of commercial chicken meal (Meadow Feeds, P.O. Box 426, Pietermaritzburg, SA) (Hinze, 2000). Bran was not used because this contains phytic acid that affects the absorption of calcium which is and essential mineral for a healthy *T. molitor* culture (Hinze, 2000). *T. molitor* larvae were reared in 350 mm x 250 mm square plastic containers containing chicken meal to a depth of 70 mm. For moisture, potatoes skins, cabbage leaves and carrot peels were added once a week (Hinze, 2000). Carrots are an essential additive because most of the micronutrients required by *T. molitor* larvae are present in carrots (Hinze, 2000). Adults and pupae were removed carefully with forceps from the cultures on a regular basis and placed into in 350 mm x 250 mm square plastic containers containing chicken meal to a depth of 70 mm. After 14 mo of rearing, a population of *T. molitor* larvae was available that was large enough for the planned bioassays. It was not possible to determine the instars' stages because meal worms have between 10 and 14 instars, and several instars occur with larvae in the same size range (25 mm in length) (Anonymous, 2008a). Therefore, larvae sized between 18-20 mm were used in the bioassays.

Test organism preparation

T. molitor larvae were carefully removed from the media by gently sifting the chicken meal through a sieve. This procedure separated the *T. molitor* larvae and the chicken meal effectively. The *T. molitor* larvae, sized between 18-20 mm, were placed in a division of a plastic ice tray, for counting purposes, prior to being inoculated with the bacterial isolates (NDR1, NDR2, NDR3, NDR5 and NDR11). Isolates NDR1, NDR3 and NDR11 were isolates of *B. thuringiensis.* NDR2 was an isolate of *B. laterosporus*, formerly *Bacillus laterosporus* (De Oliviera et al., 2004). NDR5 was an isolate of *B. cereus*.

Inoculum preparation

Bacterial isolates NDR1, NDR2, NDR3, NDR5 and NDR11 were used to inoculate 150 ml of sterile tryptone soy broth (TSB) (Biolab, Biolab Diagnostics, 259 Davidson Road, Wadeville, Johannesburg) and incubated in a shaker water bath at 250 rpm for 5 d at 30°C (Meadows et al., 1992). A viable spore count was conducted using a standard viable spore technique (Wheelis and Segel, 1979). The concentration of the viable spores was measured in colony forming units (CFUs). The final whole culture (FWC), which consisted of spores and crystal proteins, was used for inoculation. For NDR5 only spores are present because this isolate does not produce crystal proteins.

Inoculation

Discs with an 18 mm diameter were cut out of the inner leaves of freshly purchased cabbage using a pre-sterilized test tube cap. The FWC of NDR1, NDR2, NDR3, NDR5 and NDR11 were diluted with sterile distilled water to make up concentrations ranges of 20, 40, 60, 80 and 100%. Cabbage discs were dipped into the whole cell bacterial suspensions and fed to the *T. molitor* larvae in Petri dishes. The trials were conducted in sterile Petri dishes. Control discs were dipped into sterile distilled water. Twenty larvae per dose in replicates of five were used for each bioassay for NDR1, NDR2, NDR3 and NDR11. A total of 28 larvae per dose in replicates of five were used for NDR5. Dead larvae turned black and liquefied internally. Koch's postulate was used to determine if the isolates were responsible for the insect deaths (Prescott et al., 1999). The amount of cabbage disc consumption (mm) was a general observation and was not recorded because larval mortality

Table 1. (LSTATS) PROBAN analysis of three replicate bioassays of five spore-forming bacteria tested against *Tenebrio molitor*, with regression parameters displaying the efficacy of these assays.

Isolate	No of larvae affected	LC_{50} [a]	95% FI [b]	Slope	$X^2_{(df-13)}$
NDR1[n]					
Assay 1	68	7.495	7.414 - 7.548	5.45	1.42
Assay 2	55	7.391	7.256 - 7.497	2.54	3.47
Assay 3	61	7.332	7.176 - 7.436	2.59	3.13
Mean	61	7.406	7.282 - 7.494	3.53	-
NDR2[n]					
Assay 1	63	6.572	6.488 - 6.630	4.65	2.47
Assay 2	68	6.507	6.358 - 6.603	3.02	0.36
Assay 3	65	6.512	6.388 - 6.600	3.26	2.36
Mean	65	6.530	6.411 - 6.611	3.64	-
NDR3[n]					
Assay 1	49	7.219	7.092 - 7.344	2.35	2.65
Assay 2	46	7.281	7.170 - 7.403	2.56	1.32
Assay 3	53	7.207	7.084 - 7.312	2.63	5.27
Mean	49	7.236	7.115 - 7.353	2.51	-
NDR5[n]					
Assay 1	51	7.904	7.786 - 8.070	1.95	4.56
Assay 2	49	7.927	7.809 - 8.101	1.97	2.04
Assay 3	56	7.963	7.832 - 8.142	1.71	3.66
Mean	52	7.931	7.809 - 8.104	1.88	-
NDR11[n]					
Assay 1	57	7.095	6.953 - 7.199	2.57	2.62
Assay 2	48	7.178	7.087 - 7.266	3.35	2.48
Assay 3	50	7.146	7.037 - 7.248	2.79	0.63
Mean	52	7.140	7.026 - 7.237	2.90	-

[a] Lethal concentration (LC_{50}); [b] 95% fiducial limits were log transformed concentrations of bacterial spores per ml; [n] 20 larvae per dose in replicates of five (total of 100 larvae per bioassay).

was the objective of this study. Results were recorded after 5 days.

Statistical analyses

The statistical computer programs (LSTATS) P/PROBAN Version 2.1 (1992), as programmed by Van Ark (1983), and SPSS Version 11.5, were used to calculate the regression parameters that included the determination of lethal concentration (LC_{50}) and their fiducial limits. Each sample reflected three estimates of LC_{50} and fiducial limits. These were compared using a standard one-way ANOVA. A similar format to that of Hatting (2002) was used to depict the results obtained (Table 1). The LC_{50} values were converted back to concentrations of viable spores to determine the 50% mortality dose.

RESULTS

Mortality of *T. molitor* larvae in the control Petri dishes was zero, and the cabbage discs were completely consumed after 5 days. The complete consumption of the cabbage discs indicates that no toxic effects were present in the control. Cabbage discs that were inoculated with different doses of the various bacterial isolates were consumed according to dose. An overall trend observed was that the more concentrated the inocula of spore-forming bacteria applied to the cabbage disc, the less the cabbage disc was consumed. Low bacterial dilutions resulted in levels of cabbage disc consumption similar to that of the controls but with a low level of *T. molitor* mortality. No further larval deaths were found to occur after 5 days.

The minimum LC_{50} was 6.530 for NDR2 at a concentration equal to 3.388×10^6 spores ml^{-1}. The maximum LC_{50} was 7.931 (NDR5) at a concentration of 8.531×10^7 spores ml^{-1} (Table 1). NDR5 could thus be considered the most effective isolate. A comparative bioassay with *B. thuringiensis* subsp. *tenebrionis* was not conducted because this strain is not a registered biocontrol

Table 2. (LSTATS) PROBAN hypothesis test used to determine the homogeneity between the five independent bioassays using NDR1, NDR2, NDR3, NDR5 and NDR11.

Hypothesis		Slopes equal			Slopes and intercepts equal	
Isolate	X^2	**Df**	**P**	X^2	**df**	**P**
NDR1	0.341	2	0.842	5.404	2	0.065
NDR2	1.445	2	0.49	2.007	2	0.368
NDR3	0.73	2	0.699	0.121	2	0.932
NDR5	0.256	2	0.876	0.317	2	0.851
NDR11	0.846	2	0.661	0.793	2	0.678

Table 3. Results of a parallelism test between the three assays of the five bacterial isolates to determine homogeneity.

Isolate	No. of larvae affected	Slope ± SE	LC_{50} [a]	95% FI	$X^2_{(df-13)}$	G [b]
NDR1	184	2.82 ± 0.37	7.386	7.314 - 7.443	15.781	0.068
NDR2	196	3.39 ± 0.40	6.530	6.468 - 6.580	7.271	0.055
NDR3	148	2.50 ± 0.34	7.235	7.172 - 7.297	10.132	0.071
NDR5	156	1.85 ± 0.22	7.933	7.861 - 8.021	11.091	0.053
NDR11	155	2.87 ± 0.35	7.140	7.082 - 7.194	8.031	0.058

[a] The data of the three bioassays were pooled to obtain a more accurate estimation of the LC_{50} and fiducial limits. [b] The Fiducial limits calculated by Fieller's theorem as a measurement of variation.

agent in SA and is not readily available. *B. thuringiensis* subsp. *azawai* and *B. thuringiensis* subsp. *kurstaki* are the only two subsp. of *B. thuringiensis* registered in SA for use as a bio-pesticide (Anonymous, 2008b).

Numbers of larvae used in the assays were either 20 for NDR1, NDR2, NDR3, NDR5 and NDR11 (per single dose). These were the largest numbers possible at the time, in this project, due to limited insect rearing facilities, financial resources and time constraints.

One–way ANOVA was used to determine whether the slopes and intercepts of the three bioassays of each of the isolates were comparable. Deviations of the slopes and intercepts from the Probit lines were homogenous. These lines were therefore comparable (Tables 2 and 3, Figure 1).

DISCUSSION

Statistical results

The chi-squared values obtained for each of the bioassays suggested that the deviations of the observed mortalities are within the range of accepted parameters for the calculated Probit line (Table 1) (Van Ark, 1983). Therefore, it may be concluded that the calculated Probit line is an acceptable representation of insect response to *B. thuringiensis* isolates. Parallelism tests showed that no significant differences could be detected between the five bioassays for each of the isolates. This allowed for the comparison of slopes and intercepts (Table 3). The slopes of the independent bioassays showed no significant differences and were found to be homogenous hence the lines were comparable (Table 2) (Van Ark, 1983). The deviations from the observed mortalities were within the expected limitations of deviation thus rendering the Probit line acceptable (Table 2 and Figure 1) (Van Ark, 1983). Values obtained indicated that the lines for each of the five bioassays for each isolate were parallel and hence a similar response was observed for each of the isolates (Table 2 and Figure 1). The LC_{50} spore concentrations for each of the isolates were: NDR1 = 2.432×10^7, NDR2 = 3.388×10^6, NDR3 = 1.718×10^7, NDR5 = 8.579×10^7 and NDR11 = 1.380×10^7.

G is used as a measure of variation in the calculation of fiducial limits and is derived from Fieller's Theorem (Van Ark, 1983). According to Finney (1971), in a good bioassay, the value of G will lie between 0.2 and 0.05. Van Ark (1983) suggested using G values of 0.25 and 0.025. G values above 0.025 indicate that the variation of mortality is high. Values above 0.25 indicate that the experimental design is not appropriate and requires amendment. The fiducial limits cannot be calculated for assays where the G value is equal to one (Van Ark, 1983). The G values for all five isolates fell within the parameters set by Van Ark as well as by Finney. However, the experimental precision was not ideal because the G values were larger than 0.025, indicating that the variation in mortalities was large. However, the values were still acceptable because they did not exceed 0.25 (Table 3).

These results could be improved by conducting six or

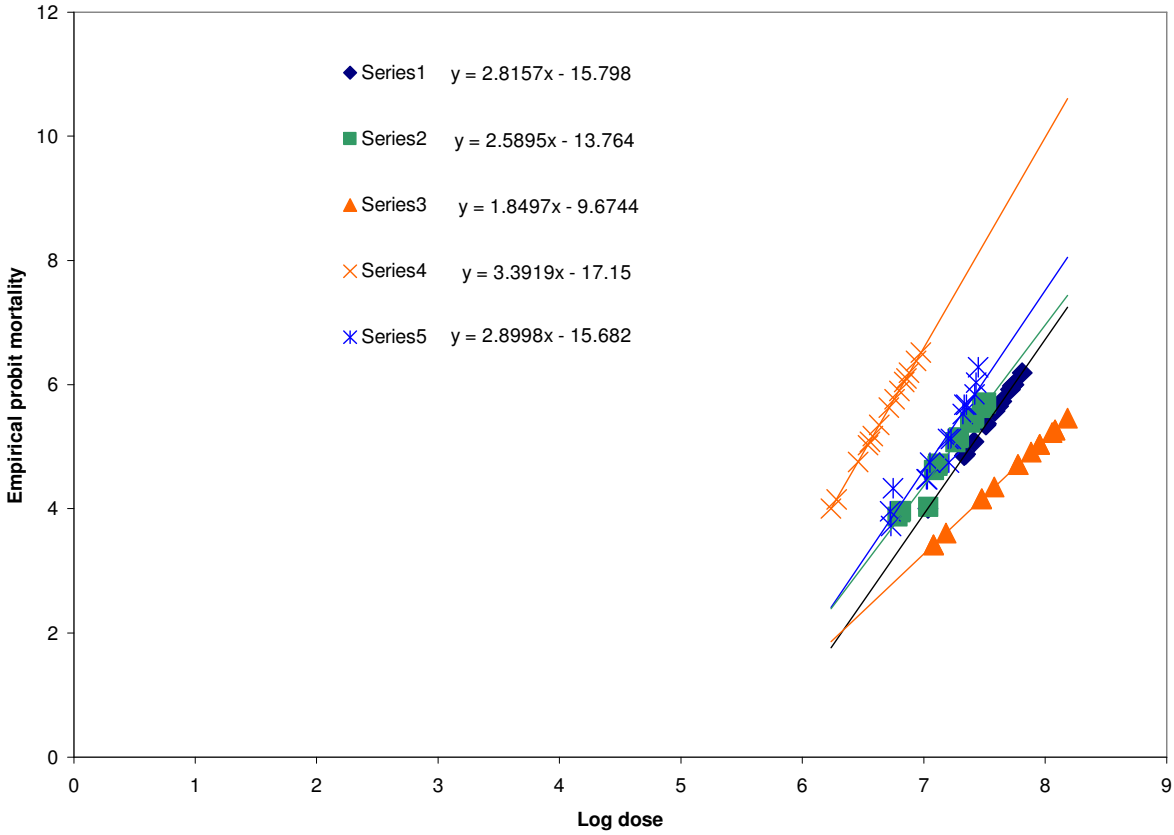

Figure 1. Separate Probit lines of the pooled data for each of the five isolates. 1. Series 1 = NDR1; Series 2 = NDR2; Series 3 = NDR3; Series 4 = NDR5 and Series 5 = NDR11.

more bioassays per isolate. This would result in a better it of line and hence a lower G value (Van Ark, 1983). The number of insects required in a bioassay for reliable results depends on the experimental procedures, as well as the species of insect (Van Ark, 1983).

Toxicity comparison of isolates

The isolate with the best LC_{50} was NDR2, with a log value of 6.530 (3.388×10^6 spores ml^{-1}) (Table 3). This isolate was identified previously as an isolate of *B. laterosporus.* Assays conducted with isolates of *B. laterosporus* did not exhibit any toxicity towards *T. molitor*. However, toxicity was evident against mosquitoes (*Culex quinquefasciatus* and *Aedes aegypti*) and a species of snail *Biomphalaria glabrata* (Favret and Yousten, 1985). Assays conducted by Rivers *et al.* (1991) on *T. molitor* with strains of *B. laterosporus* showed these strains to have toxicity values similar to those obtained from strains of *B. thuringiensis* subsp. *tenebrionis.*

The isolate with the weakest LC_{50} was NDR5, with a log value of 7.933 (8.579×10^7 spores ml^{-1}). This isolate was identified previously as an isolate of *B. cereus.* This species is not often used in entomopathogenic bioassays because it is considered an organism associated with gastrointestinal diseases and is often found as a food contaminant. However, *B. cereus* strains with insecticidal properties against coleopteran larvae have been isolated from species of white grub (Selvakumar et al. 2007; Sushil et al., 2008). The insecticidal properties of this organism lie in its ability to produce vegetative insecticidal proteins (VIP) (Estruch et al., 1996; Moar et al., 1994; Yu et al., 1997) which have been found to be effective against Western Corn Rootworm, *Diabrotica virgifera* LeConte (Coleoptera, Chrysomelidae) (Warren, 1997).

All five bacterial isolates (NDR1, NDR2, NDR3, NDR5 and NDR11) displayed toxicity against *T. molitor*. The three *B. thuringiensis* isolates (NDR1, NDR3 and NDR11) displayed high levels of toxicity to *T. molitor* but were less toxic than the *B. laterosporus* isolate (NDR2) (Table 3). The dose response of the *B. cereus* isolate (NDR5) was found to stand alone from the other two species tested (Figure 1). This is indicative of substantially greater toxicity than the other isolates.

Standardization assays often involve the use of purified or extracted crystal proteins (Cry and Cyt toxins) and do not include any of the other toxins and synergists (e.g., chitinases) produced by these species of bacteria

(Thamthiankul et al., 2001; Lee et al., 2007). *B. thuringiensis* and *B. cereus* are able to synthesize chitinases (Lee et al., 2007). Other toxins may play an important role in preventing target insects from becoming resistant because they would have to evolve resistance against more than one compound concurrently. In the bioassays used in this research, the FWC were used in toxicity determination. Hence, other unknown toxins and synergistic factors were included. Whole cultures consisting of spore and crystal suspensions are often used in bioassays to determine toxicity. The use of FWCs is applied where whole culture products will be commercialized, as opposed to pure protein crystals (De Oliviera, 2004; Lambert et al., 1992). Whole culture products have to be screened for beta-exotoxins because current registration of *B. thuringiensis* products requires the absence of these toxins (Prieto-Samsonova et al., 1997). Continuous sub-culturing of *B. thuringiensis* can be problematic because it may cause a decline in toxicity (Sachidanandham and Jayaraman, 2003).

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