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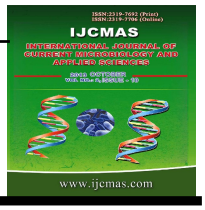

Original Research Article

Toxicity of different indigen isolates of *Bacillus thuringiensis* on mango weevil *Myllocerus undecimpustulatus* (Coleoptera: Curculionidae)

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Keywords

Bacillus thuringiensis; Bioaasay, Coleoptera; Insect resistance; Mortality; *Myllocerus undecimpustulatus.*

A B S T R A C T

Coleopteran pest control in general is entirely relied on the application of chemical insecticides. Little or no information is known about the natural presence of *Bacillus thuringiensis* species that possess insecticidal activity in the environment against coleopteran insect pests. The problems associated with resistance show how important it is to continue screening for new strains harboring toxins with high activity against pests. Moreover, the discovery of new toxins may also represent new genetic resources for developing genetically engineered technologies utilizing *B. thuringiensis* toxin genes, including integrating them into the genomes of plants of agri and horticultural interest. The present study aimed at searching for *Bt* strains encoding coleopteran active insecticidal crystal proteins that act more effectively against mango weevil. Thirty *Bt* strains were evaluated through bioassay studies. Crude protein from thirty indigen *Bt* isolates was extracted and used at different concentrations for bioassay against adults of *M. undecimpustulatus*. The per cent mortality was recorded after 24, 48 and 72 hrs of treatment. 15 native *Bt* isolates (50.00%) showed cent percent mortality followed by IIHR_HMM_MAD_KL (92.22%), IIHR_HMM_NAL_KL, (93.33%) at 600 ηg/cm^2 after treatment when compared with *Bt* subsp. *morrisoni*. pathovar *tenebrionis*. The present study aimed at searching for *Bt* strains encoding coleopteran active insecticidal crystal proteins that act more effectively against mango weevil. Thirty *Bt* strains were evaluated through bioassay studies.

Introduction

World-wide yield losses of crops due to pests and diseases have been estimated around one third of the total production with a significant proportion (15%) of the

damage attributable to insects (Oerke *et al.*, 1994). Traditional control of economically important insect pests has relied for decades on a large family of chemical insecticides. However, their broad activity spectrum and the accumulation of persistent residues have increased the demand for environmental friendly alternatives. One such near term application of the environmental friendly alternative is the insect resistant plants by the insertion of toxin gene from the bacterium *Bacillus thuringiensis*, which produces Cry proteins which could be active against certain insect species (Lepidoptera, Coleoptera, Diptera, Hemiptera, Hymenoptera etc.,).

Over 80% of all commercial biological pesticides are based on crystal proteins, produced by the bacterium *B. thuringiensis* during sporulation (Whalon and Wingerd, 2003). The importance of this class of insecticidal proteins is expected to increase in the near future due to the success of current genetically modified crops and second generation plants expressing multiple *B. thuringiensis* crystal proteins. Mango is a major horticultural crop in the world. There are more than 250 pests on record only a few groups like the Coleopterans (weevils and stem borers), Homopterans (plant and leaf hoppers, mealy bugs, scale insects), Dipterans (fruit flies), Lepidopterans (leaf webber) occurs regularly and causes severe yield loses (Tandon and Verghese 1985). Among them, leaf eating weevils, leafhoppers and fruit-flies are considered major constraints in India in terms of quality and quantity.

Of the several weevils reported on mango the weevils belonging to the genus *Myllocerus* are economically important. Hill (1987) reported *Myllocerus* weevils are polyphagous with a large number of plant hosts which make them important pest species among leaf eating weevils. Butani (1979) identified *M. undecimpustulatus* to be dominant among the three common *Myllocerus* species i.e., *M. undecimpustulatus, M. discolour* and *M. subfasciatus.* At present the control of these pests is accomplished primarily by the use of chemical insecticides. In the present study an attempt was made to study the bioefficacy of native *Bt* strains against mango weevils with the objective of increasing the host specificity.

Materials and Methods

Bacterial strains and maintenance

Thirty strains were used from a collection of entomopathogenic *Bacillus* isolates of BioPesticide Laboratory (BPL), Division of Biotechnology, IIHR, Bangalore. These isolates were obtained from soil samples from different areas of Kerala and Madhya Pradesh states regions of India and stored as glycerol stocks at -80^{0}C. The standard strain used in bioassays is *Bt* subsp. *morrisoni*. pathovar *tenebrionis* supplied by Institute Paster, France. LB medium was used to culture *Bt* strains. G-Tris medium was used for accelerating spore and parasporal crystal generation in *Bt* strains (Aronson and Thompson, 1971). BP medium was used for analyzing how ICPs change with the cultured times of *Bt* strains (Xie *et al.*, 2009). *Bt* strains were cultivated at 30^0 C temperature.

Characterization of parasporal inclusions and protein electrophoresis

For each *B.thuringiensis* strain, a single colony was inoculated into 5 ml T3 broth and incubated in a rotatory shaker, maintained at 30^{0}C at 200 rpm for nearly 48–60 h, and the bacterial sporulation was monitored through a phase contrast

microscope. When more than 90% of cells had lysed, the sporulated broth culture was transferred to 4^0C, at least half-an-hour before harvesting. The T3 broth containing spore-crystal mixture was centrifuged for 10 min at 10,000 rpm at 4^0C. The pellet was washed once with 5 ml of ice-cold Tris–EDTA buffer [Tris 10 mM, EDTA 1 mM, pH 8.0 with 1 mM phenyl methyl sulphonyl fluoride (PMSF)], once with 5 ml of ice-cold 0.5 M NaCl followed by two more washes with 5 ml of Tris–EDTA buffer with 0.5 mM PMSF by centrifuging at the same speed and time. Finally, the spore-crystal pellet was suspended in 100 µl of sterile distilled water containing 1 mM PMSF and stored in -20^0C. Gels were stained with Coomassie brilliant blue R-250 for 40 min, and de-stained in a solution containing 6.75% (v/v), glacial acetic acid and 9.45% (v/v) methanol.

Collection of test insects and maintenance

The adults of mango ash weevil *M. undecimpustulatus* were collected from the Mango fields at Indian Institute of Horticultural Research (IIHR), Bangalore. The insects were collected manually and maintained the insects on the mango leaves. The insects were maintained under the under the following conditions: 26 ± 1^0C, 70 ± 10% relative humidity and a light:dark period of 12:12 h.

Bioassays

Preliminary leaf dip bioassays with highly concentrated spore–crystal suspensions (about 600 ng of toxin per square centimetre of leaf surface) of strains containing coleopteran-active *cry* genes, were performed with the *M. undecimpustulatus* (Coleoptera: Curculionidae) adults. For each treatment, 30 adults were placed on each leaf (three replicates per treatment). To calculate LC_{50} values of the selected isolates against *M. undecimpustulatus* adults, leaflets of mango were cut to the desired size and coated with serial dilutions of spore–crystal mixtures. The solubilisation buffer and water were used as negative controls. After air drying of the leaves, they were transferred to Petri dishes lined with moistened (0.5 ml distilled water) filter paper that was replaced daily. Bioassays were conducted at 25^0C in 60–70% relative humidity with a 16:8 light/dark cycle. The percentage of mortality was scored after 5 days in comparison with parallel control in which leaflets were dipped in sterile distilled water instead of bacterial suspension. *B. thuringiensis* subsp. *morrisoni* pathovar *tenebrionis* was used as a positive control.

Statistical analysis

Bioassay experiment was conducted in a completely randomised design in a factorial and data were analysed by Probit with SAS software (SAS 1997). The 50% lethal concentrations and confidence limits were obtained by probit analysis. For all investigated parameters, the analysis of variance (ANOVA) was performed using the GraphPad Prism5 statistical software (Table 2 and 3).

Results and Discussion

The *Bt* collection described in this work was systematically screened against the adults of Mango weevil (*M. Undecimpustulatus*). The control of this pest had not been researched in the rapidly expanding mango growing areas of the world in general and in India in particular. From the *Bt* strains bioassayed, fifteen Coleopteran active *Bt* strain exhibited cent percent mortality. Notwithstanding the variability of Cry proteins described till

date, it is still necessary to search for more toxins, since a significant number of pests are not controlled with the available Cry proteins (Bravo *et al*, 1998). It is also important to provide alternatives for coping with the problem of insect resistance, especially with regard to the expression of *B. thuringiensis* genes encoding insecticidal proteins in transgenic plants (Van Rie, 1991).

The crude protein (spore crystal suspension) extracted from the 30 *B. thuringiensis* isolates was tested at six different concentrations (100, 200, 300, 400, 500 and 600ηg/cm^2) against adults of *M. undecimpustulatus* and the results are presented in Table 1 and 2. In general, the per cent mortality was found to increase with increase in concentration of crude protein of all the isolates. A cumulative mortality of 100 per cent was recorded by 15 (50) isolates *viz*.,

IIHR_HMM_PAT_KL,
IIHR_HMM_PER_KL,
IIHR_HMM_KAN_KL,
IIHR_HMM_TAL_KL,
IIHR_HMM_VEL_KL,
IIHR_HMM_PER2_KL,
IIHR_HMM_PRN_KL,
IIHR_HMM_KUR_KL,
IIHR_HMM_KUT_KL,
IIHR_HMM_MET_KL,
IIHR_HMM_AD_KL,
IIHR_HMM_TAD_KL,
IIHR_HMM_SHE_MH,
IIHR_HMM_INDA_MH,
IIHR_HMM_BHI_MH followed by IIHR_HMM_MAD_KL (93.33%), IIHR_HMM_NAL_KL (92.22%) when compared with Bt reference strain (88.88%).

No adults death was observed in the negative control. These results are not achieved in any of the previous studies and it would be good factor for screening of biological pesticides with high lethality potential that will have an important role in the control of similar pests.

The main point in establishing *B. thuringiensis* strain collections is to have a rapid and accurate characterization method. Up to now, many different methods have been developed to characterize *B. thuringiensis* strains. The toxicity analysis of the proteins against insect orders, so-called biossay, is one of them.

It is necessary to test each isolate for all target insects, thus it is a long and exhaustive process in screening large number of natural isolates (Ceron *et al.*, 1994). Biochemical tests, DNA fingerprinting, utilization of oligo nucleotide probes specific to the *B. thuringiensis* toxin genes are possible but they are very expensive and time-consuming characterization methods for the identification of new strains from large numbers of environmental samples (Bourque *et al.*, 1993). δ-endotoxins produced by different strains can vary quantitatively and qualitatively form each other and can have different activity spectra against a particular test insect. Therefore each strain has to be evaluated against each insect species through bioassay.

In the present study the *Bt* strains showed differential toxicity levels between 0 and 100% (Table 1 and Fig.1). A number of studies have shown that the presence of spores increases the toxic activity of crystal proteins (Dubois and Dean, 1995; Johnson *et al.*, 1998; Kalmykova *et al.*, 2009), spore-crystal mixtures being more toxic for larvae than either purified spores or crystals alone.

Table.1 Insecticidal activity of the selected *B. thuringiensis* isolates against *Myllocerus undecimpustulatus* (Coleoptera: Curculionidae) adults

Sl. No.	Bt isolates	Concentration	Negative control			Treatments			% of mortality
			R1	R2	R3	R1	R2	R3	
1	IIHR_HMM_PAT_KL	100	0	0	0	6	5	6	100
		200	0	0	0	12	12	13	
		300	0	0	0	14	14	15	
		400	0	0	0	18	18	20	
		500	0	0	0	22	20	24	
		600	0	0	0	30	30	30	
2	IIHR_HMM_PER_KL	100	0	0	0	4	6	5	100
		200	0	0	0	8	13	13	
		300	0	0	0	9	18	17	
		400	0	0	0	15	21	21	
		500	0	0	0	20	24	24	
		600	0	0	0	30	30	30	
3	IIHR_HMM_KAN_KL	100	0	0	0	6	7	8	100
		200	0	0	0	14	15	16	
		300	0	0	0	16	17	20	
		400	0	0	0	20	20	22	
		500	0	0	0	24	26	24	
		600	0	0	0	30	30	30	
4	IIHR_HMM_KOT_KL	100	0	0	0	4	5	3	75.55
		200	0	0	0	10	10	9	
		300	0	0	0	12	10	10	
		400	0	0	0	14	15	14	
		500	0	0	0	18	16	20	

		600	0	0	0	23	20	25	
5	**IIHR_HMM_TAL_KL**	100	0	0	0	5	5	6	100
		200	0	0	0	11	11	13	
		300	0	0	0	16	16	15	
		400	0	0	0	20	20	20	
		500	0	0	0	25	23	24	
		600	0	0	0	30	30	30	
6	**IIHR_HMM_KAL_KL**	100	0	0	0	3	4	4	82.22
		200	0	0	0	9	8	12	
		300	0	0	0	15	16	15	
		400	0	0	0	19	19	20	
		500	0	0	0	22	20	24	
		600	0	0	0	24	26	24	
7	**IIHR_HMM_NAL_KL**	100	0	0	0	7	5	6	92.22
		200	0	0	0	12	11	14	
		300	0	0	0	15	15	15	
		400	0	0	0	20	20	20	
		500	0	0	0	24	26	24	
		600	0	0	0	28	27	28	
8	**IIHR_HMM_MAD_KL**	100	0	0	0	4	5	5	93.33
		200	0	0	0	11	12	14	
		300	0	0	0	16	16	15	
		400	0	0	0	18	18	20	
		500	0	0	0	22	20	24	
		600	0	0	0	28	29	27	
9	**IIHR_HMM_VEL_KL**	100	0	0	0	6	6	6	100
		200	0	0	0	12	14	13	

		300	0	0	0	16	14	16	
		400	0	0	0	20	20	21	
		500	0	0	0	23	24	26	
		600	0	0	0	30	30	30	
10	**IIHR_HMM_EDA_KL**	100	0	0	0	3	4	3	77.77
		200	0	0	0	9	8	8	
		300	0	0	0	10	12	10	
		400	0	0	0	18	16	17	
		500	0	0	0	20	20	19	
		600	0	0	0	23	24	23	
11	**IIHR_HMM_PER_KL**	100	0	0	0	6	6	6	100
		200	0	0	0	12	12	13	
		300	0	0	0	16	14	15	
		400	0	0	0	20	18	20	
		500	0	0	0	25	23	23	
		600	0	0	0	30	30	30	
12	**IIHR_HMM_ALM_KL**	100	0	0	0	3	2	3	84.44
		200	0	0	0	9	8	9	
		300	0	0	0	12	12	10	
		400	0	0	0	15	16	14	
		500	0	0	0	20	19	22	
		600	0	0	0	26	24	26	
13	**IIHR_HMM_TIR_KL**	100	0	0	0	4	5	4	80
		200	0	0	0	9	10	9	
		300	0	0	0	12	12	13	
		400	0	0	0	16	16	16	
		500	0	0	0	18	17	18	

		600	0	0	0	24	25	23	
14	**IIHR_HMM_THRI_KL**	100	0	0	0	5	5	6	80
		200	0	0	0	10	12	12	
		300	0	0	0	12	14	14	
		400	0	0	0	18	18	16	
		500	0	0	0	22	22	24	
		600	0	0	0	26	22	24	
15	**IIHR_HMM_ERN_KL**	100	0	0	0	4	5	4	100
		200	0	0	0	10	12	11	
		300	0	0	0	13	14	14	
		400	0	0	0	18	18	18	
		500	0	0	0	22	22	22	
		600	0	0	0	30	30	30	
16	**IIHR_HMM_VAL_KL**	100	0	0	0	4	5	4	82.22
		200	0	0	0	9	10	10	
		300	0	0	0	12	12	11	
		400	0	0	0	16	18	18	
		500	0	0	0	20	20	19	
		600	0	0	0	25	23	26	
17	**IIHR_HMM_DEV_KL**	100	0	0	0	5	5	4	87.77
		200	0	0	0	11	11	10	
		300	0	0	0	12	13	12	
		400	0	0	0	17	18	18	
		500	0	0	0	21	20	3	
		600	0	0	0	26	27	26	
18	**IIHR_HMM_KUR_KL**	100	0	0	0	7	6	6	100
		200	0	0	0	13	14	13	
		300	0	0	0	17	18	17	

		400	0	0	0	20	23	21	
		500	0	0	0	26	24	24	
		600	0	0	0	30	30	30	
19	**IIHR_HMM_KUT_KL**	100	0	0	0	5	5	6	100
		200	0	0	0	12	12	2	
		300	0	0	0	14	14	14	
		400	0	0	0	18	18	18	
		500	0	0	0	22	22	22	
		600	0	0	0	30	30	30	
20	**IIHR_HMM_KOL_KL**	100	0	0	0	4	5	4	77.77
		200	0	0	0	9	10	10	
		300	0	0	0	12	14	12	
		400	0	0	0	16	16	17	
		500	0	0	0	20	19	20	
		600	0	0	0	23	24	23	
21	**IIHR_HMM_MET_KL**	100	0	0	0	6	6	6	100
		200	0	0	0	11	10	10	
		300	0	0	0	12	14	14	
		400	0	0	0	18	18	18	
		500	0	0	0	23	23	22	
		600	0	0	0	30	30	30	
22	**IIHR_HMM_PAN_KL**	100	0	0	0	4	5	5	83.33
		200	0	0	0	10	11	10	
		300	0	0	0	12	12	12	
		400	0	0	0	16	16	15	
		500	0	0	0	18	19	17	
		600	0	0	0	24	25	26	
23	**IIHR_HMM_AD_KL**	100	0	0	0	6	6	6	100
		200	0	0	0	12	12	12	

		300	0	0	0	14	14	14	
		400	0	0	0	18	18	18	
		500	0	0	0	22	21	22	
		600	0	0	0	30	30	30	
24	**IIHR_HMM_TAD_KL**	100	0	0	0	8	7	7	100
		200	0	0	0	14	13	14	
		300	0	0	0	16	16	17	
		400	0	0	0	18	18	19	
		500	0	0	0	24	26	26	
		600	0	0	0	30	30	30	
25	**IIHR_HMM_TVM_KL**	100	0	0	0	4	4	6	78.88
		200	0	0	0	10	10	10	
		300	0	0	0	11	12	11	
		400	0	0	0	15	14	15	
		500	0	0	0	19	18	18	
		600	0	0	0	24	23	24	
26	**IIHR_HMM_SOL_MH**	100	0	0	0	3	4	3	80
		200	0	0	0	9	8	8	
		300	0	0	0	11	12	10	
		400	0	0	0	15	16	14	
		500	0	0	0	18	17	18	
		600	0	0	0	22	24	26	
27	**IIHR_HMM_SHE_MH**	100	0	0	0	6	6	6	100
		200	0	0	0	12	12	11	
		300	0	0	0	14	13	13	
		400	0	0	0	17	18	18	
		500	0	0	0	23	24	24	
		600	0	0	0	30	30	30	
28	**IIHR_HMM_TEM_MH**	100	0	0	0	3	2	3	80

		200	0	0	0	6	7	6	
		300	0	0	0	9	9	8	
		400	0	0	0	12	13	15	
		500	0	0	0	18	19	19	
		600	0	0	0	22	26	24	
29	**IIHR_HMM_INDA_MH**	100	0	0	0	6	6	6	100
		200	0	0	0	10	10	11	
		300	0	0	0	14	14	14	
		400	0	0	0	19	18	19	
		500	0	0	0	22	22	21	
		600	0	0	0	30	30	30	
30	**IIHR_HMM_BHI_MH**	100	0	0	0	4	5	4	100
		200	0	0	0	10	11	10	
		300	0	0	0	14	14	14	
		400	0	0	0	20	20	20	
		500	0	0	0	24	23	24	
		600	0	0	0	30	30	30	
31	**BT sub spp. Tenebrionis**	100	0	0	0	4	5	4	88.88
		200	0	0	0	11	11	12	
		300	0	0	0	13	13	14	
		400	0	0	0	16	16	17	
		500	0	0	0	19	18	17	
		600	0	0	0	27	26	27	

Table.2 Two-way ANOVA log-dose v/s response

Table Analyzed	log-dose vs response				
Two-way ANOVA	Ordinary				
Alpha	0.05				
Source of Variation	% of total variation	P value	P value summary	Significant?	
Interaction	15.38	< 0.0001	****	Yes	
Row Factor	15.38	< 0.0001	****	Yes	
Column Factor	68.62	< 0.0001	****	Yes	
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	15905	185	85.98	F (185, 744) = 99.22	P < 0.0001
Row Factor	15905	185	85.98	F (185, 744) = 99.22	P < 0.0001
Column Factor	70961	1	70961	F (1, 744) = 81895	P < 0.0001
Residual	644.7	744	0.8665		
Number of missing values	0				

Figure.1 Log-dose v/s response of tested insects indicating the steady increase in percent of mortality with increase in concentration of protein concentration

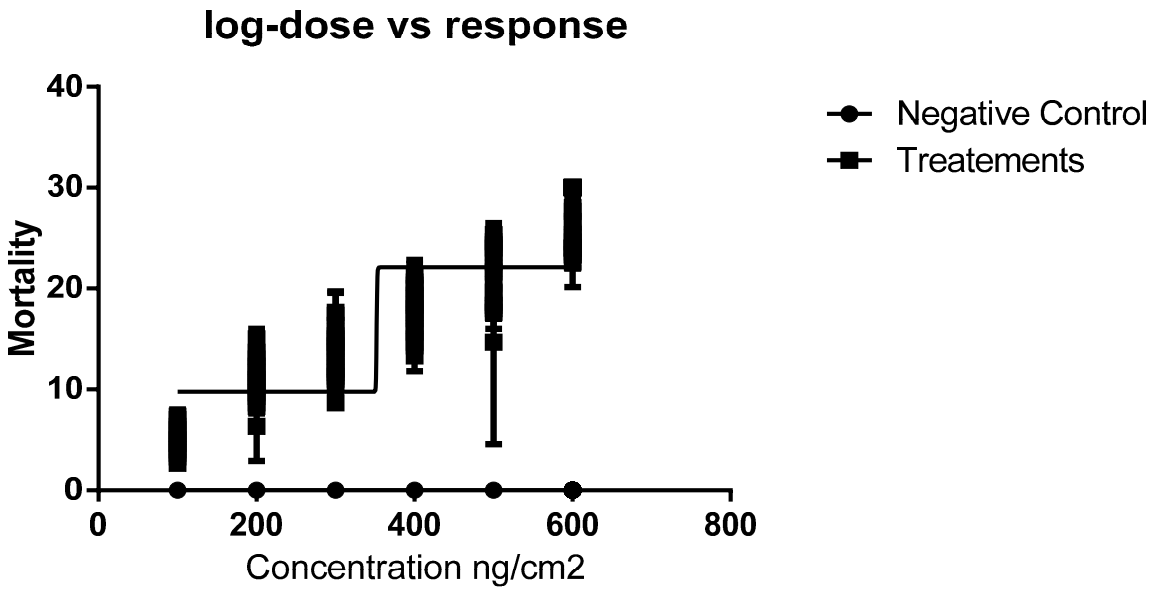

Table .3 Insecticidal activity of the selected *B. thuringiensis* isolates against *Myllocerus undecimpustulatus* (Coleoptera: Curculionidae) adults Linear regression analysis.

Best-fit values			
Slope	0.04264 ± 0.0009748	0.04160 ± 0.001020	0.04224 ± 0.001239
Y-intercept when X=0.0	0.8731 ± 0.3796	1.449 ± 0.3971	1.254 ± 0.4824
X-intercept when Y=0.0	-20.48	-34.84	-29.68
1/slope	23.45	24.04	23.67
95% Confidence Intervals			
Slope	0.04073 to 0.04455	0.03960 to 0.04360	0.03981 to 0.04467
Y-intercept when X=0.0	0.1290 to 1.617	0.6711 to 2.228	0.3083 to 2.199
X-intercept when Y=0.0	-39.54 to -2.909	-56.00 to -15.46	-54.94 to -6.939
Goodness of Fit			
R square	0.9123	0.9005	0.8634
Sy.x	2.270	2.375	2.885
Is slope significantly non-zero?			
F	1913	1664	1163
DFn, DFd	1.000, 184.0	1.000, 184.0	1.000, 184.0
P value	< 0.0001	< 0.0001	< 0.0001
Deviation from zero?	Significant	Significant	Significant
Data			
Number of X values	186	186	186
Maximum number of Y replicates	1	1	1
Total number of values	186	186	186
Number of missing values	0	0	0
Equation	Y = 0.04264*X + 0.8731	Y = 0.04160*X + 1.449	Y = 0.04224*X + 1.254

However, the difference in toxicity may also involve other factors; for example, activated and purified crystals may be more readily degraded by midgut enzymes, and therefore less toxic. Similarly, the use of purified and activated crystals entails a greater risk of decreased activity and deterioration during storage than use of a spore and crystal mixture. Indeed, the authors themselves were unable to rule out the possibility that the lack of toxicity was not due to inactivation of the crystals.

The prospects offered new isolation of *Bt* strain with improved efficacy and specificity may be benefit in developing alternative control strategies against mango ash weevil. In this context, the present *B. thuringiensis* isolates should be evaluated for activity against other insects. Novel strains or toxins may assist in pest management programs by attempting to avoid or minimise the appearance of resistance to *B. thuringiensis* in field-target insect populations. Because these isolates may represent new genetic resources that can be used to develop new technologies, the outcomes of such studies may result in the development of new microbial insecticides against pest species in integrated pest management system.

In a future work firstly verifying if Cry and/or Cyt toxins reported in this paper are really responsible for the mortality when tested against mango weevil. If not, testing for other proteins, like vip, is a possible way to find out the protein responsible for this insect mortality. The cent percent producing strains will be evaluating against different Coleopteran insect pests.

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