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Field Efficacy and Molecular Characterization of Native *Bacillus thuringiensis* Isolates against Blister Beetle, *Mylabris pustulata* (Thunberg) (Coleoptera: Meloidae) in Pigeonpea

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Over the years, blister beetle, *Mylabris pustulata* (Thunberg) has emerged as a major insect-pest of pigeonpea crop in Punjab. Efforts were made to evaluate the field efficacy of some native *Bacillus thuringiensis* (Bt) isolates against the beetle during *kharif* 2014 along with standard *Bt* and recommended insecticide. The trial compared beetle mortalities that resulted from the *Bt* treatments with those resulting from a commercial insecticide treatment. Results indicated that foliar spray of native *Bt* isolates @ 1.0% resulted in 33.33-36.66 per cent beetle mortality after 72 hours of foliar application as against the recommended insecticide, which gave 100 per cent pest control. Though the level of beetle mortality due to the native *Bt* isolates is significantly low, studies such as this have great potential in reducing the selection pressures and development of resistance to conventional insecticides in insect pests.

Key words: *Bacillus thuringiensis, Mylabris pustulata,* Pigeonpea, Field Efficacy, Molecular Characterization.

The Meloidae is a beetle family of order Coleoptera with about 120 genera and 3000 species. Among these, Blister beetle, *Mylabris pustulata* (Thunberg) has emerged as a predominant insect-pest of pigeonpea flowers in Punjab. The adult beetles are flower-feeding and polyphagous in nature. Their damage has been observed on several cucurbits, alfalfa, peanuts, soybean, ornamental flowers and agricultural crops, including potato, tomato, various leguminous plants, flax, pulses, okra, tobacco, sugarbeet, onion, spinach, pumpkin, mango, citrus fruits and some other crops in various countries[1,2,3,4,5]. Besides, adult beetles are destructive pests feeding on a wide range of host plants within families, particularly Asteraceae, Leguminosae, Compositae, Umbliferae, Solanaceae, Fabaceae, Malvaceae, Convolvulaceae and Solanaceae[6,7,8]. Infestations by the blister beetle may cause considerable damage because of the gregarious nature of adult beetles, thereby adversely affecting seed production[9]. Considerable yield losses caused by blister beetle in pigeonpea have been estimated[10].

In the present scenario, crop protection has undergone dramatic change in most parts of developed and developing countries. The emphasis has shifted from the hitherto dominant chemical pesticides to integrated pest management (IPM), where the focus is on biological control and other natural resources with reduced reliance on chemicals. Such a change became imminent mainly because of the increasing failures of chemical pesticides in controlling most of the major pests (and diseases) and also due to the ever-increasing global awareness about the undesirable side-effects of these deadly poisons such as environmental pollution, health hazards, destruction of beneficial organisms, pest

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resurgence, secondary pest outbreaks, biodiversity, deterioration of plant and soil health etc[11]. Novel pest control emphasizes the use of biological control and other control measures, and especially the chemicals must play a supportive, rather than disruptive role. In general, a strategic implementation of microbial control includes several components that require an understanding of host insects and pathogens, and the behavior of the host and pathogenicity.When incorporated into integrated pest management programs, biopesticides can greatly decrease the use of conventional pesticides or can be used in rotation or in combination with other insecticides, potentially lessening the overall quantities applied and possibly mitigating or delaying the development of resistance in pest populations.

The bacterium *Bacillus thuringiensis* is the most commonly used biopesticide globally. When ingested by pest, *Bt* releases toxins which damage the midgut of the pest, eventually killing it. The available literature contains several reported works about fungi as biopesticides against several insect pests, but there have been very few reports concerning the application of *B. thurigiensis* against blister beetle across the globe. A commercial formulation of *B. thuringiensis* was used against blister beetle in okra field in Ghana, resulting in higher yield of marketable fruit of okra than untreated plants[12]. But thereafter, no efforts were made to evaluate the efficacy of native *B. thuringiensis* isolates against the beetle. The present study deals with the field efficacy of some locally isolated *B. thuringiensis* cultures against blister beetle with an attempt to characterize the potent strains based on molecular techniques. The wider objective of the study was to ascertain the fundamental knowledge of the insect-pathogenic bacteria, *B. thuringiensis* and to use that knowledge to contribute to ecological and economic sustainability of intensified agricultural production systems through effective control of blister beetle on pigeonpea.

MATERIALS AND METHODS

Insect Collection and Rearing

For initiating the culture of insects in the laboratory, the populations comprising adults of *M. pustulata* were collected from pigeonpea fields of Ludhiana during the *kharif* season of 2014. The insects were collected and brought to the Pulses Entomology Laboratory, Department of Plant Breeding & Genetics, PAU, Ludhiana in perforated polythene bags and/or plastic containers covered with muslin and maintained on natural food in the laboratory. For providing natural food to the test insects, fresh and unsprayed pigeonpea flowers of recommended pigeonpea variety PAU 881 (sown separately in the field) were used. Populations of *M. pustulata* were reared on fresh flowers of pigeonpea in the laboratory at room temperature. The beetles were placed in plastic battery jars (measuring 10 cm x 15 cm) and covered with a piece of muslin fastened with rubber bands around their rim. Flowers were changed daily in the jars. Uniform sized adult beetles were selected for the experiments and transferred into separate plastic battery jars covered with muslin and secured with rubber bands. The selected adult beetles were pre-starved for further use in the field experiments.

Preparation of *Bt* spore crystal suspension

The *Bt* isolates were grown in Luria Bertani broth @ 1% inoculum load and incubated at 28±2° C and 200 rpm for 72-96 h till the cultures reached sporulation. The spore crystals were harvested by centrifugation. To prepare the required concentrations, the individual pellets of native *Bt* isolates were suspended in appropriate quantity of water containing CMC (1%) and Triton x100 (0.01%).

Field evaluation of *Bt* strains

Out of the 67 native *Bt* isolates evaluated against *M. pustulata* in preliminary *in-vitro* bioassays, 11 isolates proved to be toxic against the beetle. These included FDK3-3, FZR1-1, FZR1-3, HSR2-2, HSR4-1, LDH3-2, *Bt* C-2, *Bt* C-5, NSR2-3, NSR4-3 and PTA6-1. Out of these, two isolates namelyFDK3-3 and LDH3-2 were relatively more toxic to the beetle and were advanced for further field trials. Field efficacy studies were undertaken in the research area of Pulses Section, Department of Plant Breeding & Genetics, PAU, Ludhiana to examine the toxicity of two potent native *Bt* isolates against *M. pustulata*. Cultivated pigeonpea var. PAU 881 was sown during *kharif* season as per the recommended agronomic practices. Paired rows (4m row length with RTR spacing of 50 cm and interplot space of 1m) were selected for experimentation which comprised eight treatments

(including untreated control) replicated thrice in randomized block design. At flowering, foliar spray of different doses of selected *Bt* isolates (FDK3-3, LDH3-2), standard *Bt* isolate and insecticide deltamethrin 2.8 EC @ 200 ml per acre (recommended dose) was initiated using knapsack sprayer. Untreated control consisted of a water-only treatment for all replicates. Immediately after spray, each of the paired rows was covered with net cloth and 10 pre-starved adult beetles were released on the treated plants as well as untreated control inside the net cloth and confined for 72 hrs. To calculate the per cent beetle mortality from treated and control plants, the number of dead/ moribund beetles was recorded after 24 and 72 hours after treatment.Observations on the percentage of flower damaged by the beetles were recorded from three randomly selected plants per treatment at the end of 72 h. At the time of crop maturity, the grain yield was recorded from each plot and converted into kg/ha.

Protein estimation and identification of *cry* genes

Total solubilized proteins were estimated in all the indigenously isolated native *Bt* isolates showing potential against blister beetle by method of Lowry *et al* (1951)[13]. Screening for *cry* genes was carried out using PCR. Gene-specific primer pairs directed towards the identification of the two main *cry* genes specific for *cry3* and *cry9* (Table 2) were used, both of them having insecticidal activity against *M. pustulata*[14]. Amplifications were carried with the Ready to use PCR mixture (All-in-one Supermix by BR Biochem Life Sciences). In 20 µl reaction, 16 µl of PCR mix, 2 µl of template DNA and 1 µl of each forward and reverse primer were used. Amplification was done in a thermal cycler (Eppendorf, Germany) under the following conditions: a 5 min denaturation step at 95°C followed by 35 amplification cycles (1 min at 95°C, 1 min at 48°C (for *cry3*) and 56°C (for *cry9*) and 1 min at 72°C) and amplification finished with an extension step of 10 min at 72°C. PCR products were separated on 1% agarose gel[15], to which ethidium bromide was added and DNA bands were visualized in a gel documentation system.

RESULTS AND DISCUSSION

The data pertaining to the field bioefficacy of native *Bt* isolates against blister beetle have been presented in Table 1. Both the native *Bt* isolates recorded lower beetle mortality ranging from 0.00 to 13.33 per cent after 24 h of treatment. The beetle mortality in standard *Bt* isolate varied from 0.00-6.66 per cent, whereas insecticide deltamethrin 2.8 EC recorded the highest beetle mortality of 96.66 per cent after 24 h of treatment. However, after 72 h of treatment, a substantial increase in beetle mortality was observed in the native *Bt* isolate treatments. Amongst these, native *Bt* isolate LDH3-2 @ 1.0% recorded higher beetle mortality of 36.66 per cent, followed by FDK3-3 @ 1.0% (33.33% beetle mortality) after 72 h of treatment. The beetle mortality in standard *Bt* @ 1.0% and insecticide deltamethrin 2.8 EC was observed to be 16.66 and 100 per cent after 72 h of treatment, respectively.

As far as flower damage was concerned, the native *Bt* isolate LDH3-2 @ 1.0% recorded lower flower damage of 16.00 per cent, followed by FDK3-

Table 1. Field efficacy of selected native *Bt* isolates against *M. pustulata* on pigeonpea

S.No.	Treatment	Beetle mortality (%) 24 hrs	Beetle mortality (%) 72 hrs	Flower damage (%)	Grain yield (kg/ha)
T1	Native *Bt* isolate FDK3-3 @ 0.5%	0.00 (1.00)	3.33 (1.00)	26.33 (30.85)	588
T2	Native *Bt* isolate FDK3-3 @ 1.0%	6.66 (12.28)	33.33 (35.20)	18.66 (25.58)	688
T3	Native *Bt* isolate LDH3-2 @ 0.5%	3.33 (6.14)	3.33 (6.14)	27.66 (31.72)	567
T4	Native *Bt* isolate LDH3-2 @ 1.0%	13.33 (21.13)	36.66 (37.20)	16.00 (23.54)	696
T5	Standard *Bt* @ 0.5%	0.00 (1.00)	3.33 (6.14)	26.66 (31.07)	579
T6	Standard *Bt* @ 1.0%	6.66 (12.28)	16.66 (23.84)	21.66 (27.72)	642
T7	Deltamethrin 2.8 EC @ 0.2%	96.66 (83.82)	100.00 (89.96)	7.00 (15.25)	1146
T8	Untreated control	0.00 (1.00)	0.00 (1.00)	30.66 (33.61)	546
	C.D. (5%)	(13.28)	(11.99)	(1.57)	33

Figures in parentheses are transformed arcsine values

3 @ 1.0% (18.66% flower damage) which were significantly lower as compared to untreated control (30.66% flower damage). However, the treatment comprising insecticide deltamethrin 2.8 EC recorded the lowest flower damage of 7 per cent as compared to all treatments. The native *Bt* isolate LDH3-2 @ 1.0% recorded grain yield of 696 kg/ha, followed by FDK3-3 @ 1.0% (688 kg/ha) which was significantly higher as compared to untreated control (546 kg/ha), thus indicating their potential. However, the treatment comprising insecticide deltamethrin 2.8 EC recorded the highest grain yield of 1146 kg/ha as compared to all treatments.

Protein estimation studies revealed that all the native *Bt* isolates showed the presence of proteins ranging from 57.14 to 278.57 mg/g dry weight (Fig. 1), thus suggesting varied expression of *Bt* protein. Native *Bt* isolate LDH 3-2 showed highest protein content of 278.57 mg/g pellet, followed by FDK 3-3 (196.42 mg/g pellet). Out of 67 samples, 13 samples *viz*., FDK 3-3, FZR 1-1, FZR 1-2, GDP 5-1, GDP 6-1, HSR 4-1, JLD 3-1, KPT 2-1, LDH 3-2, NSR 2-2, NSR 5-2, PTA 1-1 and PTA 10-1 showed amplification for *cry3* primer. In figure 2, bands of 3 samples viz., FDK 3-3, FZR 1-1 and LDH 3-2 and Standard 4AA1 correspond to the expected product bandwidth from 652-753 bp. Likewise, 19 out of 67 samples *viz*., BTD 2-1, FZR 1-1, FZR 1-2, GDP 6-1, GDP 8-1, HSR 4-3, KPT 2-1, KPT 6-1, KPT 9-1, LDH 4-1, LDH 5-1, LDH 6-1, LDH 8-1, NSR 2-2, NSR 3-1, NSR 3-2, NSR 4-3, PTA 7-1 and RPR 2-1 showed amplification for the *cry* 9 primer. The bands of LDH 4-1, LDH 5-1 and NSR 4-3 correspond to the bandwidth of 651-654 bp in which the expected product falls (Fig. 3).

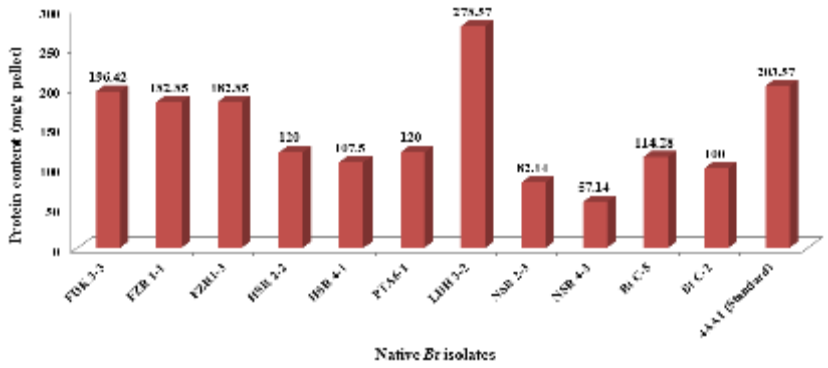

Fig. 1. Protein content (mg/g) in native *Bt* isolates

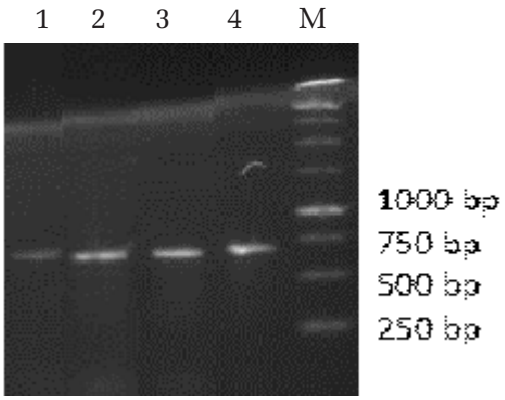

Fig. 2. Agarose gel (1%) electrophoresis of PCR products obtained with specific primers for *cry3*. Lane 1: FDK3-3, Lane 2: FZR1-1, Lane 3: LDH3-2, Lane 4: 4AA1 (Standard), M: DNA Ladder.

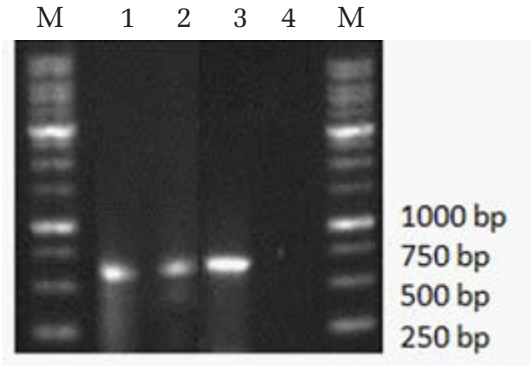

Fig. 3. Agarose gel (1%) electrophoresis of PCR products obtained with specific primers for *cry9*. M: DNA ladder, Lane 1: LDH4-1, Lane 2: LDH5-1, Lane 3: NSR4-3, Lane 4: blank, M: DNA ladder

Table 2. Sequences of the *cry*3 and *cry*9 gene primers used for PCR amplification

Primer pair	Sequence (5'-3')	Gene recognized	Product size (bp)	Annealing temperature (°C)	Reference
Cry3 F	TTAACCGTTTTCGCAGAGA	*cry3*	652-733 bp	48°C	17
Cry3 R	TCCGCACTTCTATGTGTCCAAG				
Cry9 F	GCAAATGCATTTAGCGCTGGTCAA	*cry9*	701 bp	56°C	18
Cry9 R	GTTTGAGCCGCTTCACAGCAATCC				

Entomogenous bacteria have a great promise for use as biological control agents against different insects. However, their infectivity is quite different depending on bacterial species and developmental stage of the target insects. The bioefficacy experiment has convincingly showed that both the native *Bt* isolates are pathogenic to blister beetle, however, comparatively the commercial insecticide was found to be superior. Better beetle mortality can be achieved by applying the native isolates with higher dose if it is applied only once in the crop duration. However, the modest dose may be applied splitting twice or three times during a crop season.

In the present study, management strategies were mainly concentrated on the use of bacteria. However, multiple tactics such as trapping systems and efforts to integrate the use of pathogens with other control tactics would be helpful in reducing the blister beetle problems. In inundative applications of microbial control agents, combination treatment with two entomopathogens offers an attractive biorational strategy. A bio-intensive potato pest management system developed by Gallandt *et al.* (1998) that included combined use of *B. thuringiensis* and *B. bassiana* was demonstrated to provide excellent control of Colorado potato beetle over a 6-year period. Field efficacy of *B. thuringiensis* is affected by environmental factors and takes a long time to act as compared to conventional insecticides. However, with frequent evening applications, *B. thuringiensis* might accumulate in the beetles to start an epizootic. Additional research is needed to determine what factors are presently limiting the establishment and infection of *B. thuringiensis* in the field.

Many pests have already developed resistance against chemicals and some of these compounds are largely blamed as a source of environmental pollution which also causes human health hazards. On this background, production and use of *B. thuringiensis* with indigenous strains would be one of the corner stone for sustainable pest management in the country. Clearly, studies such as this have great potential as well to reduce selection pressures and development of resistance.

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