Karshanal and Kalia
Egyptian Journal of Biological Pest Control (2023) 33:102
https://doi.org/10.1186/s41938-023-00743-7

Egyptian Journal of
Biological Pest Control

RESEARCH

Open Access

Efficacy of native *Bacillus* isolates against different larval instars of fall armyworm, *Spodoptera frugiperda* alone and in combination

J. Karshanal[1] and Vinay Kumari Kalia[1*]

Abstract

Background Fall armyworm (FAW) *Spodoptera frugiperda* (J.E. Smith) Lepidoptera: Noctuidae is an invasive polyphagous pest that causes severe damage to several agricultural crops. The use of pesticides is limited because of their mode of feeding and resistant development. Hence, the present work aimed to determine the pathogenicity of entomopathogenic bacteria (*Bacillus* spp.) against FAW in terms of mortality and growth inhibition. In this study, initially 49 native *Bacillus* isolates, isolated from diverse habitats in India, along with five reference strains, were screened for their efficacy against neonates of *S. frugiperda* under controlled laboratory conditions, followed by virulence and combinatorial bioassays.

Results Five native *Bacillus* isolates (VKK1, VKK5, S16C2, S25C1, and SOIL 20) showed mortality in the range of 35.49–65.52% against neonates of *S. frugiperda* at single concentration (1000 µg g^{-1} of diet). These five isolates, along with one reference strain *Btk*-HD1 (*Bacillus thuringiensis* serovar *kurstaki* strain HD1), were further tested to find the median lethal concentration (LC_{50}) for neonates of *S. frugiperda*. Among these, native *Bt* strain VKK5 showed the lowest LC_{50} (718.40 µg/g of diet) and HD1 showed the highest LC_{50} (3352 µg/g of diet). Combinatorial bioassay against neonate and third instar larvae showed that the combination of VKK5 and VKK1 had an additive effect. Moreover, growth inhibition was also recorded.

Conclusion The combination of *Bt* strains leads to an enhancement of pathogenicity toward FAW larvae at the initial stage of development, and in later stages, it affects their growth and development. Thus, biocontrol of FAW by entomopathogenic bacteria (*Bt*) can play a vital role in the effective management of FAW.

Keywords *Bacillus thuringiensis*, *Bacillus wiedmanni*, *Bacillus paramycoides*, *Bacillus subtilis*, Growth inhibition, Combinatorial bioassay

Background

The *Bacillus* genus has a wide genetic diversity ranging from seawater to soil, and it is even found in extreme environments (Usta 2013). Bacteria of the genus *Bacillus* are of greater agricultural importance due to their capacity to produce lipopeptides (LPs), which are active against insects, mites, nematodes, and phytopathogens (Penha et al. 2020). *B. thuringiensis* (*Bt*) is a Gram-positive, facultative anaerobic, spore-forming bacteria that produces parasporal crystals during its sporulation phase, which produce protein toxins to kill insects of different groups with high host specificity and environmental safety (Sanahuja et al. 2011). Other than *Bt*, exploration of *Bacillus* sp. against insect pests was carried out for several pests.

The fall armyworm (FAW), *Spodoptera frugiperda* (J.E. Smith) (Lepidoptera: Noctuidae), is a migratory

*Correspondence:
Vinay Kumari Kalia
vkalia_1998@yahoo.com; vkalia1998@gmail.com
[1] Division of Entomology, ICAR-Indian Agricultural Research Institute, New Delhi 110012, India

polyphagous pest native to the neotropics of the Americas and has created a great threat to food security in invaded countries (Bruce 2020). In India, the FAW was observed for the first time in 2018 (Ganiger et al. 2018). It was majorly associated with maize crops across different regions in the country (Deshmukh et al. 2020). Within a span of 2 years, FAW has established itself throughout the country and had wreaked havoc on major staple and cash crops such as maize, sorghum, sugarcane, rice, cotton, soybean, peanut, millets, and ginger (Shankar and Adachi 2019). Two types of strains were reported in FAW, referred to as rice and corn strains (Pashley 1988). In India, only the rice (R) strain was detected up to December 2018 and was found to feed on maize (Swamy et al. 2018). Later in January 2019, the occurrence of the corn (C) strain on sugarcane was also reported from India (Nayyar et al. 2021). It has affected 26 Indian states out of a total of 29 states (Delanthabettu et al. 2022). Centre for Agriculture and Bioscience International (2022) reported the occurrence of FAW in Africa (48 nations), Asia (27 nations), Europe (6 nations), North America (32 nations), Oceania (6 nations), and South America (13 nations).

Being a highly polyphagous pest, its management strategies mostly rely on the use of pesticides, which has led to the development of resistance to permethrin, cypermethrin, carbaryl, thiodicarb, chlorpyrifos, and dichlorvos (Zhang et al. 2019). Hence, biopesticides are the most prominent method for controlling this pest due to their specificity and efficacy in the management of agricultural pests. In this context, the present study was conducted on the screening of native *Bacillus* spp. isolated from diverse habitats in India against FAW, followed by combinatorial bioassays in terms of larval mortality and growth inhibition. In this context, the present study was conducted on the screening of native *Bacillus* spp. isolated from diverse habitat of India against FAW, followed by combinatorial bioassays in terms of larval mortality and growth inhibition.

Methods

Insect collection and rearing

Fall armyworm (FAW) larvae were collected from a maize field at the Indian Agricultural Research Institute, New Delhi, and reared on a kabuli gram-based semi-synthetic diet till pupation in the laboratory as per Gopalakrishnan and Kalia (2022). On emergence, five pairs of adults were transferred to a mating jar (20 cm height and 15 cm diameter) covered with muslin cloth containing a 10% fortified honey solution for adult feeding and folded paper strips for egg laying. Egg masses were collected daily and kept in a separate container for hatching. On hatching, neonates were transferred to a semi-synthetic diet in a plastic container and the lid was closed tight with tissue paper to prevent the escape of larvae. After 7 days of feeding, the larvae were separated and placed in individual plastic containers having one small cube of diet to prevent cannibalism of the larvae. Later on, pupae were collected and kept in plastic containers lined with blotting paper until adult emergence. The neonates (< 24 h old) and later instars were used for bioassays. The culture was maintained under controlled laboratory conditions, *i.e.*, at 27 ± 1 °C and 70 ± 5% relative humidity and a 14L:10D photoperiod.

Screening of *Bacillus* isolates against neonates of fall armyworm

Screening bioassays were carried out by the diet incorporation method by using acetone precipitated spore/spore crystal complexes (weight basis) of *Bacillus* isolates that were isolated from silo dust, insect cadavers, live insects, plant endophytes (neem), and various agricultural ecosystems of the north eastern region of India (Sibsagar and Jorhat, Assam), and also five reference strains were used for screening bioassays. Acetone precipitated spore crystal complex of forty nine *Bacillus* isolates, viz., *B. thuringiensis* (14), *B. subtilis* (13), *B. wiedmanni* (3), *B. cereus* (2), *B. flexus* (1), *B. megaterium* (1), *B. thermophilus* (2), *Bacillus* spp. (7), *B. paramycoides* (2), *Aneurinobacillus migulans* (1), *A. aneurinilyticus* (2), *Lysinibacillus sphaericus* (1), and 5 reference strains, viz., *B. thuringiensis* serovar *kurstaki* (HD1, HD73), *B. thuringiensis* serovar *thuringiensis* (HD2, HD14), *B. thuringiensis* serovar *aizawai* HD137 were taken from the bacterial stock of the National Facility for Insect Rearing and Xenobiotic-cum-Transgenic Bioassay, Division of Entomology, IARI, New Delhi. These isolates were screened at a single concentration (1000 μg g^{-1} of diet) by the diet incorporation method using spore/spore crystal complex as per Daravath et al (2015). The toxin-incorporated diet was transferred to small plastic containers (5 × 2 cm). Each container was served as one replicate, with three replications per treatment. Ten neonates were released on the treated diet per replicate. The diet in the control was mixed with the same volume of sterilized distilled water (SDW). All the bioassays were conducted under controlled conditions of 27 ± 1 °C, 70 ± 5% RH, and a 14L:10D photoperiod. Mortality data was recorded every 24 h till 7th day after treatment, and corrected percent mortality was calculated on 7th day using Abbott's formula (1925).

Determination of median lethal concentration (LC_{50}) with potential *Bacillus* isolates

Bacillus isolates that showed ≥ 35% mortality, *i.e.*, *B. thuringiensis* VKK5 (GenBank accession number OP743352) and VKK1 (GenBank accession number ON974875), *B. wiedmanni* S16C2 (GenBank accession number OR126911),

B. paramycoides S25C1 (GenBank accession number OR126908), *B. subtilis* SOIL20 (GenBank accession number OR126919) along with reference strain Btk-HD1, were used for virulence bioassays. Six concentrations, viz., 50, 100, 1000, 2000, 4000, and 5000 µg/g of diet of spore crystal complex of each strain, along with a control, were taken for bioassay under controlled conditions as mentioned above. Each bioassay repeated thrice with 210 neonates per treatment. Mortality data was recorded on the 7th day after the bioassay. The LC_{50} values for bioassays were calculated.

Bioassay with a combination of different *Bacillus thuringiensis* strains against neonates of fall armyworm

In this study, the Zwittermicin A-positive isolates of native *Bt i.e.*, VKK-LE1, VKK-LE2, and VKK1 (data not shown) were combined with the most potential *Bt* isolate, VKK5. Combinatorial bioassays were carried out by two methods *i.e.*, by using *Bt* cell suspensions (1×10^8 cells/g of diet in VKK5, VKK1, VKK-LE1 and VKK-LE2; 1×10^4 cells/g of each isolate in combination of VKK5 + VKK1, VKK5 + VKK-LE1,VKK5 + VKK-LE2) and another by using acetone precipitated spore crystal complex (weight basis) (1000 µg g^{-1} of diet in VKK5, VKK1, VKK-LE1 and VKK-LE2; for combination bioassay 500 µg g^{-1} of diet of each isolate (VKK5 + VKK1, VKK5 + VKK-LE1, VKK5 + VKK-LE2). For cell suspension, each *Bt* strain was inoculated in Luria Bertani broth (LB) and incubated at 30 °C for 72 h in an incubator shaker (200 rpm). After 72 h, the bacterial colonies in suspension were centrifuged, and pure colonies of bacteria collected as pellets in the centrifuge tube were re-suspended in SDW. The number of viable spores was estimated by counting colony forming units (cfu) on LA plates after overnight incubation at 30 °C. The *Bt* incorporated in the diet in the form of *Bt* cell suspensions (1×10^8 cells/g of diet) and spore crystal complexes (1000 µg g^{-1} of diet) was used for bioassay against neonates of FAW. Mortality data was recorded every day till 7th day after treatment. Corrected percent mortality on 7th day was calculated using Abbott's formula (1925).

Mortality and sublethal effects of *Bt* strains alone and their combinations on third and last-instar larvae of FAW

The combinatorial bioassays were carried out on third and last-instar larvae of FAW to examine mortality as well as growth reduction using most potential combination (VKK5 and VKK1) against neonates of FAW. This bioassay was also performed with both acetone precipitated spore crystal complex (weight basis) and cell suspensions (cfu/ml) as mentioned above. The observations were made on percent mortality and differences in larval weight in third instar and last-instar larvae after 7 and 2 days of treatment, respectively. The survived larvae were observed till adult emergence for sub-lethal effects.

Co-toxicity of a combination of different *Bt* isolates against fall armyworm larvae

The interaction between *Bt* strains in relation to larval mortality was differentiated according to the co-toxicity factor (Mansour et al 1966) as follows:

$$\%\text{Co - toxicity factor} = [(\%\text{observed mortality} - \%\text{expected mortality} / \%\text{expected mortality})] \times 100$$

where % expected mortality = the sum of % mortality in each *Bt* isolate alone and % observed mortality = % mortality in combination bioassay. A co-toxicity factor of > 20 is considered synergistic, a co-toxicity factor of < − 20 is considered antagonistic, and co-toxicity factor of an intermediate value between − 20 and + 20 is considered as additive.

Confirmation of the *Bt* strain in the infected larvae

To verify that the strains employed in the bioassays were the cause of the larval mortality, larvae that had exhibited typical symptoms were collected. Each deceased larva was packed into a 1.5 ml micro-centrifuge tube after surface sterilization with 70% ethyl alcohol and then submerged in sterile water. Larvae were then homogenized with 100 µl SDW and spread on a nutrient agar (NA) plate supplemented with ampicillin at 50 µg/ml (these *Bt* strains are resistant to ampicillin) and incubated at 30 °C for 72 h. The bacterial colonies grown on NA plates were checked with original *Bt* colonies for colony morphology; further confirmation of spore crystals inside the bacterial cells of re-isolated *Bt* colonies was done by using a phase contrast microscope.

Statistical analysis

The data on corrected percent mortality of all bioassays and larval weight were subjected to analysis of variance (ANOVA) at 5% level of significance using statistical analysis system (SAS) version 4.2 (SAS Institute Inc. Cary, USA). The significantly different means (< 0.05) were separated using Duncan's Multiple Range (DMRT) test. The LC_{50} values for bioassays were calculated using the maximum likelihood programme (MLP) 3.01 (Ross 2000).

Results

Screening of native *Bacillus* isolates against neonates of fall armyworm

Perusal of the mortality data in (Table 1) revealed that BtVKK5 attained maximum mortality (65.52%), which

Table 1 Efficacy of *Bacillus* isolates from diverse sources against neonates of *Spodoptera frugiperda* in terms of percent mortality

S.No	***Bacillus* spp**	**Strain ID**	**Source**	**Accession Number**	**Corrected percent mortality**
1.	*B. thuringiensis*	VKK-LE1	Insect cadaver	KT714048	31.04[e]
2.		VKK-LE2		KT714049	20.69[h]
3.		VKK-AG1		KT714052	24.14[g]
4.		VKK1	Silo dust	ON974875	51.73[b]
5.		VKK-GA-7		KT714039	31.04[e]
6.		VKK-GJ-3		*	20.69[h]
7.		VKK5		OP743352	65.52[a]
8.		S17C1	Tea Ecosystem	OR126907	20.69[h]
9.		S17C5		OR126913	17.24[i]
10.		S31C3		OR126915	17.24[i]
11.		S28C2		OR126916	17.24[i]
12.		S22C2		OR126920	6.9[l]
13.		S5C3	Rice Ecosystem	OR126910	13.8[j]
14.		S2C4		OR126918	10.35[k]
15.	*B. subtilis*	VKK-GA-3	Silo dust	*	31.04[e]
16.		VKK-GA-6		MF983540	20.69[h]
17.		VKK-GA-8		MF983542	24.14[g]
18.		VKK-GA-10		MF983543	31.04[e]
19.		VKK-GJ-1		MF983544	20.69[h]
20.		VKK-GJ-5		MF993341	31.04[e]
21.		VKK-GJ-6		MF993340	24.14[g]
22.		VKK-MGA-1		MF993341	20.69[h]
23.		VKK-MGA-3		MF993342	13.8[j]
24.		VKK-SL1	Insect cadaver	MF993346	20.69[h]
25.		VKK-SL3		MF993347	27.59[f]
26.		SOIL 20	Tea Ecosystem	OR126919	35.49[d]
27.		S17C2		OR126921	20.69[h]
28.	*B. wiedmanni*	S5C5	Rice Ecosystem	OR126922	24.14[g]
29.		S24C4	Rice Ecosystem	OR126914	20.69[h]
30.		S16C2	Tea Ecosystem	OR126911	37.93[c]
31.	*B. cereus*	S24C1	Rice Ecosystem	OR126912	27.59[f]
32.		S4C1		OR126909	17.24[i]
33.	*B. flexus*	VKK-PXI	Insect cadaver		24.14[g]
34.	*B. megaterium*	VKK-AG2	Live insect	MF993339	27.59[f]
35.	*B. thermophilus*	VKK-MPL	Insect cadaver	*	24.14[g]
36.		VKK-6OL	Plant endophyte (Neem)	*	13.8[j]
37.	*Bacillus* spp	J7C5	Pristine field	*	27.59[f]
38.		S14C4	Tea Ecosystem	*	31.04[e]
39.		S9C1		*	20.69[h]
40.		S7C5	Vegetable Ecosystem	*	31.04[e]
41.		S6C2		*	20.69[h]
42.		J5(1)C(1)	Vegetable Ecosystem	*	20.69[h]
43.		J1R2	Some plantation	*	27.59[f]
44.	*B. paramycoides*	S4C2	Rice Ecosystem	OR126923	31.04[e]
45.		S25C1		OR126908	37.93[c]
46.	*Aneurinobacillus migulans*	VKK-FCI4	Silo dust	*	27.59[f]
47.	*Aneurinibacillus aneurinilyticus*	VKK-ENT3	Soil	*	31.04[e]
48.		VKK-SO	Insect cadaver	*	31.04[e]

Table 1 (continued)

S.No	*Bacillus* spp	Strain ID	Source	Accession Number	Corrected percent mortality
49.	*Lysinibacillus sphaericus*	S25C2	Rice Ecosystem	OR126917	24.14 [g]
50.	*Bt* serovar *kurstaki*	HD1	Pasteur institute, Paris		17.24[i]
51.		HD73			17.24[i]
52.	*Bt* serovar *thuringiensis*	HD2			17.24[i]
53.		HD14			10.35 [k]
54.	*Bt* serovar *aizawai*	HD137			13.8[j]

Means followed by same letter within column alphabets are not significantly different at 5% level. *-Sequence of these strains yet to submit in NCBI

Table 2 Median lethal concentration (LC_{50}) of potential *Bacillus* isolates against neonates of *Spodoptera frugiperda* on 7th day after treatment

Bacillus isolate ID	LC_{50} µg/g of diet	95% Fiducial limit		Slope ± std error	Chi square value	Degrees of freedom
		Lower	Upper			
VKK5 (*B. thuringiensis*)	718.40	428.25	2661.05	0.96 ± 0.32	2.23	4
VKK1 (*B. thuringiensis*)	995.59	598.12	4788.47	1.07 ± 0.35	1.00	4
S16C2 (*B. wiedmanni*)	2246.18	1504.14	3586.18	1.23 ± 0.19	1.69	4
S25C1(*B. paramycoides*)	1733.06	1126.21	2796.37	1.13 ± 0.17	0.29	4
SOIL20 (*B. subtilis*)	3295.64	1829.60	8148.19	0.81 ± 0.16	1.89	4
HD1 (*Bt.kurstaki*)	3352.01	2304.16	5539.29	1.29 ± 0.20	0.169	4

was significantly different from all other isolates tested. The second high mortality was obtained in BtVKK1 at 51.73%, which also significantly different than all the isolates. These two native *Bt* strains were significantly superior among the *Bacillus* isolates tested against neonates of FAW. Among the *Bacillus* isolates from the north-eastern region of India, *B. wiedmanni* strain S16C2 and *B. paramycoides* strain S25C1 showed the same mortality of 37.93%, followed by *B. subtilis* strain SOIL 20, which showed a mortality of 35.49%, which was found to be statistically different than other isolates. These five isolates were showing more than 35% mortality against FAW neonates. *B. subtilis* strains VKK-GA3, VKK-GA10, and VKK-GJ5 showed 31.04% mortality and were found to be at par with *B. thuringiensis* (VKK-GA7, VKK-LE1), *A. aneurinilyticus* (VKK-SO, VKK-ENT3), *B. paramycoides* (S4C2), and *Bacillus* spp. (S14C4, S7C5). *B. subtilis* strains showed mortality in the range of 13.8 to 35.49%. The reference strains Btk-HD1, HD2, and HD73 attained a mortality rate of 17.24%, which was found to be statistically at par with *B. cereus* (S4C1) and *B. thuringiensis* (S17C3, S31C5, S28C2). Out of 49 native *Bacillus* isolates, 2 showed more than 50% mortality, 13 showed 30–50% mortality, and 5 showed less than 15% mortality. The results revealed that there was a variation in virulence among *Bt* strains as well as other *Bacillus* isolates from different habitats toward FAW neonates.

Computation of the median lethal concentration (LC_{50}) of potential *Bacillus* spp. against neonates of fall armyworm

Perusal of LC_{50} data (Table 2) showed that LC_{50} values of the spore crystal form of *Bacillus* spp isolates varied from 718.40 µg/g of diet (BtVKK5) to 3352 µg/g of diet (Btk-HD1) against neonates of FAW. Among the studied *Bacillus* spp. isolates, BtVKK5 was found to be the most toxic with a minimum LC_{50} of 718.40 µg/g of diet, followed by BtVKK1 (995.59 µg/g of diet). Btk-HD1 was found to be at par with other *B. subtilis* isolates (SOIL20) but significantly different from other native *Bacillus* isolates.

Combined effect of different *Bt* isolates on neonates of the fall armyworm

Mortality results of the combinatorial bioassay (Fig. 1) showed that in cell suspensions (1×10^8 cells/g of diet), the mortality was doubled in the combination of VKK5 + VKK1 (92.86%) as compared to VKK5 alone (46.43%). The corrected percent mortality in VKK1 (32.14%) was less than half of the percentage mortality obtained in its combination with VKK5. The VKK-LE1 and VKK-LE2 strains were able to cause 25 and 17.85%

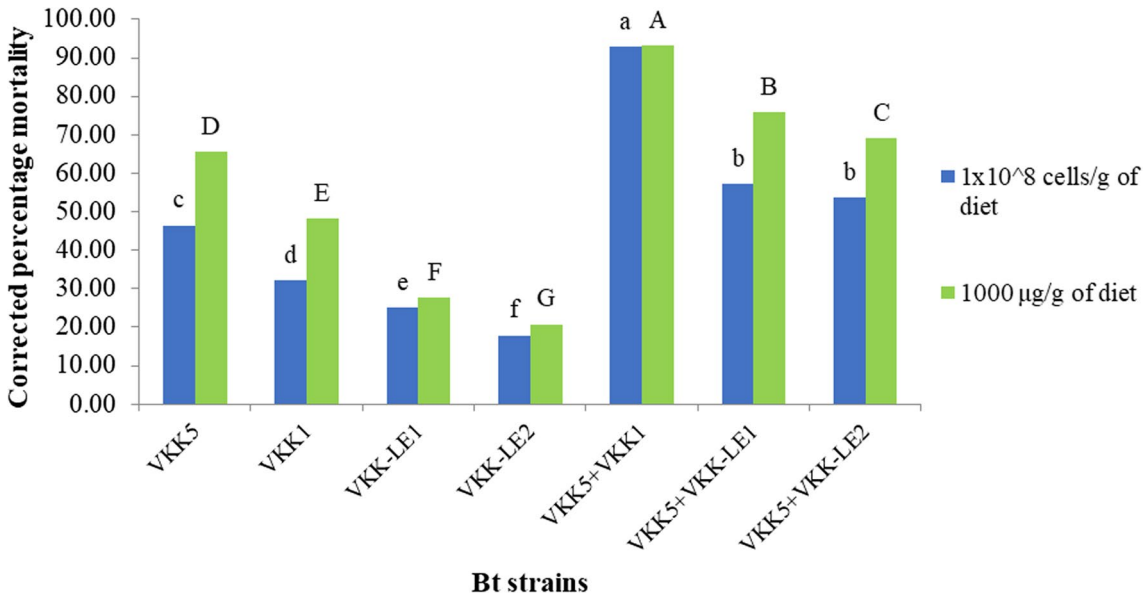

Fig. 1 Corrected percent mortality of combinatorial bioassay in spore crystal and cell suspension form of *Bacillus thuringiensis* strains against neonates of *Spodoptera frugiperda*. *Corrected percent mortality bar's followed by different small alphabets and capital alphabets are significantly different at 5% level in 1×10^8 cells/g of diet and 1000 µg/g of diet, respectively

mortality, respectively, while their combination with VKK5 increased the mortality by more than two-fold. In the cell suspension bioassay, the mortality recorded in Bt isolates alone and in combination with BtVKK5 was statistically different from each other except for combinations of VKK5 + VKK-LE1 and VKK5 + VKK-LE2.

The spore crystal complex bioassay results (Fig. 1) showed that the percent mortality obtained in combination of VKK5 + VKK1 (93.11%) was the maximum, followed by VKK5 + VKK-LE1 (75.87%), which were statistically different from each other. Comparing the results of VKK1 with the combination of VKK5 and VKK1, the percent mortality was nearly doubled. The results obtained clearly showed that the combination of VKK5 with VKK1, VKK-LE1, and VKK-LE2 increases the virulence of VKK5 against neonates of FAW. Among those, the highest mortality was observed in VKK5 + VKK1 in both cell suspension (92.86%) and spore crystal complex (93.11%) bioassays.

Mortality and growth reduction in third and last larval instars fed on a *Bt*-incorporated diet

The results of a combinatorial bioassay of VKK5 and VKK1 against third and last larval instars of FAW in both cell suspension (1×10^8 cells/g of diet) and spore crystal complex (1000 µg g^{-1} of diet) revealed that in last instar larvae no mortality was observed. Perusal of data in (Fig. 2) showed that there was a significant increase in mortality with the combination of VKK5 and VKK1 when compared to the treatments alone in third-instar larvae in both cell suspension and spore crystal complex bioassays.

Difference in larval size was observed in both cell suspension and spore crystal complex bioassays (Fig. 4A). The larval weight was significantly reduced in all the treatments, especially in the combination of VKK5 + VKK1 (180.69, 158.83 mg) when compared to control (461.35, 463.80 mg) (Fig. 3), in both cell suspension and spore crystal form, respectively. The weight of the larvae attained after bioassay was statistically at par in VKK5 (248.60 ± 25.65 mg) and VKK1 (254.71 ± 21.23 mg) in cell suspension (1×10^8 cells/g of diet) bioassay. In the last instar larval bioassay, the weight of the combination of VKK5 + VKK1 was very low in both cell suspension (162.80 mg) and spore crystal complex (135.05 mg) bioassays when compared to all other treatments and control (420.88 mg). The weight attained in larvae fed on the diet incorporated with VKK5 (206.80, 225.75 mg) was significantly different when compared to VKK1 (308.10, 272.30 mg) in both cell suspension and spore crystal complex bioassays of last instar larvae (Fig. 3). The size of pupae was reduced in all the treatments than the control. Subsequently, small pupae (Fig. 4B) and malformed adults (Fig. 4C) were formed in the larvae that fed on the combination of VKK5 and VKK1.

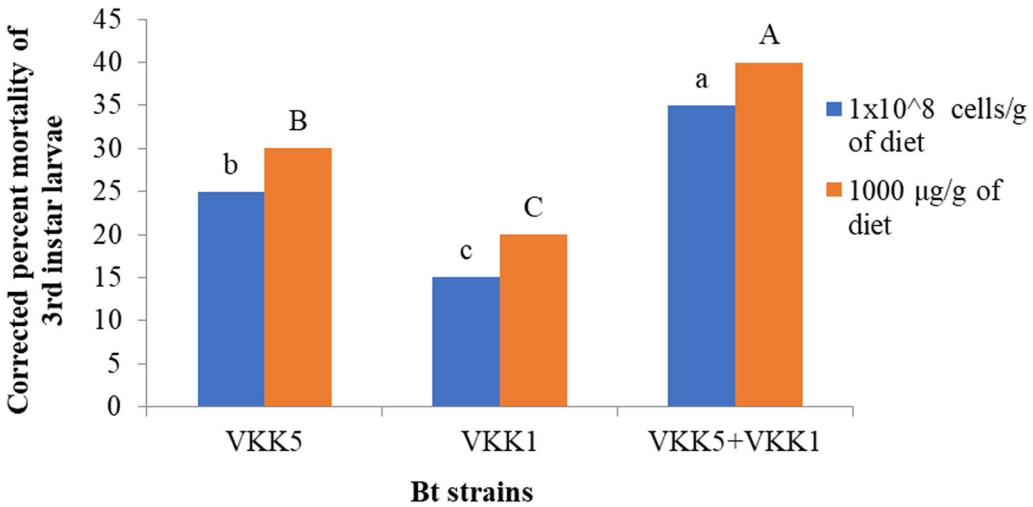

Fig. 2 Corrected percent mortality of combinatorial bioassay in spore crystal and cell suspension form of *Bacillus thuringiensis* strains against 3rd instar larvae of *Spodoptera frugiperda*. *Corrected percent mortality bar's followed by different small alphabets and capital alphabets are significantly different at 5% level in 1×10^8 cells/g of diet and 1000 µg/g of diet, respectively

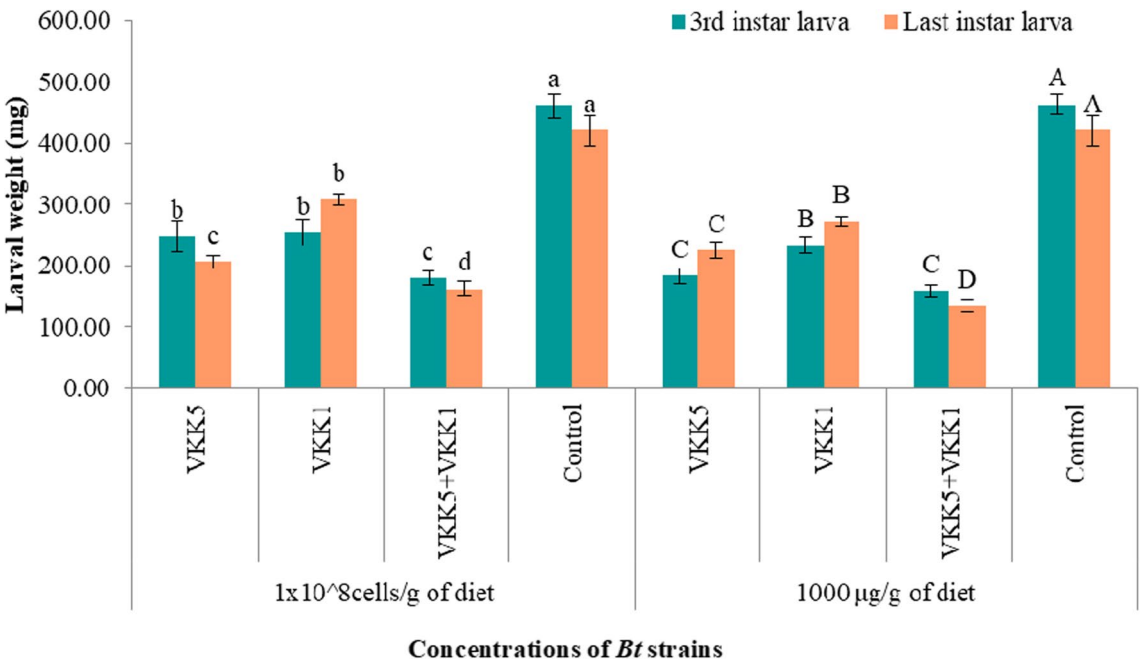

Fig. 3 Mean larval weight attained in 3rd and last larval instars of *Spodoptera frugiperda* after 4th day and 2nd day of combinatorial bioassay of *Bacillus thuringiensis* strains. *Mean larval weight bar's followed by different small alphabets and capital alphabets are significantly different at 5% level in 1×10^8 cells/g of diet and 1000 µg/g of diet, respectively

Co-toxicity factor of a combination of *Bt* isolates in neonates and third-instar larvae of FAW

The co-toxicity factor was calculated for the combined effect of different *Bt* isolates (Table 3). The results revealed that, in the neonate bioassay, the combination of VKK5 and VKK1 in cell suspension has an additive effect with a co-toxicity factor of 18.19%. All the isolates in combination with BtVKK5 in both cell suspension and spore crystal complex showed additive effects to different extents. Third-instar larvae also showed an additive effect with a co-toxicity factor of − 12.50 and − 20% in both cell suspension and spore crystal

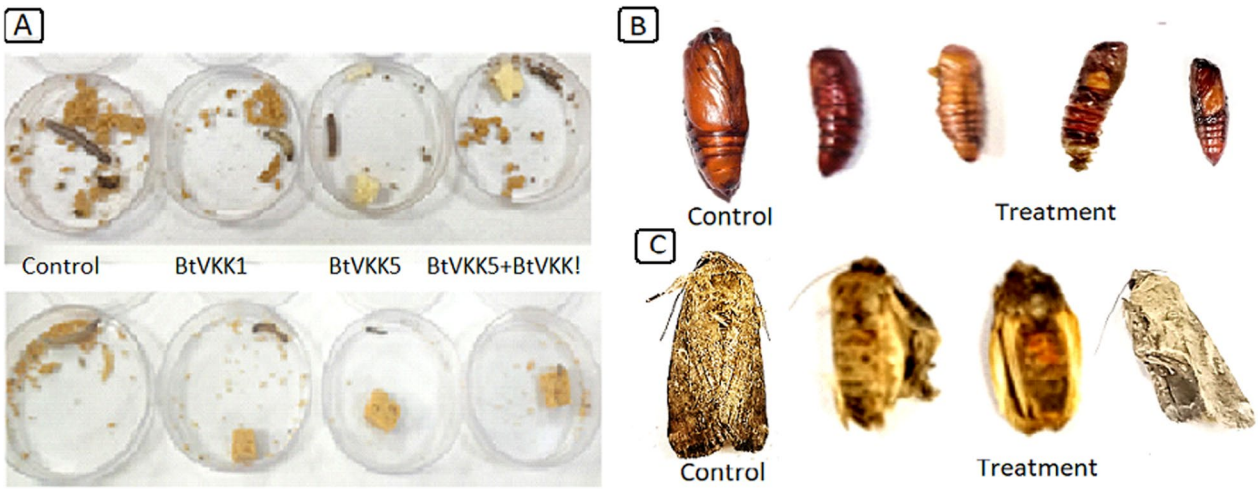

Fig. 4 Growth and development of larvae which fed with native *Bacillus thuringiensis* strains incorporated diet in combinatorial bioassay **A** Differences in larval size; **B** Differences in pupal size; **C** Malformation in adults

Table 3 Combinatorial effect of native *Bacillus thuringiensis* isolates against neonates and third instar larvae of *Spodoptera frugiperda*

Larval instars	*Bt* strains	Concentrations	% observed mortality	% expected mortality	% co-toxicity factor	Type of interaction
Neonates	VKK5 + VKK1	1×10^8 cells/g of diet	92.86	78.57	18.19	Additive
	VKK5 + VKK-LE1		57.14	71.42	− 20.00	Additive
	VKK5 + VKK-LE2		53.57	64.28	− 16.66	Additive
	VKK5 + VKK1	1000 µg $g^{-1}$of diet	93.11	113.81	− 18.18	Additive
	VKK5 + VKK-LE1		75.87	93.12	− 18.52	Additive
	VKK5 + VKK-LE2		68.97	86.22	− 20.00	Additive
3rd instar	VKK5 + VKK1	1×10^8 cells/g of diet	35	40.00	− 12.50	Additive
		1000 µg $g^{-1}$of diet	40	50.00	− 20.00	Additive

form, respectively (Table 3). The results of this study indicated that there was a strong additive effect in the toxicity of native *Bt* isolates, which produced mortality in FAW larvae. Apart from mortality, native *Bt* isolates can produce sublethal effects on the growth and development of FAW larvae, which remain until adult emergence.

Bt confirmation in the infected larvae

The neonate larvae feeding on a diet treated with native *Bacillus* isolates during the bioassay experiment became lethargic, stopped feeding, and turned black when compared to the control (Fig. 5A, B). But in the case of *Bt* treatment, in addition to the above symptoms, the gut region of larvae turned a black colour (Fig. 5C, D). The larvae were found dead on the surface of the diet and became flaccid after death. The dead larva was homogenized and plated on NA plates. The bacterial colonies obtained on NA plates (Fig. 5E) were confirmed for spore crystals inside the bacterial cells by using a phase contrast microscope (Fig. 5F). The results proved that the *Bt* strains were responsible for the mortality of neonates, and the physical changes were induced by *B. thuringiensis* infection. Koch's postulates were fulfilled by the confirmation of the *Bt* strain after re-isolation from the infected larval gut.

Discussions

Fall armyworm is a highly polyphagous pest, and its management methods are not well developed in India. *Bacillus* isolates have a range of functions in ecology, biotechnology, industrial, and clinical microbiology. Soil-associated *Bacillus* strains are the source of industrial enzymes and may be vital for the cycling of organic matter in soil environments (Avsar et al. 2017). *Bt* has been isolated from a variety of environments, including soil, dead insects, grains, deciduous and coniferous leaves, and stored product dust (Rajashekhar et al. 2017). In the present study, the susceptibility of FAW against different

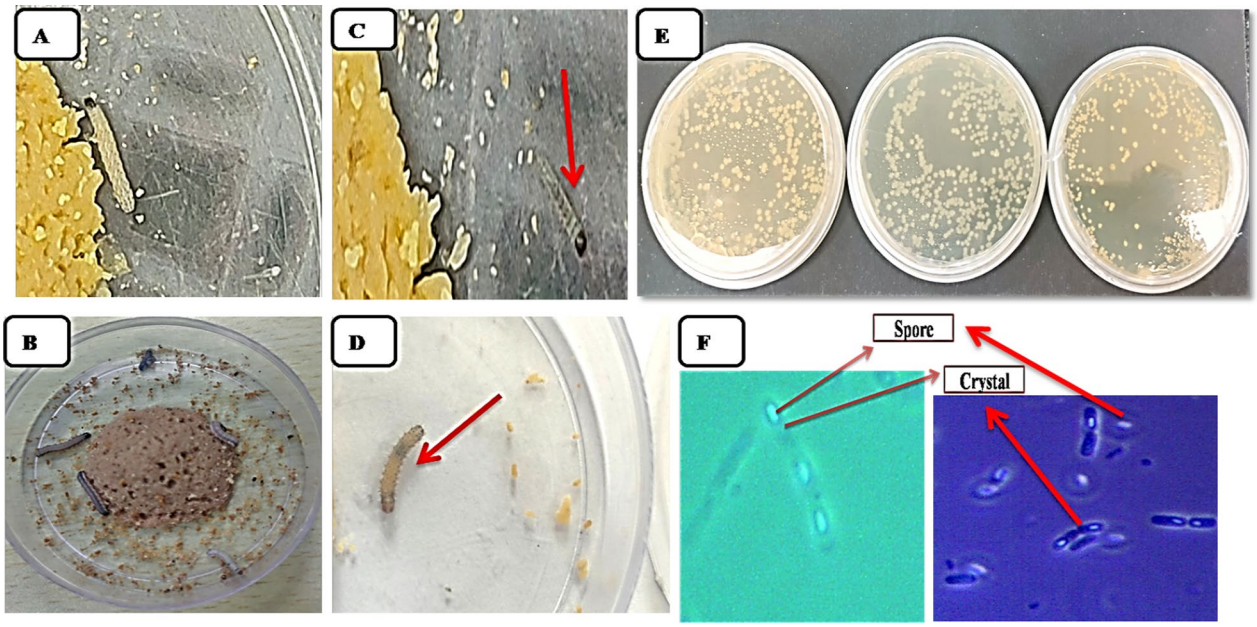

Fig. 5 Isolation of *Bacillus thuringiensis* from dead larva and visualization of spore crystal by phase contrast microscope **A**, **B** Control larvae not showing any gut discoloration; **C**, **D** Gut discoloration in fall armyworm larva due to infection of various *B. thuringiensis* strains; **E** Plates of colonies reisolated from dead larvae; **F** Spore and crystal of re-isolated *B. thuringiensis* cells from gut of dead larvae under phase contrast microscope

Bacillus sp. from diverse habitats was explored. The pathogenicity of 49 native *Bacillus* isolates, which were isolated from silo dust, insect cadavers, live insects, plant endophytes (neem), and various agricultural ecosystems of the north eastern region of India was tested against neonates of *S. frugiperda*. Two *Bt* isolates, viz., VKK5 and VKK1, recorded > 50% mortality, whereas isolates S16C2, S25C1, and SOIL 20 caused > 35% mortality in the diet incorporation bioassay. The results revealed that other than *Bt*, several *Bacillus* spp. also showed pathogenicity toward neonates of FAW. Studies observed that *Bacillus* spp., which were isolated from soil, also had a larvicidal effect against FAW (Handayani et al. 2023). There are several studies exploring the susceptibility of *S. frugiperda* to Cry toxins and Bt strains (Maheesha et al. 2021). Several other *Bt* subspecies were also reported to cause mortality in second instar FAW larvae, viz., *Bt dendrolimus* HD37, *Bt aizawai* HD68, *Bt kurstaki* HD73, and *Bt darmstadiensis* HD146, in *in-vivo* assays. According to studies performed by Hernandez (1988), subspecies of *Bt kurstaki*, *Bt aizawai*, and *Bt thuringiensis* caused death rates of 80, 100 and 70% against FAW neonates at (3×10^7 cells/ml), respectively. The composition of the crystals and their toxic potential may be associated with the variations in these strains' toxicity to *S. frugiperda* (Polanczyk et al. 2000).

Present study reported that the pathogenicity of native strains was higher than that of reference strains. Similar kinds of studies with *Bacillus* isolates stated that the commercially available *B. thuringiensis* subsp. *kurstaki* product, Dipel, was not promising against larvae of fall armyworm, with 48.86% mortality and an LC_{50} of 116.239 (Priyanka et al. 2021). Obtained results are in accordance with the results of Delanthabettu et al (2022), who found that some strains were much more efficient than the reference strain (HD1) in killing the FAW larvae. *Bt* isolates were showing mortality in the range of 6.9–65.52%.

In present study, the combined effects of different *Bt* strains are evident. Kausarmalik and Rizwana (2014) stated the combined effect of different *Bt* strains on the management of the red flour beetle. There are reports indicating that certain strains of *B. thuringiensis* produce a compound that potentiates another *B. thuringiensis* activity (Manker et al. 2002). In combination bioassays of third and last larval instars, mortality was observed only in third instar larvae but not in last instar larvae. The differences in vulnerability and death rates across developmental instar larvae may be connected to their morphological features, sizes, behaviors, and immunological defence systems, as previously stated by Elbrense et al (2021). The extent of synergism between spores and toxins of *B. thuringiensis* depends on the strain of insect, the type of spore, the set of toxins, the presence of other materials such as formulation ingredients, and the concentrations of spores and toxins (Liu et al. 1998).

In this study, the larvae fed on *Bt* incorporated diet showed significant reductions in growth and development. A study by Li et al (2015) showed that *B. amyloliquefaciens*, deterred feeding by FAW larvae and caused a significant decrease in the weight of larvae. In other investigations, sublethal dosages of *Bt* induced reductions in consumption and delays in development in *S. frugiperda* (Lambert et al. 1996), *S. littoralis* (Regev et al. 1996), and *S. exigua* (Lopes Lastra et al. 1995). However, these effects were temporary, and their intensity decreased with the growth of the larvae. This makes it evident that, in addition to having a fatal impact on hosts, entomopathogens may change an insect's physiology, which prevents them from consuming food and reproducing (Polanczyk and Alves 2005).

Conclusions

The majority of chemical insecticides now in use were unable to control FAW and they also had some negative environmental effects. Because of its effectiveness and lack of negative effects on natural enemies, the use of *Bt* against FAW is gaining interest. The biocidal property of *Bt* is caused by the spore-crystal complex. Besides *Bt*, other *Bacillus* spp. exhibit entomocidal activity against FAW, which needs further exploration. The combination of *Bt* strains increased the efficacy on *S. frugiperda* in terms of mortality and growth inhibition in neonates, third and last larval instars. The combination of *Bt* strains led to an enhancement of pathogenicity toward FAW larvae at the initial stage of development, and in later stages, it affects their growth and development. Therefore, biological control by entomopathogenic bacteria (*Bt*) and its combination is the unavoidable choice to manage the FAW in an eco-friendly way.

Abbreviations

Bt	*Bacillus thuringiensis*
FAW	Fall armyworm
hrs	Hour
°C	Degree Celsius
RH	Relative humidity
MLP	Maximum likelihood programme
LC_{50}	Median lethal concentration
cfu	Colony forming units
SDW	Sterilized distilled water
NA	Nutrient agar
µg	Microgram
LB	Luria Bertani broth
LA	Luria Bertani agar

Acknowledgements

JK is grateful to Indian Agricultural Research Institute, New Delhi for Senior Research Fellowship and Director, IARI, New Delhi for providing infrastructure to carry out the study.

Author contributions

VKK conceived the idea and designed the methodology; JK conducted experiments and collected the data; JK analyzed the data and wrote the manuscript; VKK reviewed and edited the manuscript. All authors read and approved the manuscript.

Funding

Not applicable.

Availability of data and materials

The authors confirm that the data supporting the findings of this study are available within the article.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Received: 22 June 2023 Accepted: 21 September 2023
Published online: 11 October 2023

References

Abbott WS (1925) A method of computing the effectiveness of an insecticide. J Econ Entomol 18(2):265–267. https://doi.org/10.1093/jee/18.2.265a

Avsar C, Koyuncu H, Aras ES (2017) Isolation and molecular characterization of *Bacillus* spp. isolated from soil for production of industrial enzymes. Biol Chem Res 3(9):72–86

Bruce T (2020) This "Very Hungry Caterpillar" is wreaking havoc in fields around the world. In World Economic Forum. https://www.weforum.org/agenda/2019/07/fall-armyworm-asia-devastating-crops/ (Vol. 24).

Centre for Agriculture and Bioscience International (CABI) (2018) CABI warns of rapid spread of crop-devastating fall armyworm across Asia, https://www.cabi.org/news-and-media/2018/cabi-warns-of-rapid-spreadof-crop-devastating-fall-armyworm-across-asia/

Daravath V, Anubhav M, Mandla R, Kalia VK (2015) Distribution and insecticidal activity of *Bacillus thuringiensis* strains isolated from warehouses in India. Biopesticides Int 11(2):96–107

Delanthabettu A, Narasimhappa NS, Ramaswamy A, Mallesh MH, Nagarajappa N, Govind G (2022) Molecular Characterization of Native *Bacillus thuringiensis* Strains from Root Nodules with Toxicity Against the Fall Armyworm (FAW, *Spodoptera frugiperda*) and Brinjal Ash Weevil (*Myllocerus subfasciatus*). Curr Microbiol 79(9):1–2. https://doi.org/10.1007/s00284-022-02951-0

Deshmukh S, Pavithra HB, Kalleshwaraswamy CM, Shivanna BK, Maruthi MS, Mota-Sanchez D (2020) Field efficacy of insecticides for management of invasive fall armyworm, *Spodoptera frugiperda* (JE Smith) (Lepidoptera: Noctuidae) on maize in India. Fla Entomol 103(2):221–227. https://doi.org/10.1653/024.103.0211

Elbrense H, Elmasry A, Seleiman MF, Al-Harbi MS, El-Raheem A, Ahmed M (2021) Can symbiotic bacteria (*Xenorhabdus* and *Photorhabdus*) be more efficient than their Entomopathogenic nematodes against *Pieris rapae* and *Pentodon algerinus* larvae? Biology 10(10):999. https://doi.org/10.3390/biology10100999

Ganiger PC, Yeshwanth HM, Muralimohan K, Vinay N, Kumar AR, Chandrashekara K (2018) Occurrence of the new invasive pest, fall armyworm, *Spodoptera frugiperda* (JE Smith) (Lepidoptera: Noctuidae), in the maize fields of Karnataka, India. Curr Sci 115(4):621–623. https://doi.org/10.18520/cs/v115/i4/621-623

Gopalakrishnan R, Kalia VK (2022) Biology and biometric characteristics of *Spodoptera frugiperda* (Lepidoptera: Noctuidae) reared on different host plants with regard to diet. Pest Manage Sci 78(5):2043–2051. https://doi.org/10.1002/ps.6830

Handayani K, Janah SM, Ekowati CN, Kanedi M (2023) Larvicide effects of *Bacillus* sp isolated from soil against fall armyworm *Spodoptera frugiperda* (Lepidoptera: Noctuidae). GSC Biol Pharm Sci 22(03):1028–1034. https://doi.org/10.30574/gscbps.2023.22.3.0088

Hernandez JLL (1988) Évaluation de la toxicité de *Bacillus thuringiensis* sur *Spodoptera frugiperda*. Entomoph 32:163–171

Lambert B, Buysse L, Decock C, Jansens S, Piens C, Saey B, Seurinck J, Van Audenhove K, Van Rie J, Van Vliet A, Peferoen MA (1996) *Bacillus thuringiensis* insecticidal crystal protein with high activity against members of the family Noctuidae. Appl Environ Microbiol 62:80–86. https://doi.org/10.1128/aem.62.1.80-86.1996

Li H, Soares MA, Torres MS, Bergen M, White JF Jr (2015) Endophytic bacterium, *Bacillus amyloliquefaciens*, enhances ornamental hosta resistance to diseases and insect pests. J Plant Interact 10(1):224–229. https://doi.org/10.1080/17429145.2015.1056261

Liu YB, Tabashnik BE, Moar WJ, Smith RA (1998) Synergism between Bacillus thuringiensis spores and toxins against resistant and susceptible diamondback moths (*Plutella xylostella*). Appl Environ Microbiol 64:1385–1389. https://doi.org/10.1128/aem.64.4.1385-1389.1998

Lopes Lastra CC, Boucias DG, Soares GG Jr (1995) *Bacillus thuringiensis* endotoxin effects on *Spodoptera exigua* and *Spodoptera frugiperda* larva infected with baculovirus. Env Entomol 24:239–242. https://doi.org/10.1093/ee/24.2.239

Maheesha M, Balasubramani V, Murugan M, Raveendran M, Rajadurai G, Tamilnayagan T, Kokiladevi E, Sathiah N (2021) Characterisation of native *Bacillus thuringiensis* isolates toxicity to fall armyworm, *Spodoptera frugiperda* (JE Smith). J Biol Control 35(3):171–180

Manker DC, Lidster WD, MacIntosh SC, Starnes RL (2002) Potentiator of *Bacillus* pesticidal activity. Patent Number US Patent: 6406691.

Mansour NA, Eldefrawi ME, Tappozada A, Zied M (1966) Toxicological studies on the Egyptian cotton leafworm *Prodenia litura* F.VI. Potentiation and antagonism of organophosphorus and carbamates. J Econ Entomol 59:307–311. https://doi.org/10.1093/JEE%2F59.2.307

Nayyar N, Gracy RG, Ashika TR, Mohan G, Swathi RS, Mohan M, Chaudhary M, Bakthavatsalam N, Venkatesan T (2021) Population structure and genetic diversity of invasive Fall Armyworm after 2 years of introduction in India. Sci Rep 11(1):1–2. https://doi.org/10.1038/s41598-021-87414-5

Pashley DP (1988) Current status of fall armyworm host strains. Fla Entomol 1:227–234. https://doi.org/10.2307/3495425

Penha RO, Vandenberghe LP, Faulds C, Soccol VT, Soccol CR (2020) *Bacillus* lipopeptides as powerful pest control agents for a more sustainable and healthy agriculture: recent studies and innovations. Planta 251:1–5. https://doi.org/10.1007/s00425-020-03357-7

Polanczyk RA, Alves SB (2005) Biological parameters of *Spodoptera frugiperda* (JE Smith) (Lepidoptera: Noctuidae) assayed with *Bacillus thuringiensis* Berliner. Scientia Agricola 62:464–468. https://doi.org/10.1590/S0103-90162005000500009

Polanczyk RA, Silva RF, Fiuza LM (2000) Effectiveness of *Bacillus thuringiensis* strains against *Spodoptera frugiperda* (Lepidoptera: Noctuidae). Braz J Microbiol 31:164–166. https://doi.org/10.1590/S1517-83822000000300003

Priyanka M, Yasodha P, Justin CG, Ejilane J, Rajanbabu V (2021) Biorational management of maize fall armyworm, *Spodoptera frugiperda* (J. E. Smith) (Lepidoptera: Noctuidae) using *Bacillus thuringiensis* (Berliner) enriched with chemical additives. J Appl Nat Sci 13(4):1231–1237. https://doi.org/10.31018/jans.v13i4.2999

Rajashekhar M, Mittal A, Veeranna D, Kalia V (2017) Characterization of potential native *Bacillus thuringensis* strains isolated from insect cadavers against cotton aphid *Aphis gossypii* Glover (Hemiptera: Aphididae). Indian J Entomol. https://doi.org/10.5958/0974-8172.2018.00032.9

Regev A, Keller M, Strizhov N, Sneh B, Prudovsky E, Chet I, Ginzberg I, Koncz-Kalman Z, Koncz C, Schell J, Zilberstein A (1996) Synergistic activity of a *Bacillus thuringiensis* d-endotoxin and a bacterial endochitinase against *Spodoptera littoralis* larvae. Appl Environ Microbiol 62:3581–3386. https://doi.org/10.1128/aem.62.10.3581-3586.1996

Ross GJS (2000) MLP: maximum likelihood program, user manual. Numerica Algorithms Group, Oxford

Sanahuja G, Banakar R, Twyman RM, Capell T, Christou P (2011) *Bacillus thuringiensis*: a century of research, development and commercial applications. Plant Biotechnol J 9:283–300. https://doi.org/10.1111/j.1467-7652.2011.00595.x

Shankar G, Adachi Y (2019) First report of the occurrence of fall armyworm, *Spodoptera frugiperda* (JE Smith) (Lepidoptera: Noctuidae) on Ginger (*Zingiber officinale*) in Haveri district, Karnataka. India J Entomol Zool Stud 7(5):78–80

Swamy HM, Asokan R, Kalleshwaraswamy CM, Prasad YG, Maruthi MS, Shashank PR, Devi NI, Surakasula A, Adarsha S, Srinivas A, Rao S (2018) Prevalence of "R" strain and molecular diversity of fall army worm *Spodoptera frugiperda* (JE Smith) (Lepidoptera: Noctuidae) in India. Indian J Entomol 80(3):544–553

Usta C (2013) Microorganisms in biological pest control-a review (bacterial toxin application and effect of environmental factors). Curr Progress Biol Res 13:287–317

Zhang L, Liu B, Zheng W, Liu C, Zhang D, Zhao S, Xu P, Wilson K, Withers A, Jones CM, Smith JA (2019) High-depth resequencing reveals hybrid population and insecticide resistance characteristics of fall armyworm (*Spodoptera frugiperda*) invading China. BioRxiv. https://doi.org/10.1101/813154

Publisher's Note