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RESEARCH

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Endophytic establishment of native *Bacillus thuringiensis* strain in maize plants and its efficacy against *Spodoptera frugiperda* (J.E. Smith) (Lepidoptera: Noctuidae)

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Abstract

Background *Bacillus thuringiensis* (*Bt*) is known as the most successful microbial insecticide worldwide used against lepidopteran insect pests in agriculture. Native *Bacillus* isolate VKK5 showing insecticidal activity against *Spodoptera frugiperda* (FAW) (J.E. Smith) (Lepidoptera: Noctuidae) was characterized as *B. thuringiensis* (BtVKK5) on a morphological and molecular basis. Recent research has shown that *Bt* can be established as an endophytic organism for controlling insect pests. The present work aimed at assessing the colonization of BtVKK5 as an endophyte in five maize cultivars by seed treatment (ST), soil drenching (SD), foliar application (FA) and combination of all methods (ST + SD + FA) and its bioefficacy against neonates of FAW.

Results Establishment of inoculated BtVKK5 as endophytes in five maize cultivars, viz. Pusa HQPM7 Improved, Pusa Jawahar Hybrid Maize 1, Pusa Vivek Hybrid 27 Improved (PVH27I), Pusa HQPM5 Improved and DMRH 1301, was confirmed by re-isolating from the leaves of the plant on ampicillin-selected agar plates. Estimation of colony-forming units per gram of leaf showed that there was a significant difference in colonization of the *Bt* strain among maize cultivars by different inoculation methods. The colonies were further substantiated by the amplification of *cry1A* and *cry1E* genes. Bioefficacy studies showed the highest mortality (50%) in the ST + FA + SD inoculation method, followed by ST (40%) in PVH27I. Moreover, growth inhibition was observed in survived larvae on inoculated plants vis-a-vis control.

Conclusion Establishment of *Bt* strain as an endophyte in maize plants, complemented with insecticidal activity, could possibly lead to an innovative approach to the management of *S. frugiperda* and other borers.

Keywords *Bacillus thuringiensis*, Maize plants, Endophyte, Colonization, Fall armyworm, Bioefficacy, Seed treatment, Foliar application, Soil drenching

Background

Maize (*Zea mays* L.) is one of the most versatile emerging crops having wider adaptability and highest genetic yield potential among the cereals. It is the third most important cereal crop in India after rice and wheat. It accounts for around 10% of total food grain production in India (APEDA 2019). The fall armyworm (FAW) *Spodoptera frugiperda* (J.E. Smith) (Lepidoptera: Noctuidae) is an invasive polyphagous insect pest engender significant

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damage to several crops, including maize, cotton, rice, soybean, tomato, potato, onion, bean, cabbage, sorghum, etc. (Day et al. 2017). It is an economically important insect pest and poses major threat to food security due to its rapid spread and ability to cause substantial distortion to multiple crops. FAW causes maize yield reduction of up to 70%, when maize plants are attacked during early stages (Hruska 2019). FAW attacks all stages of maize crop from seedling emergence to ear development (Sisay et al. 2019). After the invasion of FAW in India, the pest scenario in maize drastically changed, and it quickly spread to all maize-growing regions, posing a serious threat to maize productivity throughout India (Suby et al. 2020). The evaluation and exploitation of resistant genotypes for inclusion in an IPM schedule is the first line of strategy available for the control of FAW. The search for resistant maize genotypes against FAW is just beginning in India (Soujanya et al. 2022). The chemical insecticides anthranilic diamides, avermectins and spinosyns have been registered in India for urgent management of FAW based on their exclusive mode of action. Moreover, the efficacy of these insecticides on mature FAW larvae is limited due to its cryptic feeding behavior (De Groote et al. 2020). Thus, management of FAW is challenging as currently relies on chemical insecticides and indiscriminate use of various insecticides can adversely affect the ecosystem and its inhabitants (Harrison et al. 2019). Besides, FAW has become resistant to many chemical insecticides and transgenic *Bt*-maize (Prasanna et al. 2018). Entomopathogens, the naturally occurring population regulators of insect pests, are another option for controlling FAW. Of that *Bacillus thuringiensis* (*Bt*) is known as the most successful entomopathogen used as microbial insecticide worldwide (Bravo et al. 2011).

Bacillus thuringiensis is a Gram-positive bacterium which produces protein toxins to kill insects of different groups with high host specificity and environmental safety (Sanahuja et al. 2011). But their commercial application as formulation is limited due to their susceptibility to abiotic factors, viz. temperature, humidity and UV rays. To overcome these bottlenecks, a hidden ecological role played by entomopathogenic bacteria (EPB), as endophytes, was sought. Endophytes are nonpathogenic microbial symbionts residing within the plant for the majority of their life cycle (Brader et al. 2014). Endophytic association with the plant boosts up the plant growth by synthesizing phytohormones, nitrogen fixation, phosphorous solubilization, modulation of plant metabolism and confers resistance to biotic and abiotic stresses (Irizarry and White 2018). Some investigations demonstrated this novel approach of pest management (Pola et al. 2022). Plant–bacteria mutualistic symbiosis as endophytes can benefit plants by increasing its fitness by inducing resistance to insect pests. The present work aimed to assess the colonization potential of native *Bt* strain as endophytes in maize plant as well as its insecticidal activity against *S. frugiperda*.

Methods

Bacterial strain

Native *Bacillus* isolate VKK5 used in this work was taken from the bacterial stock of the National Facility for Insect Rearing and Xenobiotic-Transgenic Bioassays, Division of Entomology, Indian Agricultural Research Institute (IARI), New Delhi. Forty-nine native *Bacillus* isolates were evaluated against neonates of *S. frugiperda* for its insecticidal activity using bacterial suspensions of 1×10^8 cells/ml by diet incorporation methods under controlled laboratory conditions, i.e., at 27 ± 1 °C and 70 ± 5% RH.

Morphological and molecular characterizations of Bacillus isolate VKK5

Phase contrast and electron microscopy

Morphological characterization of *Bacillus* isolate VKK5 was initially performed using phase contrast microscopy. For electron microscopy, the *Bacillus* isolate was grown overnight in Luria broth and the cell pellet was washed with 0.1N NaCl solution to remove the cell debris. Then, the cell pellet was suspended by adding 0.1 M sodium phosphate buffer twice and centrifuged at 10,000 rpm for 5 min to remove the buffer. Then, the cell pellet was fixed with Karnovsky's fixative overnight and again washed with 0.1 M sodium phosphate buffer. The final cell pellet was submitted at Transmission Electron Microscope (TEM), Sophisticated Analytical Instrumentation Facility (SAIF), All Indian Institute of Medical Science (AIIMS), New Delhi, for cutting the transection of cell pellet, and the thin section was viewed through TEM (CRYO-TEM (TALOS) from Thermo Scientific company) at different magnifications to visualize the cell and spore crystal structure of *Bt*.

16S rRNA gene sequencing and phylogenetic analysis

Using Quick-DNATM Fungal/Bacterial Miniprep by ZYMO RESEARCH, the genomic DNA was isolated from Bacillus strainVKK5 in accordance with the manufacturer's instructions and verified on a 0.8% agarose gel. The 16S rRNA gene was amplified with 16S primer (27F: AGAGTTTGATCTGGCTCAG and 1492R: GGTTAC CTTGTTACGACTT). All PCR reactions were carried out in 50 µl reaction volumes as per Rajashekhar et al. (2017). The PCR amplified product was verified with 1.2% agarose gel. Gels were documented using Alphaimager™ documentation and analyzed with AlphaEase™ software. Subsequently, ~ 1.5 kb PCR products were purified using a PCR purification kit (Qiagen, Germany). Purified

samples were sent for sequencing at Barcode Biosciences Pvt. Ltd. (Bengaluru, India). Sequences obtained were processed by trimming the poor quality bases manually using, Bioedit (Version 7.2.6.1) and submitted in GenBank (NCBI).

Amplification and characterization of cry genes

Bacillus thuringiensis strains were grown for 12 h on Luria agar plates and a loopful cell was transferred to 100 µl of sterile distilled water, and the mixture was frozen for 20 min at − 80 °C and then transferred to boiling water for 10 min to lyse the cells. The resulting cell lysate was centrifuged at 10,000 rpm for 30 s, and supernatant was used as a DNA template in the PCR as per Sarkar and Kalia (2021). The presence of *cry1* and *cry2* genes was performed to identify the toxin-encoding genes using oligonucleotide primers specific for genes (Additional file 1: Table S1).

Antibiotic susceptibility

In order to characterize BtVKK5 further, antibiotic susceptibility test was performed by the standard disk-diffusion method on Luria agar plates with antibiotic disks (HI media- OD043-IPK). This Hi-media octa disk contains 8 antibiotics, viz. co-trimoxazole (COT) (25 mcg), streptomycin (S) (10 mcg), tetracycline (TE) (30 mcg), chloramphenicol (C) (30 mcg), amikacin (AK) (30 mcg), ampicillin (AMP) (10 mcg), gentamicin (GEN) (10 mcg) and kanamycin (K) (30 mcg). The overnight grown culture of BtVKK5 in Luria broth was spread plated on Luria agar plate before placing the antibiotic disks and allowed to incubate for 24 h at 30 °C. After 24 h zone of inhibition was observed around the antibiotic disk.

Plant material

Seeds of five cultivars of maize, viz. Pusa HQPM 7 Improved (PHQPM7I), Pusa Jawahar Hybrid Maize 1 (PJHM1), Pusa Vivek hybrid 27 improved (PVH27I), Pusa HQPM 5 Improved (PHQPM5I) and DMRH 1301, were procured from Division of Genetics, IARI, New Delhi, and Indian Institute of Maize Research, New Delhi. The potting mixture was prepared by mixing soil, vermiculite and cocopeat in 1:1:1 ratio and autoclaved. The seeds of all the five cultivars were surface sterilized by washing with tap water thrice followed by 70% ethanol for 3 min and 2% sodium hypochloride (NaOCl) solution for 5 min thrice and finally washed with autoclaved distilled water (ADW) (Pola et al. 2022). The surface sterilized seeds were used for inoculation of BtVKK5 in seed treatment and sowing.

Inoculation methods of bacteria into plants

Four methods, viz. seed treatment (ST), foliar application (FA), soil drenching (SD) and combination (ST + FA + SD) of all methods, were used for inoculation of BtVKK5 into plants. Nine sterilized seeds of each cultivar for seed treatment and combination treatment were soaked in VKK5 @1×10^8 cfu/ml for 48 h. Total 50 ml culture of VKK5 in Luria broth was used in each treatment for seed soaking. For foliar application, soil drenching and control, the sterilized seeds were soaked in ADW for 48 h. Equal quantity of sterilized potting mixture was filled in the pots of 17 × 12.5 cm size (capacity 2 l). Seeds were sown in pots at the rate of 3 seeds/pot and 3 pots/treatment. The plants were maintained in net house. For foliar application, the bacterial culture at the concentration of 1×10^8 cfu/ml was sprayed at 15 days after emergence (DAE) until the leaf became wet (~ 5 ml/plant). For soil drenching, the bacterial culture was poured near the root zone of 15 days old plant at the rate of 5 ml/plant. For combination treatment, the plants in which seed treatment was given were sprayed on 15 DAE with 1×10^8 cfu/ml until they became wet (~ 5 ml/plant) as well as bacterial culture was poured near the root zone @ 5 ml/plant (Pola et al. 2022). In all the treatments, respective controls were treated with ADW.

Retrieval of *Bacillus thuringiensis* strain VKK5 from inoculated maize plants over time

The leaf was taken from control and ST plants after 7, 14, 21, 28 and 35 days after emergence (DAE). But for FA, SD and ST + FA + SD-treated plants, leaf was collected after 7, 14, 21, 28 and 35 days after inoculation (DAI). For each treatment, leaves were collected from three plants. 100 mg of leaf from each plant was surface sterilized by washing with 70% ethanol and 2% sodium hypochlorite solution followed by 3 washes of ADW. A 100 µl volume of the last wash water was plated on Luria agar plates and incubated at 30 °C for 24 h to ensure free from epiphytic bacteria. Then, the surface sterilized leaf was homogenized in 1000 µl of ADW with the help of pestle and mortar. Supernatant was collected, and serial dilutions of 1:10, 1:100 and 1:1000 were prepared. A 100 µl volume of 1: 1000 dilutions was plated on selective Luria agar plates containing ampicillin (50 µg/ml). Three replicates per treatment were taken. The whole process was carried out in aseptic condition in laminar air flow. The plates were incubated at 30 °C for 24 h. Bacterial colonies growing in each plate were counted, and the cfu/gm was calculated. Further colonies were confirmed by both colony morphological and molecular basis (colony PCR).

Confirmation of *cry1A* and *cry1E* gene in *Bacillus thuringiensis* strain VKK5 colonies retrieved from leaves

To ensure the presence of cry genes in recovered colonies from leaves, colony PCR was performed with specific *cry1A* primers F: 5'CCGGTGCTGGATTTG TGTTA-3' and R: 5'AATCCCGTATTGTACCAGCG-3' (Carozzi et al. 1991) and *cry1E* primers F: 5'AGGGCA TCGTGTAACTTCTC 3' and R: 5'CCACCACCAAGT CTAAATCC3'. A loopful cell was transferred to 20 µl nuclease-free water, 2.5 µl of Taq buffer (10X) with $MgCl_2$, 0.33 µl of Taq DNA polymerase, 0.2 µl of dNTPs and 1 µl of each forward and reverse primer. The PCR condition were, initial denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 52 °C (*cry1A*) and 52.3 °C (*cry1E*) for 1 min, extension at 72 °C for 2 min and a final extension at 72 °C for 5 min in a thermocycler (FlexigeneTechne, UK). After amplification, 2 µl of loading buffer (0.5% bromophenol blue in 50% glycerol) was added to 5 µl of each amplified PCR product and was electrophoresed (at 70 V for 10 min, followed by 90 V for 45 min) on a 1 × Tris–acetate-EDTA (TAE with ethidium bromide) buffer in 1.2% agarose gel. Gels were visualized in a gel documentation system (AlphaimagerTM) and analyzed with AlphaEaseFC.

Bioassay of inoculated plants against fall armyworm *Spodoptera frugiperda*

FAW larvae were collected from a maize field of the IARI, New Delhi, and were reared on Kabuli gram-based semi-synthetic diet till pupation in the laboratory at 27 ± 1 °C, 70 ± 5% RH and 14:10 L/D photoperiod as per Gopalakrishnan and Kalia (2022). On emergence, five pairs of adults were transferred to mating jar (20 cm height and 15 cm diameter) containing 10% fortified honey solution for adult feeding and folded blotting paper strips for egg laying. Egg masses were collected daily and kept in a separate container. Neonates (< 24 h old) were used for conducting bioassay. Leaf bits (1″ size) of 14 and 21 DAE in seed treatment and 14 and 21 DAI from FA, SD, ST + FA + SD inoculated plants, and their respective control plants were collected for leaf bioassay. Each treatment was replicated three times with 10 larvae per replicate. Leaf bits were changed on daily basis to overcome food shortage and dryness of leaf. The mortality was taken daily till 7 days. After 7 days of surviving, larvae were collected from each treatment as well as control and weighed individually to compare the weight attained on control with regard to inoculated maize plants by different methods, viz. seed treatment, foliar application, soil drenching and combination of all inoculation methods.

Statistical analysis

Data of cfu/g of leaf among different maize cultivars, different methods and different days after inoculation and average weight of the larvae after leaf bioassay were analyzed by ANOVA (SAS version 9.2, SAS Institute). The significantly different means were separated using Duncan's Multiple Range (DMRT) test.

Results

Morphological and molecular characterization of *Bacillus* isolate VKK5

Phase contrast microscopy of sporulated cultures of *Bacillus* isolate VKK5 showing typical parasporal inclusion bodies (crystal) (Fig. 1). Further, transmission electron microscopy revealed the presence of polymorphic crystals, viz. bipyramidal, cuboidal, trapezoidal, irregular, spherical and ovoid shapes (Fig. 2), which suggested a variety of endotoxin generation. Additionally, a homology search was performed on the 16S rRNA gene sequence (1479 bp) using the BLAST tool of the National Centre for Biotechnology Information (NCBI), and a phylogenetic tree was constructed via https://www.ncbi.nlm.nih.gov/blast/treeview/treeView.cgi?request=page&blastRID=2D1XT12R013&queryID=gb|OP743352| (Additional file 1: Fig. S1). The sequence was submitted to NCBI (GenBank accession number OP743352), and based upon taxonomic identity as well as the presence of crystal, VKK5 was characterized as *B. thuringiensis* strain VKK5, and henceforth, BtVKK5 was used throughout the paper. PCR amplification using *cry* gene-specific primers showed that the BtVKK5 possesses *cry*1A, *cry*1B, *cry*1E, *cry*1I, and *cry*2 genes (Fig. 3). Antibiotic sensitivity tests showed that BtVKK5 was resistant to only one antibiotic (ampicillin) out of eight tested (Additional file 1: Fig. S2).

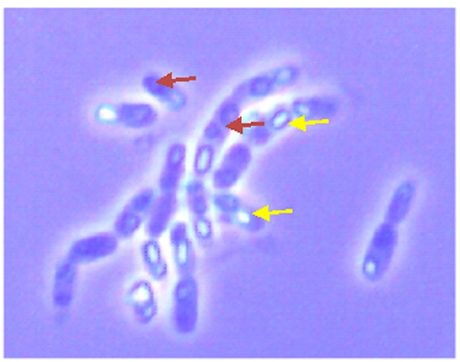

Fig. 1 Photomicrographs of *Bacillus thuringiensis* strain VKK5 viewed by phase contrast microscope (1000 × magnification) showing rod shaped cells with the parasporal crystals (red arrow) of insecticidal toxin, which are less phase bright than the spores (yellow arrow)

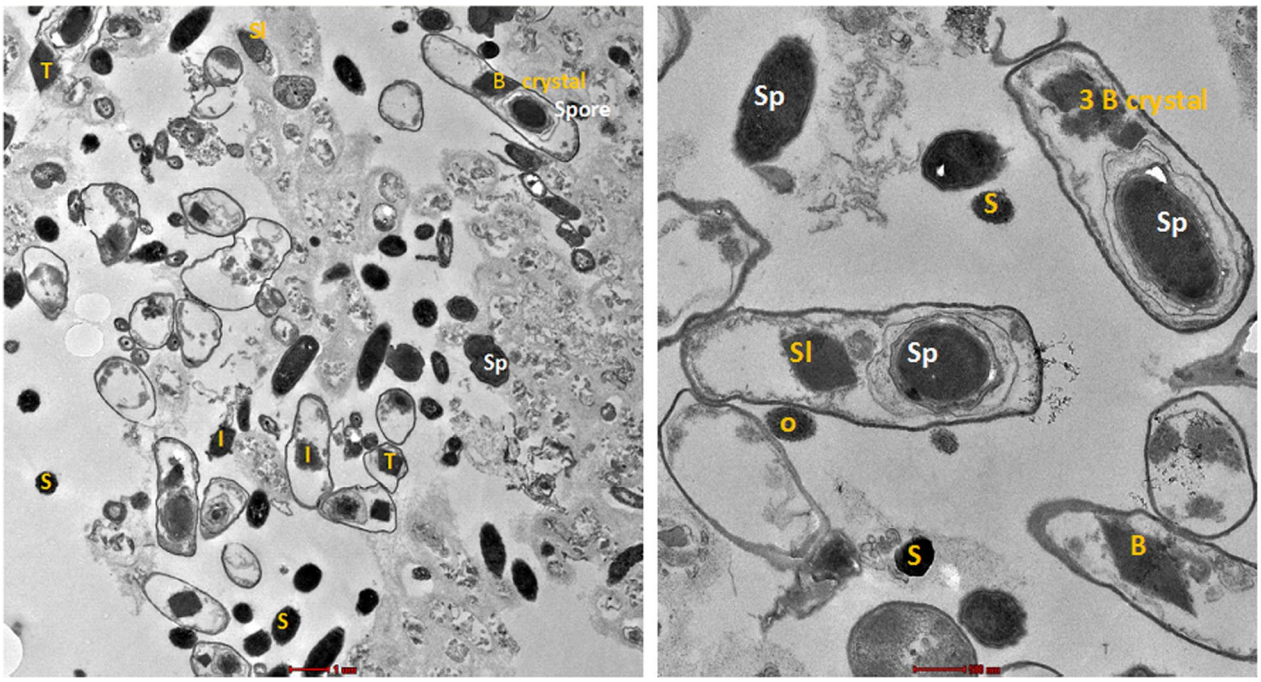

Fig. 2 Transmission electron microscopy showing spore crystal morphology of *Bacillus thuringiensis* strain VKK5. B, bipyramidal; S, spherical; Sl, spindle O, ovoid; I, irregularly; T, trapezoid shaped; and Sp, spore

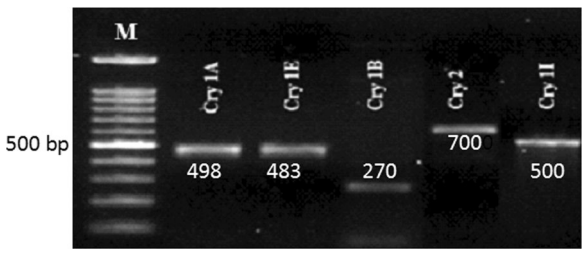

Fig. 3 PCR product amplified with *cry1A, cry1B, cry1E, cry1I* and *cry2* gene primers in *Bacillus thuringiensis* strain VKK5. M- Marker (100 bp DNA marker)

Colonization of native *Bacillus thuringiensis* strain VKK5 into plants and confirmation of retrieved bacteria from plants

BtVKK5 was found to be the most effective, with a mortality rate of 65% out of 49 native *Bacillus* isolates tested against neonates of *S. frugiperda* and shortlisted for colonization into maize plants. Pot culture experiments containing five cultivars of maize in net house condition which were inoculated with BtVKK5 shown in Fig. 4A. Retrieval of colonies from inoculated plants was successful in all the cultivars. The retrieved colonies were morphologically similar to the inoculated BtVKK5 (Fig. 4B), and no *Bt* colonies were observed in control plants. PCR with *cry1A* and *cry1E* gene-specific primer produced an expected amplicon size of 498 bp and 483 bp, respectively (Fig. 4C, D).

Quantification of colonization in different maize cultivars

Perusal of data in Table1 shows that on 7 DAI no cfu/g of leaf was found in DMRH 1301, PHQPM5I and PHQPM7I in SD inoculation method and found to be at par with control. The lowest mean cfu/g of leaf (0.33×10^4) was recorded in PJHM1 by SD method. The highest (1.0×10^5) cfu/g was observed in PVH27I followed by PHQPM7I (9.67×10^4) with ST + FA + SD method. The mean cfu/g was at par for PHQPM7I (ST + FA + SD) and PVH27I (ST, FA, ST + FA + SD) method but was significantly different from all other cultivars and all other inoculation methods as well. On 14 DAI, the mean cfu/g of leaf was the lowest (1.33×10^4) in DMRH 1301 with SD method, and it was at par with PHQPM5I (SD), PHQPM7I (SD), PJHM1 (ST, FA, SD), PVH27I (SD). The highest (6.63×10^5) cfu/g was observed in PVH27I with ST method and it was at par with ST + FA + SD method of same cultivar.

The mean cfu/g of leaf on 21 DAI was the lowest (3×10^4) in DMRH 1301 with SD method, and it was at par with SD method of all cultivars. The highest (1.11×10^6) cfu/g was observed in PVH27I with ST + FA + SD method, and it was significantly different from all other treatments except ST method of same cultivar. On 28 and 35 DAI, the highest cfu/g was observed

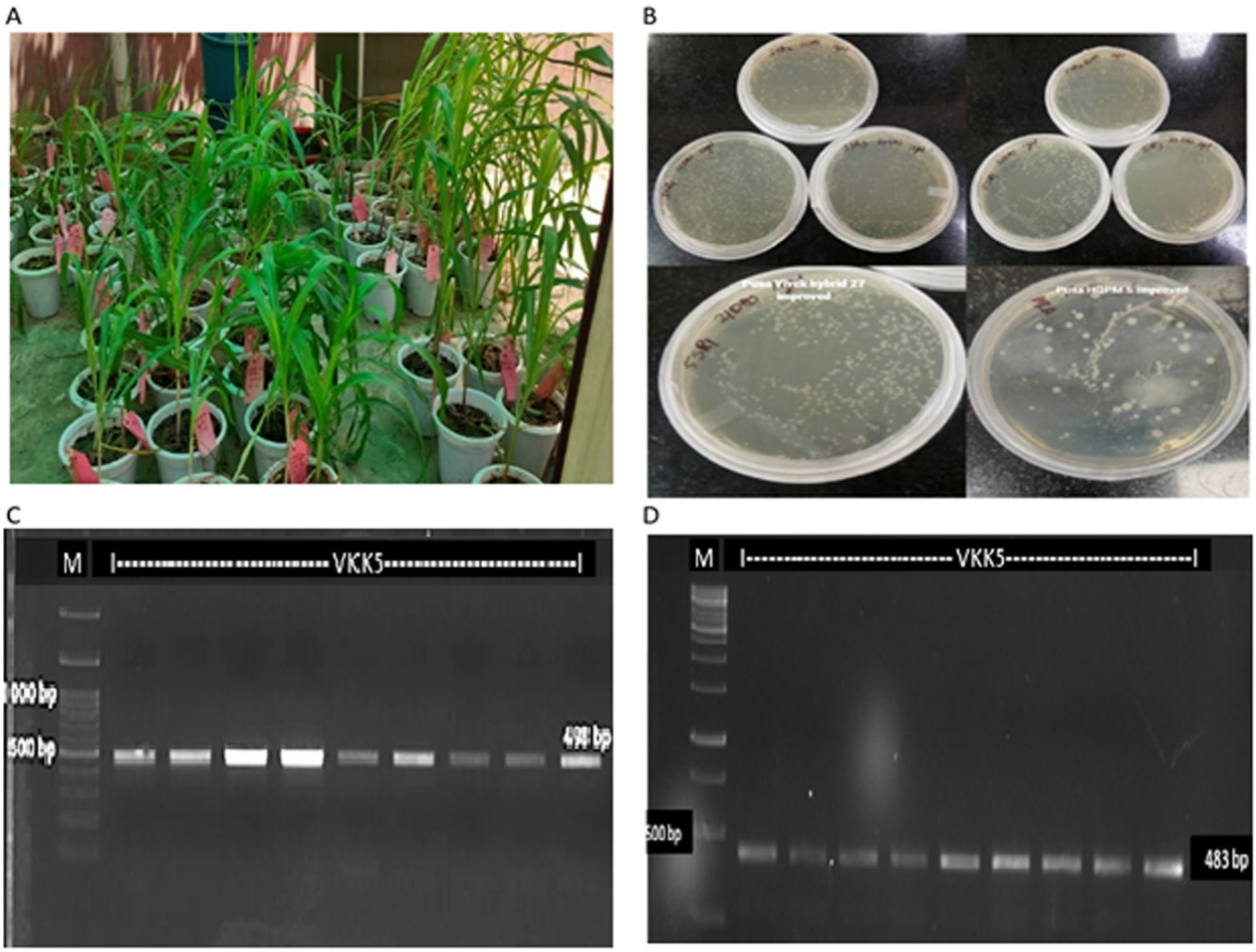

Fig. 4 **A.** Pot culture experiments of five maize cultivars inoculated with *Bacillus thuringiensis* strain VKK5 by different methods; **B**. *B. thuringiensis* strain VKK5 colonies retrieved from inoculated plant; **C**. Confirmation of *cry1A* gene in retrieved colonies; and **D**. Confirmation of *cry1E* gene in retrieved colonies (M- Marker)

in PVH27I with ST + FA + SD method (1.04×10^6 and 1.07×10^6, respectively), and it was at par with ST method of same cultivar but was significantly different from all other treatments. The lowest (2.67×10^4) mean cfu/g of leaf on 35 DAI was observed in DMRH 1301 with SD method and it was at par with DMRH 1301(ST, FA), PHQPM5I (FA, SD), PHQPM7I (SD), PJHM1 (SD) and PVH27I (SD).

The results of cfu/g of leaf on different DAI from different inoculation method of different maize cultivars show that highest colonization was occurred in PVH27I with ST + FA + SD method on 21, 28 and 35 DAI and the lowest colonization occurred in DMRH 1301 hybrid with SD method on 14, 21, 28 and 35 DAI (Table 1). Furthermore, it was found that the colonization was low in 7 DAI irrespective of methods and cultivars. Colonization was reached maximum at 21 DAI, and further there is no increase in cfu. However, more or less stable mean cfu/g of leaf was recorded in 28 and 35 DAI in all cultivars with regard to methods. In accordance with cultivars, PVH27I showed maximum colonization with respect to all methods and the lowest was observed in DMRH 1301. Among all the inoculation methods ST and combination of all method (ST + FA + SD) showed at par colonization, followed by FA in all the cultivars. The soil drenching is the less efficient method for colonization when compared to all other methods. The colonization and establishment of BtVKK5 was depended on the cultivar, inoculation methods and days after inoculation.

Evaluation of efficacy of *B. thuringiensis* strain VKK5 colonized plants against *S. frugiperda* by detached leaf bioassay

The efficacy of colonized BtVKK5 as endophyte in maize plants against neonates of FAW was validated by leaf bioassay. The observations on mortality were taken till 7th day after releases. The results indicated that on 14 DAI the highest mortality was observed in PVH27I

Table 1 Mean colony-forming unit per gram of leaf in different maize cultivars with respect to different inoculation methods at different days after inoculation

Maize Varieties	Inoculation Method	Mean cfu/g of leaf ± SEM				
		7 DAI	14 DAI	21 DAI	28 DAI	35 DAI
DMRH 1301	Control	0.00 ± 0.00^{h}	0.00 ± 0.00^{h}	0.00 ± 0.00^{j}	0.00 ± 0.00^{j}	0.00 ± 0.00^{i}
	ST*	$2.33 \pm 0.88 \times 10^{4fgh}$	$9.00 \pm 1.73 \times 10^{4defg}$	$2.13 \pm 3.18 \times 10^{5\,g}$	$1.5 \pm 2.08 \times 10^{5efg}$	$9.33 \pm 1.20 \times 10^{4efghi}$
	FA	$1.00 \pm 0.58 \times 10^{4gh}$	$8.33 \pm 1.86 \times 10^{4defg}$	$1.76 \pm 2.60 \times 10^{5gh}$	$9.00 \pm 1.53 \times 10^{4fghij}$	$8.33 \pm 0.67 \times 10^{4efghi}$
	SD	0.00 ± 0.00^{h}	$1.33 \pm 0.33 \times 10^{4gh}$	$3.00 \pm 0.58 \times 10^{4j}$	$2.00 \pm 0.58 \times 10^{4j}$	$2.67 \pm 0.67 \times 10^{4hi}$
	ST + FA + SD	$2.67 \pm 0.88 \times 10^{4fgh}$	$1.13 \pm 0.88 \times 10^{5ed}$	$2.20 \pm 2.52 \times 10^{5\,g}$	$1.60 \pm 1.00 \times 10^{5ef}$	$1.37 \pm 1.20 \times 10^{4defg}$
Pusa HQPM 5 improved	Control	0.00 ± 0.00^{h}	0.00 ± 0.00^{h}	0.00 ± 0.00^{j}	0.00 ± 0.00^{j}	0.00 ± 0.00^{i}
	ST*	$6.67 \pm 2.23 \times 10^{4bdec}$	$2.73 \pm 2.33 \times 10^{5b}$	$4.03 \pm 2.73 \times 10^{5de}$	$2.87 \pm 1.86 \times 10^{4d}$	$1.47 \pm 1.45 \times 10^{4def}$
	FA	$4.00 \pm 1.15 \times 10^{4efg}$	$1.23 \pm 3.76 \times 10^{5ed}$	$2.70 \pm 3.46 \times 10^{5\,fg}$	$2.07 \pm 4.84 \times 10^{5ed}$	$9.67 \pm 0.88 \times 10^{4efghi}$
	SD	0.00 ± 0.00^{h}	$2.67 \pm 0.33 \times 10^{4fgh}$	$4.33 \pm 1.45 \times 10^{4ij}$	$3.33 \pm 0.88 \times 10^{4ij}$	$3.33 \pm 0.33 \times 10^{4ghi}$
	ST + FA + SD	$6.33 \pm 2.19 \times 10^{4cde}$	$2.83 \pm 2.19 \times 10^{5b}$	$3.60 \pm 3.06 \times 10^{5ef}$	$2.93 \pm 2.40 \times 10^{5d}$	$2.13 \pm 1.45 \times 10^{4d}$
Pusa HQPM 7 improved	Control	0.00 ± 0.00^{h}	0.00 ± 0.00^{h}	0.00 ± 0.00^{j}	0.00 ± 0.00^{j}	0.00 ± 0.00^{i}
	ST*	$4.00 \pm 1.53 \times 10^{4efg}$	$9.67 \pm 1.20 \times 10^{4def}$	$7.43 \pm 7.31 \times 10^{5b}$	$6.37 \pm 5.55 \times 10^{5b}$	$6.30 \pm 7.94 \times 10^{4b}$
	FA	$1.67 \pm 0.33 \times 10^{4gh}$	$8.67 \pm 0.88 \times 10^{4defg}$	$4.83 \pm 3.53 \times 10^{5\,cd}$	$4.00 \pm 2.52 \times 10^{5c}$	$4.43 \pm 2.19 \times 10^{5c}$
	SD	0.00 ± 0.00^{h}	$2.67 \pm 0.67 \times 10^{4fgh}$	$8.67 \pm 0.88 \times 10^{4hij}$	$6.00 \pm 1.15 \times 10^{4hij}$	$6.00 \pm 1.53 \times 10^{4efghi}$
	ST + FA + SD	$9.67 \pm 1.45 \times 10^{4ab}$	$2.30 \pm 5.57 \times 10^{5bc}$	$5.50 \pm 4.58 \times 10^{5c}$	$5.57 \pm 5.70 \times 10^{5b}$	$5.23 \pm 5.36 \times 10^{5c}$
Pusa Jawahar Hybrid Maize 1	Control	0.00 ± 0.00^{h}	0.00 ± 0.00^{h}	0.00 ± 0.00^{j}	0.00 ± 0.00^{j}	0.00 ± 0.00^{i}
	ST*	$1.33 \pm 0.88 \times 10^{4gh}$	$4.67 \pm 1.45 \times 10^{4efgh}$	$2.20 \pm 4.04 \times 10^{4\,g}$	$1.60 \pm 2.08 \times 10^{4ef}$	$1.50 \pm 1.15 \times 10^{5ed}$
	FA	$2.00 \pm 1.15 \times 10^{4fgh}$	$7.33 \pm 1.20 \times 10^{4defgh}$	$1.57 \pm 3.18 \times 10^{4ghi}$	$1.20 \pm 1.73 \times 10^{4efghi}$	$1.07 \pm 1.20 \times 10^{4efgh}$
	SD	$0.33 \pm 0.33 \times 10^{4\,g}$	$2.33 \pm 0.88 \times 10^{4fgh}$	$4.67 \pm 0.88 \times 10^{4ij}$	$4.67 \pm 0.33 \times 10^{4hij}$	$4.33 \pm 0.67 \times 10^{4fghi}$
	ST + FA + SD	$5.33 \pm 1.86 \times 10^{4def}$	$1.30 \pm 2.65 \times 10^{5d}$	$1.53 \pm 2.60 \times 10^{4ghi}$	$1.40 \pm 2.31 \times 10^{4efgh}$	$1.43 \pm 1.20 \times 10^{5def}$
Pusa Vivek Hybrid 27 improved	Control	0.00 ± 0.00^{h}	0.00 ± 0.00^{h}	0.00 ± 0.00^{j}	0.00 ± 0.00^{j}	0.00 ± 0.00^{i}
	ST*	$9.00 \pm 1.15 \times 10^{4abc}$	$6.63 \pm 6.33 \times 10^{5a}$	$1.11 \pm 7.51 \times 10^{6a}$	$1.02 \pm 7.26 \times 10^{6a}$	$1.02 \pm 6.89 \times 10^{6a}$
	FA	$8.00 \pm 0.58 \times 10^{4abcd}$	$2.00 \pm 3.06 \times 10^{5c}$	$4.63 \pm 3.53 \times 10^{5cde}$	$4.43 \pm 2.85 \times 10^{5c}$	$4.57 \pm 8.29 \times 10^{5c}$
	SD	$1.67 \pm 0.88 \times 10^{4\,g}$	$3.33 \pm 0.88 \times 10^{4fgh}$	$9.33 \pm 0.88 \times 10^{4hij}$	$9.67 \pm 1.45 \times 10^{4fghij}$	$7.67 \pm 1.45 \times 10^{4efghi}$
	ST + FA + SD	$1.00 \pm 1.15 \times 10^{5a}$	$6.33 \pm 3.84 \times 10^{4a}$	$1.11 \pm 8.84 \times 10^{4a}$	$1.04 \pm 6.23 \times 10^{6a}$	$1.07 \pm 3.48 \times 10^{6a}$
F value		10.53	55.41	78.88	97.65	94.62
P value		< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001

Means within a column followed by the same letters are not significantly different;

ST- Seed Treatment, FA- Foliar Application, SD- Soil Drenching, SEM- standard error of the mean;

ST*- Mean cfu (10^4)/g of leaf after DAE (Days after emergence); and DAI- Days after inoculation

with ST + FA + SD method (28%), followed by PVH27I with ST (26.7%) ($F = 6.59$, $p < 0.0001$). Combination of all inoculation methods (ST + FA + SD) of PJHM1, PHQPM5I, PHQPM7I and ST method of PHQPM5I showed only up to 13.33% mortality. On 21 DAI, the highest mortality (50%) was observed in ST + FA + SD inoculation method, followed by ST (40%) in PVH27I, whereas 23.33% mortality was recorded in DMRH 1301 with ST and ST + FA + SD method ($F = 10.29$, $p < 0.0001$). No mortality was observed in respective controls (Fig. 5).

Perusal of data in Fig. 6 showed that weight attained on 7th day after bioassay by surviving larvae on ST, FA and ST + FA + SD inoculated plants was significantly low (9.82–18.21 mg) as compared to control (26.4–35.7 mg) in all the cultivars on 14 DAI ($F = 243.14$, $p < 0.0001$). SD method showed high larval weight (20.20–32.80 mg) as compared to all other methods of inoculation in all the maize cultivars on 14 DAI. Lowest larval weight (9.82–20.20 mg) was attained in all the inoculated methods on PVH27I than all other cultivars (Fig. 6). Bioassay conducted on 21 DAI showed that weight attained on 7th day after bioassay by surviving larvae on ST, FA and ST + FA + SD inoculated plants was significantly low (4.40–16.22 mg) as compared to control (27.7–36.10 mg) in all the cultivars ($F = 284.73$, $p < 0.0001$). Higher larval weight (15.15–31.30 mg) was recorded on SD method as compared to other method of inoculation in all the maize cultivars. Similar to 14 DAI, lowest larval weight (4.40–12.40 mg)

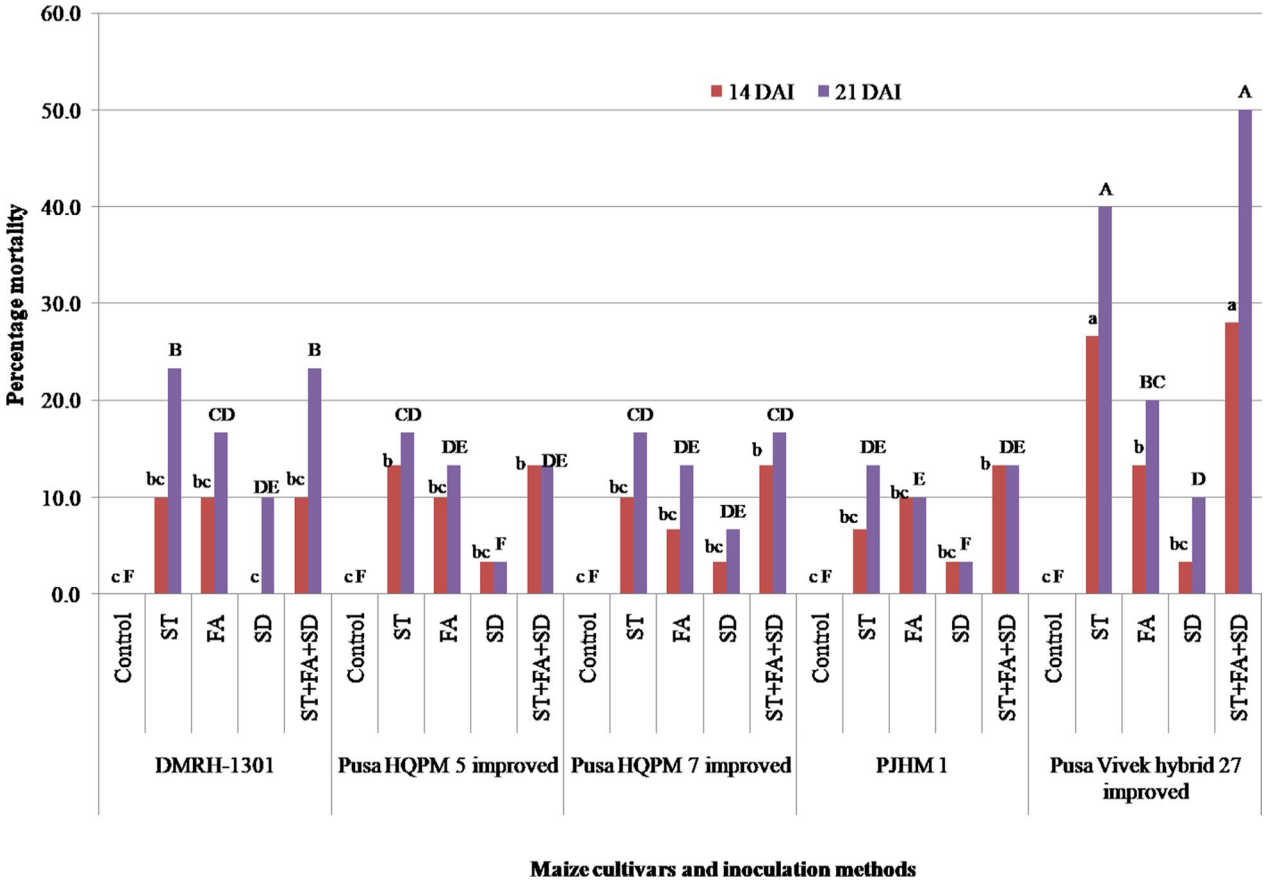

Fig. 5 Bioefficacy of *Bacillus thuringiensis* strain VKK5 inoculated five maize cultivars by different inoculation methods, viz. seed treatment (ST), soil drenching (SD), foliar application (FA) and combination of all methods (ST + SD + FA), against neonates of *Spodoptera frugiperda* on 14 and 21 days of inoculation in terms of percent mortality after 7th day of bioassay. *14DAI bars followed by different small alphabet and 21DAI bars followed by different capital alphabets are significantly different at 5% level

was attained on ST, FA and ST + FA + SD inoculated PVH27I plants.

Discussion

The *S. frugiperda* is a devastating polyphagous pest, recent invasion of this pest in India and other countries (CABI 2018) leads to significant loss of maize crop thus created a great threat to the food security in invaded countries (FAO 2020; Jing et al. 2021). Mostly insecticides are used to manage the FAW at early as well as later stage of infestation that will increase the cost of cultivation and even pose risk to environment and non-target organism (Gutierrez-Moreno et al. 2019). Moreover, it has developed resistance against many insecticides (Chen et al. 2023) as well as against *Bt*-crops with Cry toxins, viz. Cry1F, Cry1Ac, Cry1Ab, etc., in the USA and Brazil (Suby et al. 2020). Thus, there is a need to look for novel bacterial strains. In the present study, *Bacillus* strain VKK5 was characterized as BtVKK5 morphologically based upon presence of crystal along with spore and at molecular level based upon 16S rRNA gene sequence homology as well as amplification of *cry1* and *cry2* genes. BtVKK5 showed polymorphic crystals which are similar to study of Mukhija and Khanna (2018). The analysis of the nucleotide sequences of the 16S rRNA gene is often used for identification of the bacterial species (Rajashekhar et al. 2017). Similarly, *cry* gene profiling using gene-specific primers in *Bt* strains were reported by Sarkar and Kalia (2021).

To overcome the resistance development in FAW and improve the field efficacy of entomopathogens against internal borer, the endophytic colonization of entomopathogens in maize plants may be an alternative to insect resistant *Bt* transgenic crops. The most of the studies were related to colonization of EPF into maize plant against FAW (Ramos et al. 2020). But the studies are limited with respect to the endophytic ability of EPB against FAW. Earlier study on association of *Bt* with host plants reported the ability of *Bt* to colonize interior of plants (Pola et al. 2022) similar to present study. Present

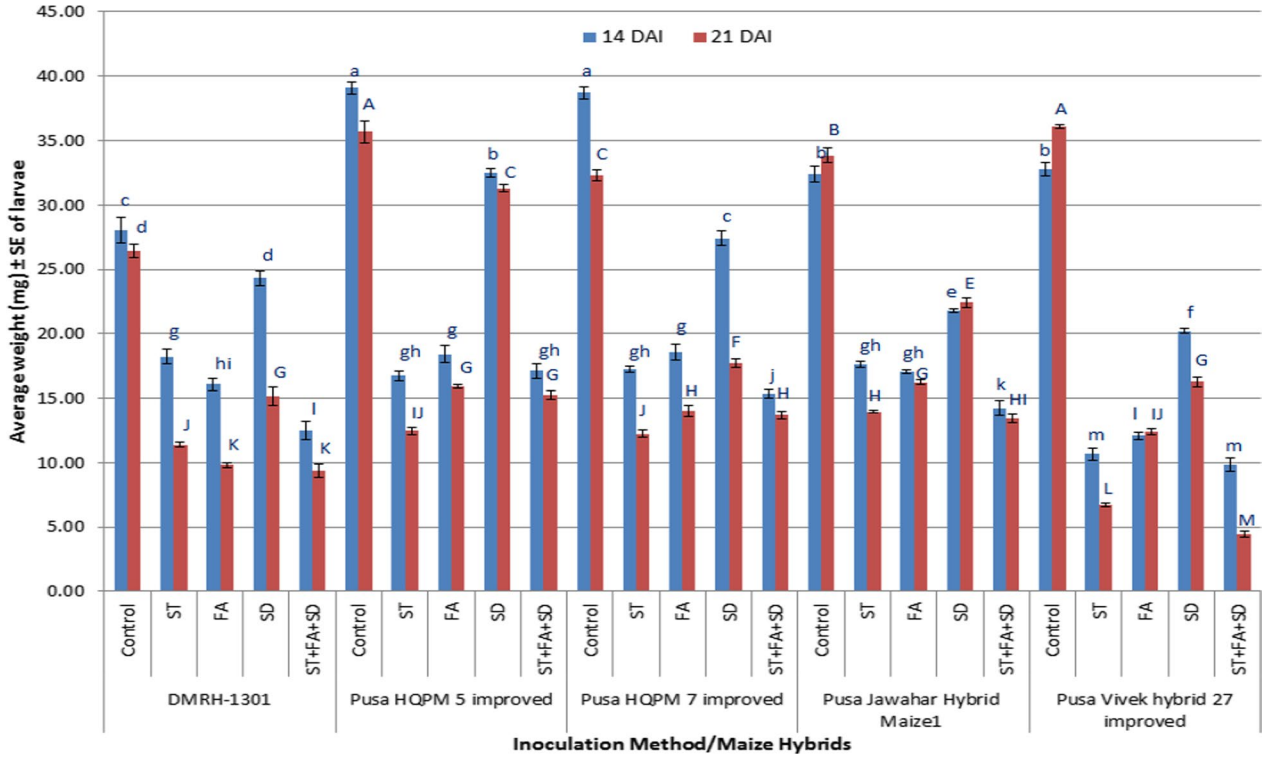

Fig. 6 Average weight attained by survived larvae of *Spodoptera frugiperda* on 7th day after bioassay on 14 and 21 days after inoculation of *Bacillus thuringiensis* strain VKK5 in five maize cultivars by different inoculation methods, viz. seed treatment (ST), soil drenching (SD), foliar application (FA), combination of all methods (ST + SD + FA) and control (uninoculated). *14DAI bars followed by different small alphabet and 21DAI bars followed by different capital alphabets are significantly different at 5% level

study mainly focused on establishment of native *Bt* strain as endophytes in maize plant to give protection against FAW.

Understanding the ability of colonization of *Bt* by different inoculation methods is the first step for application strategy of *Bt* as endophyte in field conditions. The establishment of *Bt* as an endophyte may occur via natural openings in seeds, leaf and root reaching various tissues and systemically colonizing the plant. The present study demonstrated that there was a significant difference in endophytic colonization into different maize cultivars by different inoculation methods. Thus, it was shown that the genotype of host plant plays an imperative role in establishment of bacteria as endophytes. Likewise, other studies also suggested that plant genotypes play a significant role on the microbiome in the plant endosphere (Ding and Melcher 2016). Recent studies showed that successful colonization as endophytes was regulated by plant tissue type, plant genotype, microbial taxon and strain type as well as environmental conditions (Mengistu 2020). The inoculated bacteria take its time to colonize and multiply inside the plants. Present study reported that on 7th day after inoculation the colonies were less among all the cultivars and inoculation methods. Significant increase in cfu was observed on 14, 21 and 28 days after inoculation. Among different inoculation methods, seed treatment (ST) and combination of all method (ST + FA + SD) were found to be more suitable.

Observation on different methods of inoculation showed that soil drenching (SD) is the least effective method when compared to other methods in terms of cfu/gm of leaf. For seed inoculation methods, seeds were soaked for 48 h in *Bt* inoculum as the seed imbibe water for their germination which may be responsible for more colonization in seed treatment. Moreover, during germination process significant amount of metabolites in the form of exudate gives the bacteria inoculated on the seeds a better chance to be the first to use these substrates, thus improving their chances of establishment (Pelzer et al. 2011). The leaf anatomy and structure may be responsible for more colonization by foliar application as of its C4 nature. Normally, plant roots are used to act as gatekeepers to screen soil bacteria from the rhizosphere. The most probably colonized endophytes spread from roots to aerial plant parts are due to the mobility of bacterial cells and production of cellulolytic enzymes by the bacteria (Santi et al. 2013).

Bioassay conducted with leaves of BtVKK5 inoculated maize cultivars against neonates of *S. frugiperda* showed insecticidal activity. Inoculated plant leaf bioassay showed higher mortality in PVH27I with ST + FA + SD method as of more colonization of bacteria inside the plants than other treatments. In overall, the leaf bioassay mortality results were consistent with quantification of colonization in plants (cfu/g). The reduction in larval weight which fed on inoculated plants showed that there was a great effect on growth and development of larvae occurred because of *Bt* as endophyte. This may enhance the susceptibility of larvae to other control strategies which needs further investigation.

The outcome of the present study is that colonization was significantly different among maize cultivars, inoculation methods and days after inoculation. Collectively, endophytic bacteria and host plants may have a genetic basis to their differential colonization patterns, which may further correlate with their interaction patterns within plants. The long-term persistent of bacteria in the leaf of maize plant clearly shows that they were able to colonize rapidly and can compete or relish with other internal microbes inside the plants. Apart from this, it is still a matter of research to find out the strategies of plants to uptake the bacterial cells from seed treatment, foliar application and soil drenching. Applicability of colonized *Bt* as endophyte has to be further explored against larval herbivores especially internal borers to enhance its biocontrol potential which will control the insect pests in a sustainable way. Establishment of *Bt* as an endophyte can shed more light on biocontrol of FAW and play an imperative role in sustainable IPM practices. Hopefully, present approach will be gradually adopted toward application of entomopathogenic bacteria as endophytes in many more crops to protect them from insect pests in the near future.

Conclusions

Establishment of native *Bt* strain(s) as endophyte in maize plants, complemented with insecticidal activity, could possibly lead to an innovative approach to the management of *S. frugiperda* and can be an alternative to transgenic plants.

Abbreviations

Bt	*Bacillus thuringiensis*
FAW	Fall armyworm
CFU	Colony-forming unit
DAI	Day after inoculation
DAE	Day after emergence
h	Hour
min	Minute
sec	Second
Adw	Autoclaved distilled water
µg	Microgram
µl	Microliter
Fig.	Figure

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s41938-023-00726-8.

Additional file 1. Table S1. List of cry1 and cry2 genes primer sequences and its annealing temperature with amplicon size. **Fig. S1** Phylogenetic analysis of Bacillus thuringiensis strain VKK5 based on 16S rRNA gene sequence. **Fig. S2** Antibiotic sensitivity pattern of native Bacillus thuringiensis strain VKK5.

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Author contributions

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

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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.

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