Insecticidal activity of native *Bacillus* species against brinjal shoot and fruit Borer, *Leucinodes orbonalis* (Lepidoptera: Crambidae)

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ABSTRACT: Brinjal (*Solanum melongena* L.), is an economically important vegetable crop cultivated across the world. Shoot and fruit borer, *Leucinodes orbonalis* (Guenée) is the major biotic stress in brinjal crop. The insecticidal activities of the twenty six strains of *Bacillus* comprising of *Bacillus thuringiensis* (17), *B. subtilis* (5), *B. pumilus* (2), *B. atrophaeus* (1) and *B. amyloliquifaciens* (1) along with reference strain *i.e. B. thuringiensis* sub species *kurstaki*HD1were screened at single concentration of 10 µgg^{-1}of diet against neonates of *L. orbonalis* using diet incorporation method. On 7[th] day after treatment, the highest mortality was observed in *B. thuringiensis* reference strain HD1 (100%) followed by 96% (VKK-13 and VKK-BB2) and 80% (VKK-BB1) mortality with native *Bt* strains. Moreover, endophytic bacteria *B. atrophaeus*, VKK-6OL and *B. subtilis*strain resulted in 68% and 52% mortality respectively. Median lethal concentration (LC_{50}) the potential *Bt* strains revealed that Btk HD1 (LC_{50}=0.49µg/g of diet) and BtVKK-BB2 (LC_{50}=0.59µg/g of diet) were found to be at par as their fiducial limits are overlapping. Results suggested that besides *B. thuringiensis*, *B. atrophaeus* and *B. subtilis* strains also have insecticidal activity against BSFB and could be suitable for development of bio formulations in future.

Key words: *Bacillus strains, Bacillus thuringiensis*, *Bacillus subtilis*, *Bacillus atrophaeus, Leucinodes orbonalis*, Brinjal

INTRODUCTION

Brinjal (*Solanum melongena* L.) or eggplant is grown in India and many other parts of the world. It is a very popular and nutritious vegetable rich in minerals (Choudhary and Gaur, 2009). India holds second rank next to China in brinjal production with major brinjal producing states being West Bengal, Gujarat, Madhya Pradesh and Bihar. This crop is prone to a number of insect pests but among these, the monophagous pest, brinjal shoot and fruit borer (BSFB), *Leucinodes orbonalis* Guenee has been reported to be the most destructive pest which causes a yield loss of up to 60-70% and imposes the immense loss in production (Singh and Nath 2010). Farmers usually spray synthetic chemicals to manage this pervasive borer but it has developed resistance to commonly used insecticides *viz.,* deltamethrin, fenvalerate, chlorpyriphos and profenofos (Shirale*et al.* 2017) and synthetic pyrethroids (Murali *et al*. 2017). Considering the detrimental effects of chemical control on the environment, food safety issues and legal restrictions on the usage of conventional pesticides especially in vegetables, research has been shifted towards green chemicals and microbial control. Among the entomopathogenic microbes, the well-known and successful insect pathogen *Bacillus thuringiensis* (*Bt*) is a spore-forming rod-shaped, aerobic gram-positive bacterium belonging to Bacillaceae family. It has been extensively used for biological control of noxious pests in different crops because it has the ability to produce crystalline (Cry) and cytotoxic (Cyt) proteins during its sporulation phase with unique activity against lepidopteran, coleopteran and dipteran pests(Aranda *et al*. 1996; Kumar *et al*. 2019). The *Bacillus* species like *B. popilliae, B. lentimorbus, B. larvae,B. sphericus, B. subtilis*, are other than Bt commonly recognized as insect pathogens [de Barjac, 1985; Gorashi *et al*., 2016; Tripathi *et al*., 2016; Rajeshekar *et al*., 2017]. At present, *Bacillus thuringiensis* (Bt) is the only microbial insecticide in widespread use but with the development of resistance in some insects, there is need to explore other Bacillus spp for pest control management. Present studies were carried out during 2019-20 deals with the efficacy of native *Bacillus* strains isolated from various habitats against neonates of *L. orbonalis*.

MATERIALS AND METHODS

Insect collection and rearing

Brinjal shoot and fruit borer infested brinjal fruits were collected from Indian Agricultural Research Institute field. Larvae were collected by cutting the fruits and allowed to grow on brinjal till pupation in the laboratory at 27±2 ºC and 65±5% RH and 14L: 10D photoperiod. Pupae were collected and placed in plastic containers for adult emergence. Newly emerged adults

Table 1. List of native *Bacillus* strains used for evaluation of insecticidal activity against neonates of *Leucinodes orbonalis*

***Bacillus* spp.**	**Strain ID**	**NCBI Accession number**
B. thuringiensis	VKK-PX2	*
	VKK-LE1	KT714048
	VKK-LE2	KT714049
	VKK-BB1	KT714044
	VKK-BB2	KT714045
	VKK-SL2	KT714055
	VKK-LO	KT714050
	VKK-EV	KT714046
	VKK-MPW	KT714054
	VKK-ENT-1	KT714053
	VKK-ENT-2	*
	VKK-ENT-3	*
	VKK-13	MW380680
	VKK-HA2	*
	VKK-GJ2	KT714041
	VKK-GJ4	KJ000210
	VKK-AC1	*
B. subtilis	VKK-SL1	MF993346.1
	VKK-AC2	*
	VKK-GJ3	MF983545
	VKK-3OL	JX852576
	VKK-2NL	KJ000212.1
B. pumilus	VKK-1OL	KJ000216.1
	VKK-4NL	JX852571.1
B. atrophaeus	VKK-6OL	KJ000214.1
B. amyloliquifaciens	VKK-3NL	KJ000213.1
B. *thuringiensis* kurstaki	*Btk*-HD-1	Reference strain

*Sequence of these strains yet to submit in NCBI

were transferred into mating jars (20 cm X 15cm)having 10% honey solution and a brinjal twig/tender leaf kept in glass vial filled with water for egg laying. The twigs having white coloured eggs were replaced every day and kept in plastic jars for hatching. Upon hatching, the neonates were considered as F1 generation and were reared on semi-synthetic diet till pupation. The BSFB culture was maintained in the laboratory for subsequent generations and newly hatched larvae (<24 h old) were used for bioassays.

Screening of *Bacillus* strains against *Leucinodes orbonalis*:

Twenty six *Bacillus* strains comprising of *Bacillus thuringiensis*(17), *Bacillus subtilis* (5), *Bacillus pumilus* (2), *Bacillus atrophaeus* (1) and *Bacillus amyloliquifaciens* (1) along with reference strain *i.e. Bacillus thuringiensis* sub species *kurstaki*HD1 (Table 1)were screened at single concentration (10 μgg^{-1}of diet) by diet incorporation method as per Dharavath*et al.*, 2016. The test concentration 10 μgg^{-1}diet was prepared for each test strain and mixed thoroughly. Diet was transferred to small plastic containers (5 × 2cm). Each container served as one replicate, with six replications per test strain. Five neonates were released on the treated diet (3 g diet) per replicate. The control consisted of diet (without toxin). A minimum of 60 neonates were used for each strain bioassay. All the bioassays were conducted under controlled conditions of 27±2 ºC, 65±5% RH, and 14L: 10D photoperiod. Mortality data was recorded on 7th day after treatment and corrected percent mortality was calculated by using Abbott's formula (1925).

Virulence bioassays with shortlisted *Bacillus* strains

Bacillus strains which showed ≥80% mortality i.e., VKK-BB1, VKK-BB2, VKK-13 and reference strain *Btk*HD-1 at 10 μg g^{-1}dietassays were used for virulence bioassays. Four concentrations *viz.*, 0.1, 1.0, 5.0, and 10 μg g^{-1} of each strain were taken for full bioassay under controlled conditions as mentioned above. For each bioassay 150 neonates were used. Mortality data were recorded on 7th day.

Statistical Analysis

The corrected percent mortality data of single concentration obtained in screening bioassays was subjected to analysis of variance (ANOVA) at 5% level of significance using Statistical Analysis System (SAS) version 4.2 (SAS Institute Inc. Cary, USA) to compare the insecticidal activities among different *Bacillus* strains. The significantly different means (<0.05) were separated using Duncan's Multiple Range test ((DMRT). Median lethal concentration (LC_{50}) was calculated using maximum likelihood programme (MLP) 3.01(Ross, 1987). The significance of difference between strains were determined on the basis of overlap of 95% fiducial limits of LC_{50}.

RESULTS AND DISCUSSION

Perusal of mortality data in Fig.2 showed that reference strain *Btk.* HD-1 attained maximum mortality (100%) followed by native *Bt* strains VKK-BB2 and VKK-13 with a mortality of 96% which were found to be statistically at par with reference strain *Btk*-HD1.While *Bt* strains VKK-BB1 exhibited 80% mortality followed by *Bt* strain, VKK-ENT1 and VKK-PX2 attained(76%) and *Bt* strain VKK-HA2and endophyte strain *Bacillus*

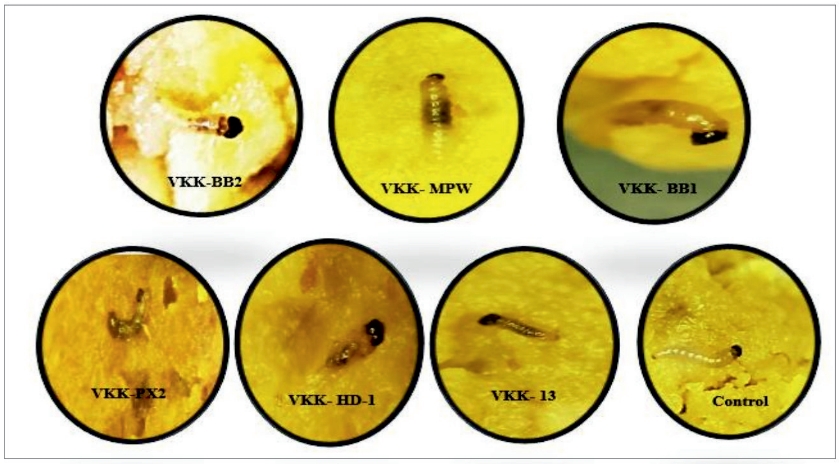

Fig. 1. Gut discoloration in *Leucinodes orbonalis* due to infection of various *B. thuringiensis* strains

atrophaeus, VKK-6OL (68%)Among the other *Bacillus* spp. leaf endophyte, *B.subtilis* strain VKK-2NL attained 52% mortality followed by VKK-1OL and VKK-SL1 strains with 43.98%mortality of neonates of *L. orbonalis*. Based on the present findings, it was evident that endophytic strains viz., *B. atrophaeus*VKK-6OL and *B. subtilis* (VKK-2NL) were found to be potential strain besides *B. thuringiensis* strains. Similarly, neonates of cotton boll worm, *Helicoverpa armigera* attained40% mortality at 10 µg/g concentration with *B. subtilis* strain Sh-3 (Gorashi *et al.,* 2014).Out of three *B. subtilis* strains (GTG-57, GTG-59 and GTG-69), GTG-59 collected from North East India caused 50% mortality against the neonates of *H. armigera* and *Spodoptera litura.* Further, *B.pumilus* strain (GTG-11) also caused 36% and 26% mortality against *S.litura* and *H. armigera*respectively (Tripathi *et al*., 2016).Correspondingly, Van Zijll *et al*. (2016) reported that *Brevibacillus laterosporus* isolates from brassica were proved to cause mortality of the larvae of diamondback moth, *P. xylostella* due to declined larval feeding and one isolate was found to be comparable to that of *B. thuringiensis*. Khedher *et al*., 2017 indicated that biosurfactant produced by *Bacillus amyloliquefaciens* AG1 has shown adverse effect on the first instar larvae of *Spodoptera littoralis* with an LC_{50} of 245 ng/cm^2 and histopathology examination showed that vacuolization, necrosis and disintegration of the basement membrane in the larval midgut. Similarly, toxicity of *B. amyloliquefaciens*(GTG-4) has been reported against neonates of *S. litura*(43%) and *H. armigera*(13%) on 7th day after treatment (Tripathi *et al*., 2016). Afriani *et al*., 2018 revealed that *Bt* isolates collected from soil (KJ_3K_4 and $KJ_3D_{3)}$ were proved to be pathogenic to larvae of *Spodoptera litura* similar to commercial *Bt* formulation (Dipel) while *Bt* isolate KJ_3BW_5 which was reported to be more effective when compared to Dipel.

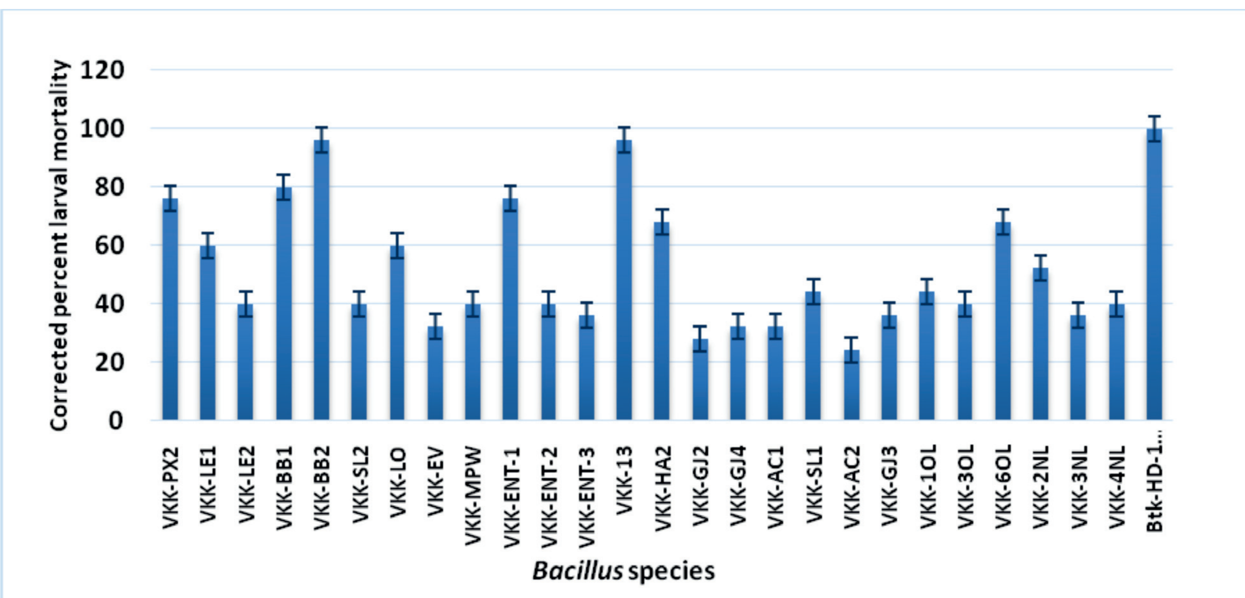

Fig. 2. Efficacy of native *Bacillus* strains against neonates of *Leucinodes orbonalis* on 7th day after treatment

Efficacy of *Bt* isolates in the spore crystal form against first instar larvae of lesser cornstalkborer, *Elasmopalpus lignosellus* indictaed that twelve isolates caused mortality above 85% and *Bt* isolates BR83, BR145, BR09, BR78, S1534, and S1302 had the lowest LC_{50} values and did not differ from the standard HD-1 strain (Zorzetti *et al*., 2017).Among the *B.thuringiensis* isolates Kb-29,St-6 and Wh-1 showed above 50% mortality on 4th day after treatment whereas, on 7th day Kb-29,St-2, St-6, St-22 and Wh-1 showed 50-70% larval mortality which were found to be comparable with reference strain HD-1 (Gorashi *et al.,* 2014). *Bt* isolates AUG-5 and GTG-7 produced above 80% mortality of neonates of *H.armigera* and in case of *S.litura, Bt* isolate AUG-5 caused 70% mortality at 1 µg/g (toxin content basis) on 7th day after treatment (Tripathi *et al.,*2016) .

Perusal of LC_{50} data showed that LC_{50} values of spore crystal form of *Bt* strains varied from 0.49µgg^{-1}of diet (Reference strain, *Btk*-HD1) to 2.69µgg^{-1}of diet (VKK-BB1) against neonates of *L.orbonalis.*Among the three native *Bt* strains VKK-BB2 was found to be most toxic with a minimum LC_{50} (0.59µgg^{-1}diet) followed by VKK-13 (1.65 10 µgg^{-1}of diet).*Btk* -HD1 was found to be significantly at par with VKK-BB2 but significantly different from two native *Bt* strains VKK-13 and VKK-BB2 as their fiducial limits were not overlapping. VKK-BB2 strain was found to be 2.8 folds and 4.6 folds more toxic than VKK-13 and VKK-BB1 strains respectively, against neonates of *L.orbonalis.*Similar to the present findings, five *Bacillus* strains were short listed after preliminary evaluation against *A.gossypii*and the LC_{50}values showed that VKK-AC2 and VKK-BB1 were the most toxic strainsfollowed by VKK-BB2 against adults of *A.gossypii* (Rajashekar *et al.*, 2018).

***Bacillus thuringiensis* confirmation in the infected larvae**

On the 7th day after inoculation of *Bt* strains in the diet, dead larvae were seen on the surface of diet with typical symptoms. However, in treatment with VKK-MPW, dead larvae occurred on day 5 and were collected to determine whether the evain the bioassays were the cause of larval mortality. The dead larvae were placed individually in a 1.5 ml microcentrifuge tube and surface sterilized with 70% ethyl alcohol and followed by sterile water. Then larvae feeding on treated diet were homogenized with sterilized distilled water (100µl), inoculated in to 5ml Luria broth which contains selective antibiotics, and incubated at 30°C at 180rpm for 72 h. Further, cells were streaked on selective nutrient plate and incubated at 30°Cfor overnight. The bacterial colonies grown on NA plate were checked with original *Bt* colonies for colony morphology, further confirmation of spore crystal inside the bacterial cells of both original VKK-MPW strain and re-isolated colonies of *Bt* was done by using phase contrast microscope (Fig 3).

The results proved that the *Bt* strains were responsible for the mortality of neonates and the physical changes were induced by *B. thuringiensis* infection; and Koch's postulates were fulfilled by the confirmation of *Bt* strain after re-isolation from the infected larval gut. Likewise, Pena *et al*. 2006 isolated colonies of *B. thuringiensis* from dead *Epilachna varivestis* Mulsant (Coleoptera: Coccinellidae) and compared with the original *Bt* colonies; proved that the strains were pathogenic. Torres-Quintero*et al*., 2015 documented the signs of infection caused by *B.thuringiensis* strains and confirmed that the mortality of green peach aphid, *Myzus persicae* (Sulzer) was due to *B. thuringiensis* strains (GP780, GP139, GP209, GP528, GP782, GP300, GP777, and GP402).*Bacillus* strains may be suitable for biocontrol of the notorious pest of brinjal, *L. orbonalis* because they caused mortality of neonates which was found to be comparable with larval mortality shown by commercial *Bt* strain *Btk*-HD1.The present study proved the potential of some indigenous *Bacillus* species, which could be

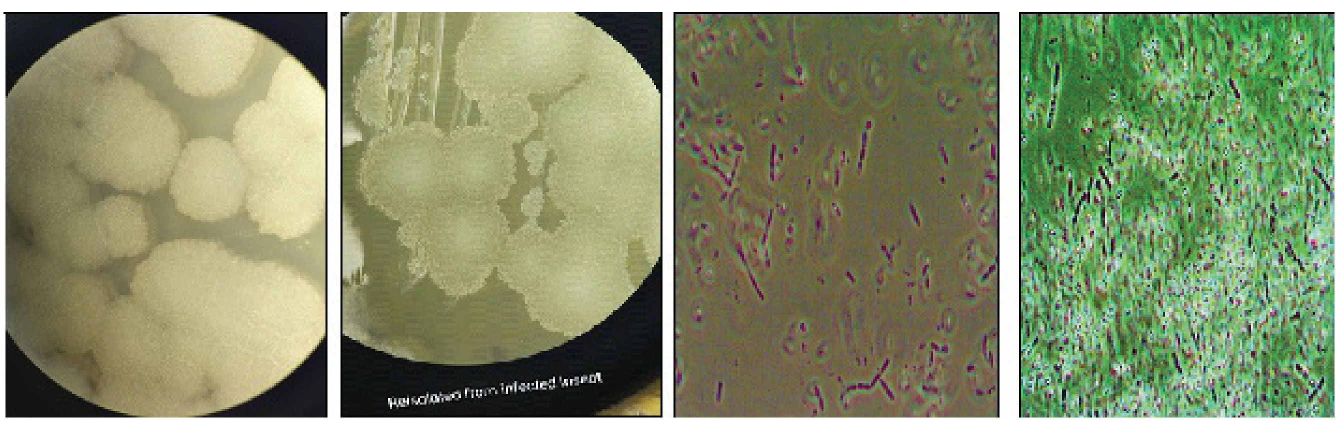

Fig. 3. Isolation of *Bacillus thuringiensis* strain from infected neonate of *Leucinodes orbonalis* after treatment and it's confirmation

suitable for development of bioformulations in future or for colonization of these potential entomopathogenic *Bacillus* spp. in brinjal plants as endophytes to manage BSFB.

The feeding of larvae on semi-synthetic diet incorporatedwith spores of *Bacillus* species and spore crystal toxins affected their survival and development. The neonate larvae feeding on diet treated with native *Bacillus* strains became sluggish, stopped feeding and larval body turned black. While in case of *Bt* treatment in addition to above symptoms turning of gut region of larvae in to black colour was observed (Fig.1).The larvae were found dead on the surface of inoculated diet and became flaccid after death.

ACKNOWLEDGMENTS

First author is grateful to the Director, ICAR-IARI, New Delhi for the financial assistance provided during the research.

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MS Received 21 October 2021
MS Accepted 23 November 2021