



Antagonistic and plant-growth promoting novel *Bacillus* species from long-term organic farming soils from Sikkim, India

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Abstract

Three bacteria namely *Bacillus luciferensis* K2, *Bacillus amyloliquefaciens* K12 and *Bacillus subtilis* BioCWB possessing plant growth promotion and biocontrol potential against phytopathogens and rice leaf folder were identified from organic soils of Sikkim, India. The results revealed significant higher production of phytohormones IAA ($97.1 \mu\text{g mL}^{-1}$) and GA_3 ($10.6 \mu\text{g mL}^{-1}$) was found in K2, whereas BioCWB had higher phosphate solubilization ($570.0 \mu\text{g mL}^{-1}$) efficacy and also possessed nitrogen fixation ability ($5.34 \log$ copy number mL^{-1} culture). All these bacteria had higher antagonistic activities against phytopathogens viz. *Rhizoctonia solani*, *Fusarium proliferatum*, *Athelia rolfsii* and *Colletotrichum gloeosporioides* and also had higher larvicidal activity against rice leaf folder *Cnaphalocrocis medinalis* (Guenne) under in vitro conditions. Molecular insights into the antagonistic mechanisms of *Bacillus* strains deciphered the presence of several antimicrobial peptides (ericin, subtilin, surfactin, iturin, bacilysin, subtilosin, fengycin and bacillomycin), volatiles (dimethyl disulphide, methyl-Furan, acetic acid, Z-1,3-pentadiene and 3-hexyn-2-ol) and soluble metabolites (9-octadecenamide, E-15-heptadecenal, E-3-eicosene and 5-octadecene). Furthermore, liquid microbial inoculum prepared using the bacterial strains (K2, K12 and BioCWB) were evaluated under glass house (rice) and field condition (capsicum), which significantly enhanced plant growth in rice and yield in capsicum compared to control. The present study revealed the combination of *Bacillus* spp. (K2, K12 and BioCWB) can be used as bio-inoculants for improving agricultural production in Sikkim. Moreover, for the first time, we demonstrated plant growth promoting (PGP) traits, antifungal and insecticidal properties of *B. luciferensis*.

Keywords PGP traits · Biocontrol · *Bacillus* spp. · Sikkim organic soil

Introduction

Sikkim an agrarian state of India situated at 27°N – 28°N latitude and 88°E to 89°E longitude with terrestrial elevations ranging from 300 to 7000 feet from mean sea level in the Eastern Himalayan range, is among the highest and richest biodiversity spots of the world (Maharana et al. 2000). Organic farming is predominantly practiced across this region (Yadav et al. 2013). Generally, nutrient depletion in soil due to leaching, volatilization and fixation are some of the problems related to decreased plant growth and development (Pathak et al. 2010). Several previous reports have shown soil bacteria to play an important role in plant growth and development by producing phytohormones such as indole-3-acetic acid (IAA) and gibberellic acid (GA_3) (Egamberdieva et al. 2017), nutrient solubilization

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(Kumar et al. 2012) and nitrogen fixation (Fisher 1999). In addition to plant growth promoting (PGP) potential, several endospore forming *Bacillus* species such as *B. subtilis*, *B. amyloliquefaciens*, *B. thuringiensis* and *B. megaterium* have been reported for their effectiveness in suppressing activity of several plant pathogens and insect pests (Prabhukarthikeyan et al. 2014; Elanchezhian et al. 2018). Among the different modes of antagonism, antimicrobial peptides (AMPs) such as surfactin, iturin, bacillomycin and fengycin produced by *Bacillus* spp. have demonstrated to play important role in suppressing several pathogens in plant (Koumoutsi et al. 2004; Elanchezhian et al. 2018). Apart from the AMPs, bacteria secreted metabolites such as volatile organic compounds (VOCs) and soluble metabolites play key roles in plant growth regulation, development of stress resistance and suppression of diseases (Tyc et al. 2017). Specifically, VOCs are known to confer swift action in plants rather than soluble metabolites due to their low molecular weights (< 300 Da) and high vapor pressure (10 Pa at 20 °C) that readily evaporates and diffuses through cell surfaces (Schulz and Dickschat, 2007). A detailed description of different types of VOCs secreted by bacteria has been well documented by Thorn and Greenman (2012).

India is one of the major agrarian Asian country comprising 29 states, of which Sikkim has become the first organic state with the adoption of 100% organic farming (Kumar et al. 2018a, b). It has been reported that climate variability and erratic precipitation are the major drivers of pest incidence (Macfadyen et al. 2018). The prevalence of plant diseases caused by rice blast fungus (Sridhar and Singh, 2001), sheath blight, stem rot fungus (Kumar et al. 2017a) and soil-borne fungal pathogens have brought severe constraints in the productivity of organic rice and horticultural crops in this area. The insect pest particularly rice leaf folder *Cnaphalocrocis medinalis* (Guenne) has turned into major pest causing leaf defoliation which has been shown to cause 61.9% yield loss in rice under epidemic situations. Apart from cereals, Sikkim is one of the major repositories of the “*Capsicum annum* complex” which comprises three cultivated species namely *C. annum*, *C. frutescens*, and *C. chinense* of which *C. annum* is widely cultivated. One of the major limitations in the cultivation of *C. annum* has been the poor development of morphological characters largely due to nutrient deficiency (Jha and Saha 2017).

Currently, there are separate management practices available for improving plant growth and disease management, especially in Sikkim’s organic farming. However, the development of an effective microbial strategy comprising insecticidal, fungicidal properties with plant growth promotion abilities could be a viable option for plant health management practices. Additionally, as supported by previous studies it is vivid that indigenous microbes are better at promoting plant growth and managing pest and diseases than alien

microbes (Kumar and Gopal 2015). Keeping this in mind, attempts were made in the present study to identify some of the novel multi-potential PGP bacteria from soils of Sikkim and assess their antagonistic potential against disease-causing phytopathogens.

Materials and methods

Sample collection and isolation of plant growth promoting bacteria

The soil sampling was conducted at 23 different agricultural fields (cropping system of rice followed by vegetable crops) across Sikkim, India. Ten soil samples were collected from each site at depth of 15–20 cm, completely homogenized and transported to laboratory in cool pack for further analysis. The collected soil samples were passed through 2 mm sieve to remove coarse rock and plant material, thoroughly mixed for uniformity and stored at 4 °C. Bacteria from collected soil samples were isolated by serial dilution technique using different agar media viz. Nutrient, Pikovskaya’s, Jensen’s and King’s B agar medium and preserved in respective media at 4 °C. The fungal pathogens used in this study viz. *Rhizoctonia solani* (MH60071), *Fusarium proliferatum* (MF033170) and *Athelia rolfsii* (MH636611), which causes sheath blight, sheath rot and seedling blight, respectively in rice were collected from Crop Protection Division, NRRI, Cuttack and *Colletotrichum gloeosporioides* a pathogen causing anthracnose in *Capsicum* plants was collected from microbial repository at NRRI, Cuttack and preserved in potato dextrose agar medium at 4 °C. The third instar rice leaf folder *Cnaphalocrocis medinalis* (Guenne) was collected from NRRI research farm, Cuttack.

Estimation of IAA and GA₃ production

Production of IAA by bacteria was quantified by spectrophotometric method (Gordon and Weber 1951), wherein the bacterial cultures were grown in tryptophan (100 mg L⁻¹) amended nutrient broth, incubated at 30 ± 2 °C for 5 days in the dark. Following which IAA was estimated by determining the colour change using Salkowski reagent. GA₃ production by bacteria was assessed as per the spectrophotometric method described by Borrow et al. (1955).

Phosphate solubilization, nitrogen fixation, and siderophore production

Phosphate solubilization potential of the bacterial isolates was estimated in NBRIP medium enriched with tricalcium phosphate as an insoluble phosphate source. For the qualitative estimation of phosphate solubilization, 24 h

old bacterial culture was spotted in NBRIP agar medium, plates were incubated at 30 ± 2 °C for 7 days and efficacy was assessed based on clear zone formation. Similarly, quantitative estimation of phosphate solubilization by bacterial isolates was assessed using NBRIP liquid medium (Panda et al. 2016) and soluble P content in the supernatant was estimated as per methodology adopted from Murphy and Riley (1962). The nitrogen-fixing ability of bacterial isolates was assessed based on their growth on N-free Jensen's medium by repeated culturing (Kumar et al. 2017b). Further, siderophore production of bacteria isolates was qualitatively determined by the method suggested by Schwyn and Neilands (1987).

Ammonia, HCN production, protease and chitinase activity

Ammonia production by bacterial isolates was qualitatively determined using Nessler's reagent which was added to culture supernatant of five-day old bacteria from peptone broth and development of brown to yellow colour indicated the production of ammonia (Cappuccino and Sherman 1992). HCN production by bacteria was detected using the filter paper method as described by Bakker and Schippers (1987). Protease activity was qualitatively determined by visualizing halo zone formation around bacteria colonies in 3% agar skim milk medium, incubated at 30 ± 2 °C for 4 days (Abo-Aba et al. 2006). Similarly, halo zone formation around bacteria colonies using colloidal chitin agar medium was used for determining chitinase activity (Hsu and Lockwood 1975).

Assessment of antifungal activities of bacterial isolates

The antifungal assay was performed by dual culture technique using potato dextrose agar medium (Lahlali and Hijri 2010). The following fungal pathogens viz., *R. solani*, *F. proliferatum*, *A. rolfsii* and *C. gloeosporioides* were used for the assay. Agar disc (5 mm) of phytopathogens from 5 days old culture was placed at one pole of the Petri's dish and 24 h old bacteria culture was streaked on the opposite pole. Antifungal activity of bacterial strains was determined by comparison with control plates inoculated only with fungus. The fungal growth was monitored for 7 days at 30 ± 2 °C and three replications per isolate were maintained. Fungal growth (colony diameter) was measured and percentage inhibition calculated according to the formula described by Lahlali and Hijri (2010).

$$\text{Percentage inhibition} = (C^d - T^d / C^d) \times 100$$

where C^d colony diameter (mm) of the control and T^d colony diameter (mm) of the test plate

Antagonistic activity assay was also performed under broth conditions wherein initially the mycelia dry weight was calculated from which the percentage inhibition by bacteria was calculated according to the formula described by Lahlali and Hijri 2010.

$$\text{Percentage inhibition} = (C^w - T^w / C^w) \times 100$$

where C^w mycelia weight (g) in control and T^w mycelia weight (g) in the treatment broth

Screening for larvicidal activities of bacterial isolates

Bacterial isolates were cultured in 100 mL nutrient broth, incubated at 30 ± 2 °C for 24 h at 150 rpm, and was further subjected to centrifugation at 10,000 rpm for 10 min to obtain cell pellets. The cell pellets were washed three times with sterile distilled water and the cell loads were adjusted to 10^1 – 10^6 bacterial cells per mL in sterile distilled water and used to calculate LC_{50} against *C. medinalis* (Guenne) third instar larvae using leaf piece bioassay (Panneerselvam et al. 2018).

DNA extraction and amplification of the antimicrobial peptides and 16S rRNA genes of bacterial isolates

Genomic DNA was extracted using the bacterial DNA isolation kit manufactured by Zymo™, USA. Approximately 5 mL of 24 h old bacterial culture grown on nutrient broth was centrifuged at 8000 rpm for 15 min and DNA extraction from cell pellet was carried as per protocol available in the kit. The quantity and purity of isolated DNA were determined by UV spectrophotometer (Nanodrop; Thermo Scientific). 27f (5'-AGAGTTTGATCTGGCTCAG) and 1492r (5'-GGTTACCTTGTTACGACTT) primers were used to amplify 16S rRNA (Suzuki and Giovannoni 1996) and the purified PCR samples were sequenced through Sanger sequencing by Eurofins, Bengaluru, India. Contigs were generated from the resulting forward and reverse read sequences using CAP3 assembly program (Huang and Madan 1999) and the presence of chimera was checked using DECIPHER v2.0 (Wright 2016). Further, sequences were submitted to NCBI database after identifying the bacteria using nBLAST. The phylogenetic tree was constructed using the molecular evolution and genetic analysis (MEGA) software version 10 (Kumar et al. 2018b). Initially, MUSCLE multiple alignment program was used for the alignment of the sequences and Neighbor-Joining phylogenetic tree was constructed using the following parameters viz. 1000 bootstrap replications, maximum composite likelihood substitution model and uniform rate of nucleotide changes with partial deletion.

The genes of antimicrobial peptides viz. ericin, subtilin, surfactin, iturin, bacilysin, subtilosin, fengycin, bacillomycin, mycosubtilin and mersacidin were amplified using specific primers provided in supplementary Table 1. A 50 μL reaction mixture containing approximately 200 μM each dNTPs, 20 pmol each of both forward and reverse primers, 0.5 U of Taq DNA polymerase (NEB) and 50 ng of total DNA was used for PCR amplification in thermocycler. The PCR program followed was: initial denaturation at 96 °C (3 min), followed by 35 cycles of denaturation at 96 °C (1 min), annealing at 55 °C (1 min) and extension at 72 °C (3 min) with final 10 min extension at 72 °C (Vinodkumar et al. 2017). The nitrogen-fixation abilities of bacterial isolates were compared with *Azotobacter tropicalis* and *Azotobacter chroococcum* by quantifying *nifH* gene abundance using the qPCR technique as reported by Kumar et al. (2017b).

Estimation of bacterial volatiles through TD-GC/MS

The nutrient broth was used for growing bacterial isolates in 500 mL flasks having 70% headspace. Volatiles emitted by bacterial isolates were captured using Tenax TA coated stainless steel desorbing column (Perkin Elmer HO244966) that was inserted through rubber stoppers. Uninoculated nutrient broth served as the negative control. Volatiles captured were analyzed with GC–MS fitted with Thermal Desorber turbomatrix 150 (Perkin Elmer, USA) as per conditions suggested by (Mishra et al. 2017) and the detected compounds were identified using mass spectral database (NIST, 2014).

Estimation of bacterial metabolites through GC/MS

The bacterial cultures were grown in 250 mL nutrient broth and subjected to centrifugation at 10,000 rpm for 10 min, and the pH of the supernatant was adjusted to 2.0. After pH adjustment, supernatant was mixed with an equal quantity of ethyl acetate and kept for overnight agitation in a shaker. The solvent fraction was separated and dried in a vacuum flash evaporator, during which the non-volatile

compounds present in the solvent phase was concentrated. The crude extract was dried overnight in sterile Petri plate and extracted using 1.0 ml methanol and filtered through 0.2 μm syringe filter for GC–MS analysis as per the method described by Anantha et al. (2016).

Antagonistic potential of *Bacillus* spp. against rice sheath blight caused by *R. solani* under glasshouse condition

Pot experiment studies were conducted in the glasshouse at ICAR-NRRI, India and evaluated in two rice cropping season's trial-I (kharif) and trial-II (rabi). Surface-sterilized seeds of *Oryza sativa* var. Naveen (rice) were used in this experiment. Liquid-based bioformulation was prepared using three *Bacillus* spp. (K2, K12, BioCWB) using the modified method of Manikandan et al. (2010), wherein the selected bacterial isolates were grown separately in nutrient broth for 48 h and then equally mixed along with osmoprotectants (glycerol and polyvinylpyrrolidone). The surface-sterilized rice seeds were soaked for 24 h separately in sterile distilled water containing bacterial formulation (2.0×10^9 CFU ml^{-1}) at the rate of 5 ml L^{-1} . After soaking, the seeds were dried and sown in pots (45 \times 60 cm) filled with sterilized soil (121 °C, 15 psi for 2 h for 2 consecutive days) collected from ICAR-NRRI, research farm. Twenty days rice seedlings were uprooted and their roots were dipped in the liquid formulation (5 ml L^{-1}) for about 2 h, which were later transplanted in plastic pots with three hills per pot. After 35 days of transplanting, *R. solani* was inoculated by placing agar disc (5 mm) from 7 days old fungal culture on the sheath of rice plant which was secured by wrapping absorbent cotton and sealed using parafilm. After 24 h of pathogen inoculation, plants were sprayed with bacterial liquid formulation (5 ml L^{-1}). The fungicide treated seeds was maintained as the negative control, wherein seed was treated with carbendazim (2 g kg^{-1}) followed by plant foliar spray (0.1% w/v). Untreated plants served as the positive control. The percentage disease index (PDI) was calculated using the formula:

Table 1 Biochemical estimations and plate assays of *Bacillus* strains

Strain	IAA ($\mu\text{g mL}^{-1}$)	GA ($\mu\text{g mL}^{-1}$)	Phosphate solubilization ($\mu\text{g mL}^{-1}$)	N-fixing activity	Siderophore production	HCN production	Ammonia production	Protease activity	Chitinase activity
K2	97.1 \pm 1.4 ^a	10.6 \pm 0.5 ^a	417.3 \pm 4.3 ^b	–	+	+	+	+	+
K12	49.9 \pm 1.1 ^b	4.6 \pm 0.8 ^b	364.1 \pm 2.1 ^c	–	+	+	+	+	+
BioCWB	10.4 \pm 0.7 ^c	3.9 \pm 0.5 ^c	570.0 \pm 2.6 ^a	+	+	+	+	+	+
CD (0.05)	12.3	0.56	23.4	NA	NA	NA	NA	NA	NA

Lowercase letters represent significant variations among the data at $p < 0.05$

+ positive, – negative

$$\text{PDI} = \frac{\text{Sum of all individual ratings}}{\text{Total number of tillers observed}} \times \frac{100}{\text{Maximum disease grade}}$$

whereas the disease grade was given with the following descriptions: 0, no infection observed; 1, lesions limited to lower 20% of the plant height; 3, (20–30%); 5, (31–45%); 7, (46–65%); 9, more than 65% (IRRI 2013).

Evaluation of plant growth promoting potential of *Bacillus* spp. in rice plant

The plant growth promoting potential of all the three *Bacillus* spp. were evaluated in rice variety Naveen. Bacterial inoculations and rice transplantation in pots were carried as per previously described methodology. After 60 DAT (days after transplanting), the agronomic parameters particularly plant height, shoot length, root length, total biomass and tiller number were measured to understand the plant growth promoting potential of *Bacillus* spp.

Evaluation of plant growth promoting potential of *Bacillus* spp. in *Capsicum* plant

The field experiment was conducted for one cropping season at Ralap Farm (27.3°N 88.6°E), East Sikkim, India. The *C. annuum* var. Krishna was used in this experiment. The field experiment was laid out in complete randomized block design with the plot size of 3.0 × 1.0 m² area. The following five treatments viz., T1, uninoculated control; T2, *B. subtilis* (BioCWB); T3, *B. amyloliquefaciens* (K12); T4, *B. luciferensis* (K2) and T5, combination of BioCWB + K12 + K2 were imposed and replicated five times. For the experiment, 25 days old seedlings were transplanted with the spacing of 30 × 40 cm in a raised bed. Bacterial inoculants (2.0 × 10⁹ CFU ml⁻¹) were applied after 1 week at the rate of 2.0 L acre⁻¹. Before application, microbial cultures were suspended in water at the rate of 1.0 L per 100 L of water and drenched basally. The data on plant growth parameters i.e. plant height, fresh and dry weight of root and shoots, fruits number were observed at regular intervals. In this experiment, farm yard manure (FYM) 10 tonnes ha⁻¹ and vermicompost 5 tonnes ha⁻¹ were applied uniformly.

Statistical analysis

The data obtained were statistically analyzed using Web-Based Agricultural Statistical Software Package (WASP 2.0) developed by Central Coastal Agricultural Research Institute (ICAR), Ela Goa (www.ccari.res.in/waspnew.html). PCA analysis was performed using R software (Team 2000).

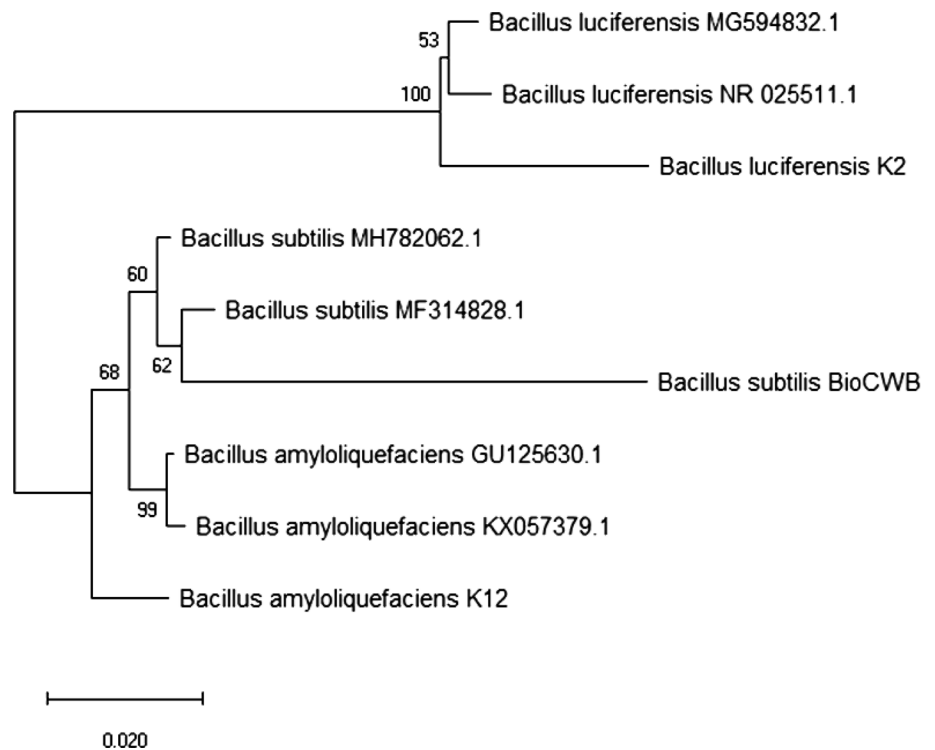
Calculation of LC₅₀ was based on probit analysis carried using Polo Plus, LeOra software version 2.0 (LeOra 2007). The data generated on the antagonistic potential of bacterial strains against rice sheath blight was analyzed using IRRISTAT v.92-1 program developed by International Rice Research Institute (IRRI), Philippines. The larval mortality was verified by using Abbott's formula (Abbott 1925) and the difference between treatments was evaluated using the least significant difference (LSD) at $p < 0.05$.

Results and discussion

Plant growth promoting traits and biochemical characterization

In this study, 63 bacteria were isolated from Sikkim's long-term organic farming soil, of which three efficient isolates were selected based on their PGP traits, in particular, the production of phytohormones such as IAA and GA₃, phosphate solubilization, nitrogen fixation and siderophore production (Supplementary Table 2). The selected three isolates were identified as *B. luciferensis* K2, *B. amyloliquefaciens* K12 and *B. subtilis* BioCWB with the following Genbank accession numbers MN087224, MG490140 and MN087225, respectively (Fig. 1). Additionally, these three bacterial strains have also been deposited at microbial culture repository at ICAR-National Bureau of Agriculturally Important Microorganisms (ICAR-NBAIM), Mau, UP, India having the following accession numbers viz. *B. subtilis* strain BioCWB (NAIMCC-B-02285), *B. luciferensis* strain K2 (NAIMCC-B-02286) and *B. amyloliquefaciens* strain K12 (NAIMCC-B-02288). The quantitative estimation of IAA and GA₃ production (Table 1) indicated that *B. luciferensis* K2 produced significantly higher GA₃ (10.6 µg mL⁻¹) and IAA (97.1 µg mL⁻¹) compared to other strains. Besides, phosphate solubilization studies showed that *B. subtilis* BioCWB could solubilize significantly higher phosphate (570 µg mL⁻¹) than *B. luciferensis* K2 (417.3 µg mL⁻¹) and *B. amyloliquefaciens* K12 (364.1 µg mL⁻¹). However, out of three *Bacillus* spp., only *B. subtilis* BioCWB had nitrogen-fixing ability (Table 1). Further, all the three strains exhibited positive results for siderophore production, ammonia production, HCN production, protease and chitinolytic activities (Table 1). There are multiple previous research findings which have proved the PGP traits of *Bacillus* through the production of phytohormones such as gibberellin (Bottini et al. 2004) and IAA (Idris et al. 2007). In our present study, based on the growth of *B. subtilis* BioCWB after repeated streaking (15 times) in nitrogen-free Jensen's medium, it was presumed to have nitrogen fixing capabilities, which was further confirmed by detecting the presence of *nifH* gene through *nifH* specific primers. Another comparative analysis based on *nifH*

Fig. 1 A phylogenetic neighbor-joining tree showing the relationship of *Bacillus* strains. The bar represents five nucleotide substitutions per 1000 nucleotides of the 16S rRNA gene sequence



gene abundance in *B. subtilis* BioCWB, *Azotobacter tropicalis* and *A. chroococcum* showed gene abundance was 5.34 log copy number mL^{-1} in *B. subtilis* BioCWB as compared to *A. tropicalis* and *A. chroococcum* which had 5.97 and 6.92 log copy number mL^{-1} culture, respectively. Previous workers have used *nifH* gene to underpin the nitrogen-fixing potential of *Bacillus* species (Ding et al. 2005) and have proved the mechanism of nitrogen fixation in *B. subtilis* (Fisher 1999). Although we isolated N-fixing *Bacillus* species, the N-fixing ability was presumed to be lower than that of other diazotrophs, based on the *nifH* gene abundances.

Antagonistic activity against plant pathogenic fungi

Both the dual plate and broth culture studies revealed that *B. subtilis* BioCWB had higher antagonistic activity against *R. solani* (79.6–84.5%), *F. proliferatum* (76.6–86.7%), *A. rolfsii* (86.7–87.8%) and *C. gloeosporioides* (78.0–83.2%) followed by *B. luciferensis* K2 and *B. amyloliquefaciens* K12 (Tables 2, 3). In addition, biocontrol potential of *Bacillus* spp. were evaluated against rice sheath blight under glasshouse studies and results showed (Table 4) that application of *B. subtilis* BioCWB significantly suppressed (69.5–72.6%) rice sheath blight incidence followed by *B. luciferensis* K2 (52.2–54.4%) and *B. amyloliquefaciens* K12 (26.2–28.7%). In chemical treatment studies, there was 82.6–83.55% disease suppression whereas the control treatment recorded 82.0–85.2% rice sheath blight incidence.

Table 2 Antagonistic activity of *Bacillus* strains against *R. solani*, *F. proliferatum*, *A. rolfsii* and *C. gloeosporioides* under dual culture plate method

Strain	<i>R. solani</i>		<i>F. proliferatum</i>		<i>A. rolfsii</i>		<i>C. gloeosporioides</i>	
	Mycelial growth diameter (cm)	Percent inhibition over control	Mycelial growth diameter (cm)	Percent inhibition over control	Mycelial growth diameter (cm)	Percent inhibition over control	Mycelial growth diameter (cm)	Percent inhibition over control
K2	2.0 ^c	75.0 ^a	1.3 ^c	83.8 ^a	1.1 ^c	86.3	2.5 ^b	68.7 ^c
K12	6.2 ^b	22.9 ^b	2.2 ^b	72.5 ^b	1.4 ^b	82.9	2.2 ^b	72.2 ^b
BioCWB	1.6 ^d	79.6 ^a	1.1 ^c	86.7 ^a	1.1 ^c	86.7	1.7 ^c	78.0 ^a
Control	8.0 ^a	–	8.0 ^a	–	8.0 ^a	–	8.0 ^a	–
CD (0.05)	0.322	3.420	0.228	3.224	0.224	NS	0.221	2.36

Lowercase letters represent significant variations among the data at $p < 0.05$

Table 3 Antagonistic activity of *Bacillus* strains against *R. solani*, *F. proliferatum*, *A. rolfsii* and *C. gloeosporioides* under broth assay

Strain	<i>R. solani</i>		<i>F. proliferatum</i>		<i>A. rolfsii</i>		<i>C. gloeosporioides</i>	
	Mycelial dry weight (g)	Percent inhibition over control	Mycelial dry weight (g)	Percent inhibition over control	Mycelial dry weight (g)	Percent inhibition over control	Mycelial dry weight (g)	Percent inhibition over control
K2	0.20 ^c	80.8 ^b	0.32 ^c	75.9 ^a	0.42 ^c	78.0 ^b	0.33 ^c	82.1 ^a
K12	0.79 ^b	25.3 ^c	0.46 ^b	65.9 ^b	0.68 ^b	65.1 ^c	0.39 ^b	78.9 ^b
BioCWB	0.15 ^d	84.5 ^a	0.27 ^c	76.6 ^a	0.25 ^d	87.8 ^a	0.31 ^c	83.2 ^a
Control	1.04 ^a	–	1.34 ^a	–	1.93 ^a	–	1.85 ^a	–
CD (0.05)	0.042	1.983	0.059	2.497	0.045	1.608	0.0521	2.731

Lowercase letters represent significant variations among the data at $p < 0.05$

Table 4 Evaluation of antagonistic potential of *Bacillus* strains against *R. solani* in potted rice plants

Treatments	Trial I		Trial II	
	PDI ^a	% Reduction over control	PDI ^a	% Reduction over control
K2	40.6 (39.58) ^c	52.2	37.35 (37.69) ^c	54.5
K12	62.8 (52.43) ^d	26.2	58.50 (49.90) ^d	28.7
BioCWB	23.3 (28.86) ^b	72.6	25.00 (29.99) ^b	69.5
Chemical	14.81 (22.63) ^a	82.6	13.50 (21.56) ^a	83.5
Control	85.2 (67.51) ^e		82.10 (65.07) ^e	

Lowercase letters represent significant variations among the data at $p < 0.05$

^aValues are the mean of five replications. Values in the parenthesis are arcsine transformed values. Means followed by a common letter are not significantly different at 5% level by DMRT

Many studies have proved that *Bacillus* is known for biocontrol of plant diseases by different mechanisms, which include antibiosis, competition for nutrients and induction of defense responses in the host plant (Prabhukarthikeyan et al. 2014; Stein 2005). Further, compatibility assays conducted on nutrient agar medium deciphered *B. luciferensis* K2, *B. amyloliquefaciens* K12 and *B. subtilis* BioCWB to have no antagonistic effect on each other.

Larvicidal activities of *Bacillus* spp. against rice leaf folder

The larvicidal potential of *Bacillus* species against third instar leaf folder larvae (Table 5) showed *B. luciferensis* treated leaves showed 50% (LC₅₀) leaf folder larval mortality with the cell loads of 2.00×10^4 CFU ml⁻¹,

Table 5 Toxicity of *Bacillus* strains against third instars larva ($n=30$) of rice leaf folder *C. medinalis* (Guenee)

Bacteria	X ² (DF) ^a	Slope	LC ₅₀ ^b	LC ₅₀ confidence interval	
				Lower	Upper
K2	0.69 (4)	2.60 ± 0.4	2.00×10^4	4.22×10^3	2.22×10^5
K12	3.21 (4)	3.47 ± 0.5	2.97×10^4	5.68×10^3	1.01×10^5
BioCWB	2.98 (4)	4.40 ± 0.6	2.10×10^4	1.01×10^4	1.09×10^5

^aDegree of freedom, ^bLC₅₀ of one treatment is significant different if the both lower and upper fiducial limit does not include LC₅₀ value of other treatment

whereas *B. subtilis* and *B. amyloliquefaciens* treated leaves showed 50% larval mortality with 2.10×10^4 CFU ml⁻¹ and 2.97×10^4 CFU ml⁻¹ inoculum load, respectively. However, there was no significant variation in larvicidal activity among the *Bacillus* spp. Additionally, in the present study, *Bacillus* spp. were identified to produce siderophore, ammonia, HCN, protease, and antimicrobial metabolites, which indicates the combined action of enzymatic activity along with other antimicrobial components might be responsible for the strong larvicidal activity against rice leaf folder (Panneerselvam et al. 2018).

Antimicrobial peptide genes present in *Bacillus* spp

To understand the mechanisms of the antagonistic potential of *Bacillus* spp., specific genes responsible for antimicrobial peptides (AMPs) production were assessed through PCR. Among the ten antimicrobial peptides (AMP) assayed (Fig. 2), *B. subtilis* strain BioCWB was found to possess the maximum of eight AMPs namely ericin, subtilin, surfactin, iturin, bacilysin, subtilosin, fengycin and bacillomycin except mycosubtilin and mersacidin. The isolate *B. amyloliquefaciens* strain K12 and *B. luciferensis*

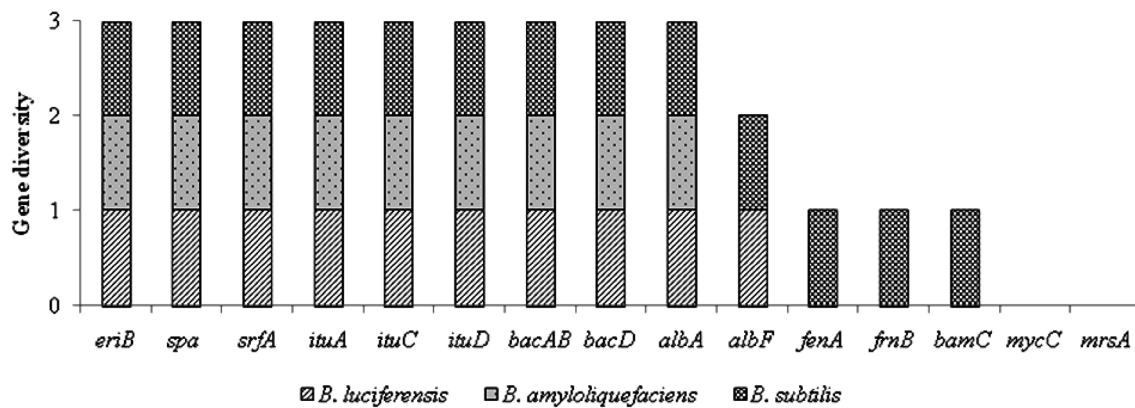


Fig. 2 Results of PCR amplification of AMP genes from *Bacillus* strains

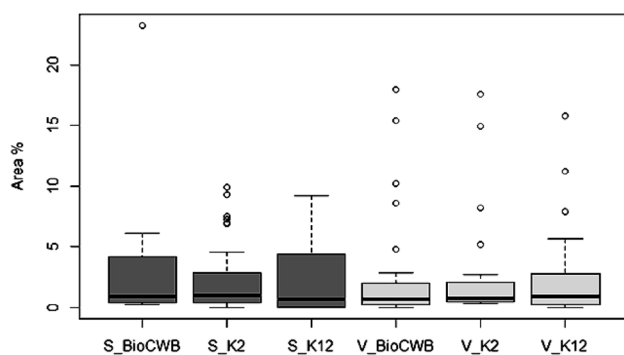


Fig. 3 Comparisons of volatiles and soluble metabolites released by *Bacillus* spp. The figure represents total number of soluble metabolites (denoted “S”; dark-grey) and volatile metabolites (denoted “V”; light-grey) released by *B. subtilis* BioCWB, *B. luciferensis* K2 and *B. amyloliquefaciens* K12

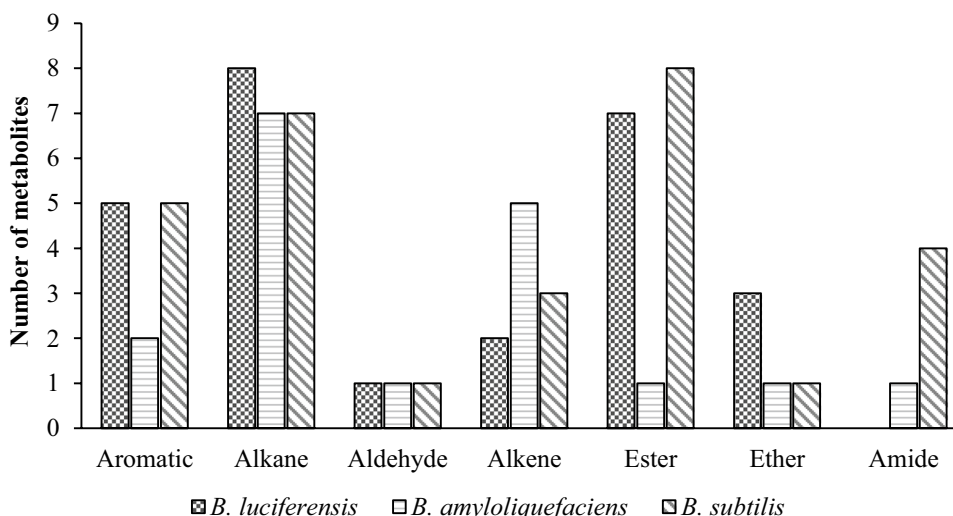
strain K2 were found to possess six AMPs viz., ericin, subtilin, surfactin, iturin and bacilysin and subtilisin, respectively. In a previous study Vater et al. (2002) opined that antibiotics production with wide antimicrobial spectrum provided strong active biocontrol activities against plant fungal pathogens, for examples cyclic lipopeptides production by *Bacillus* spp. such as bacillomycin, fengycin, iturin, and surfactin, dipeptides (bacilysin) and lantibiotic (subtilin) played a major role in bio-control activity (Stein 2005; Bongers et al. 2005).

Volatiles and soluble metabolites released by *Bacillus* spp

The release of volatiles by microorganisms such as bacteria and fungi has been studied by several researchers (Kai et al. 2009). In our study, many aliphatic, aromatic, sulfur-containing and nitro compounds were characterized using TD-GC-MS technique and the results showed that 29, 26 and 23 different volatile compounds were observed in *B.*

subtilis BioCWB, *B. luciferensis* K2 and *B. amyloliquefaciens* K12, respectively (Fig. 3). The compounds having maximum area percentage in *B. subtilis* BioCWB were 1,3-Pentadiene, (Z)- (17.98%), 3-Hexyn-2-ol (15.44%) and disulfide dimethyl (10.25%). Similarly, abundant volatiles quantified in *B. luciferensis* K2 were 2-methyl-Furan (17.56%), acetic acid (14.94%) and disulfide dimethyl (8.18%), whereas *B. amyloliquefaciens* K12 had only two abundant volatiles namely disulfide dimethyl (15.79%) and 3-methyl-Furan (11.24%). Huang et al. (2012) reported that VOCs produced by *Bacillus* spp. play important roles in the plant, such as preventing the colonization of fungal phytopathogens, triggering induced systemic resistance (ISR) by activating NPR1 and SA/ethylene mediated pathways (Rudrappa et al. 2010) and participating in growth regulations of the plant (Ryu et al. 2003). Among the soluble metabolites, disulfide dimethyl (DMDS) has been previously reported to play important role in control of several fungal diseases by triggering induced systemic resistance (ISR) in plant (Huang et al. 2012) and also improves plant growth by modifying root architecture thereby making this VOC an alternative to agricultural chemicals (Tyagi et al. 2019). Additionally, DMDS has been used as an alternative to methyl bromide which has been used as a fumigant (Fritsch 2004) for controlling phytopathogenic fungi (Zou et al. 2007) and nematodes (Coosemans 2004). Although reports have claimed regarding the production of VOCs by the rhizobacteria *B. subtilis* and *B. amyloliquefaciens* and their involvement in plant growth stimulation and ISR (Farg et al. 2006), this is the first report of DMDS quantification in *B. luciferensis*. Similarly, the soluble metabolites secreted by bacteria were quantified by GC-MS which showed the presence of aromatic, alkane, aldehyde, alkene, ester, ether and amide compounds. In our analysis, higher amount of alkane and ester compounds were present in *B. subtilis* BioCWB and *B. luciferensis* K2 as compared to *B. amyloliquefaciens* K12 (Fig. 4). Also *B. subtilis* BioCWB

Fig. 4 Different types of soluble metabolites detected in individual *Bacillus* spp



had 30 metabolites with 9-octadecenamide (23.256%) being the most abundant followed by 5-octadecene (6.074%) and 3-eicosene (5.735%) (Supplementary Fig. 1). Based on previous studies it was found the compound 9-Octadecenamide was reported to have antimicrobial properties (Gumgumjee and Hajar 2015). The other strain, *B. luciferensis* K2 had 26 metabolites among which E-15-Heptadecenal (9.89%) was found to be maximum followed by E-3-Eicosene (9.31%) (Supplementary Fig. 2). Similarly, *B. amyloliquefaciens* strain K12 recorded least number of 18 metabolites with E-15-heptadecenal (9.20%) being the maximum followed by E-3-eicosene (8.41%) (Supplementary Fig. 3). The compound E-15-heptadecenal had higher relative abundance in comparisons to other compounds as found in *B. luciferensis* and *B. amyloliquefaciens* which have earlier been reported to also have antimicrobial properties (Yogeswari et al. 2012). From the PCA analysis, it was observed that the volatiles

released by *B. luciferensis* K2 had more variations in comparisons with *B. subtilis* BioCWB and *B. amyloliquefaciens* K12 whereas soluble metabolites had more variations in *B. subtilis* BioCWB as compared to *B. amyloliquefaciens* K12 and *B. luciferensis* K2 (Fig. 5).

Evaluation of plant growth promoting potential of *Bacillus* spp. in rice and *Capsicum* plants

Based on agronomic growth parameters, there was a significant difference in all plant growth parameters except tiller number in rice plants treated with *Bacillus* spp. as compared to uninoculated control. All three *Bacillus* spp. (K2, K12 and BioCWB) significantly ($p=0.05$) improved plant dry biomass in rice (6.2–7.3 g per plant) as compared to uninoculated control (Table 6). However, among the three bacterial strains, *B. luciferensis* K2 inoculated rice plants

Fig. 5 PCA analysis of volatiles and soluble metabolites from *Bacillus* spp. The principal component analysis separates the distribution of volatile and soluble metabolites across two dimensions namely first principal component (Dim 1) and first principal component (Dim 2) representing 27% and 19.2% respectively. Soluble released by bacterial isolates, are denoted by prefix “S” whereas volatiles are denoted by prefix “V”

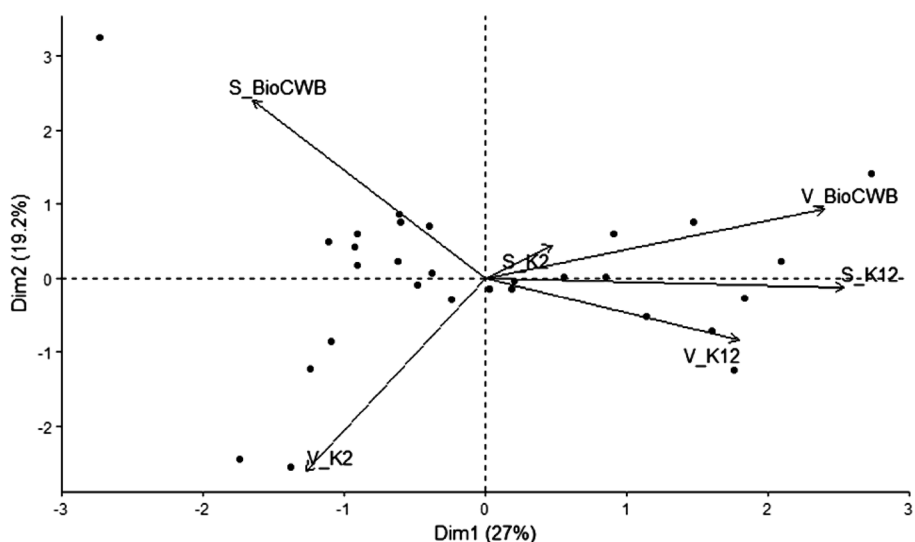


Table 6 Effect of *Bacillus* spp. inoculation on plant growth promotion in rice after 60 days of transplanting

Treatment	Plant height (cm)	Root length (cm)	Fresh shoot wt. plant ⁻¹ (g)	Fresh root wt. plant ⁻¹ (g)	Dry shoot wt. plant ⁻¹ (g)	Dry root wt. plant ⁻¹ (g)	Total dry biomass plant ⁻¹ (g)	Tiller number plant ⁻¹
K2	117.3 ± 3.12 ^b	19.3 ± 1.26 ^a	26.5 ± 0.42 ^a	2.5 ± 0.09 ^b	6.2 ± 0.06 ^a	1.1 ± 0.03 ^a	7.3 ± 1.15 ^a	7.8 ± 1.14
K12	131.2 ± 1.46 ^a	16.9 ± 0.71 ^b	24.3 ± 0.18 ^b	2.3 ± 0.12 ^c	5.7 ± 0.29 ^c	1.03 ± 0.02 ^c	6.7 ± 0.47 ^c	8.2 ± 1.34
BioCWB	129.8 ± 1.21 ^a	19.1 ± 0.35 ^a	25.6 ± 0.19 ^a	2.8 ± 0.09 ^a	6.0 ± 0.13 ^b	1.09 ± 0.05 ^b	7.1 ± 0.93 ^{ab}	8.7 ± 0.89
Control	119.4 ± 2.05 ^b	16.7 ± 0.50 ^b	22.6 ± 0.28 ^b	2.2 ± 0.05 ^c	5.2 ± 0.10 ^d	1.01 ± 0.01 ^c	6.2 ± 0.37 ^d	7.0 ± 0.81
CD (0.05)	2.7	1.07	0.38	0.12	0.23	0.04	0.25	NS

Lowercase letters represent significant variations among the data at $p < 0.05$

Table 7 Evaluation of *Bacillus* strains at field level in capsicum plant

Treatments	Plant height (cm)	Fruits number	Dry shoot weight (g)	Dry root weight (g)	Total plant dry biomass (g)
T1—uninoculated control	29.3 ± 2.6 ^c	6.0 ± 0.5 ^c	5.3 ± 0.2 ^d	0.4 ± 0.1 ^c	6.0 ± 0.5 ^d
T2—BioCWB	33.7 ± 3.5 ^{bc}	6.0 ± 0.5 ^c	7.0 ± 0.5 ^c	0.7 ± 0.1 ^{ab}	7.4 ± 0.6 ^c
T3—K12	46.0 ± 3.1 ^{ab}	10.7 ± 1.5 ^b	6.9 ± 0.2 ^c	0.6 ± 0.2 ^b	7.5 ± 0.5 ^c
T4—K2	41.3 ± 2.3 ^{abc}	8.7 ± 1.0 ^{bc}	8.2 ± 0.5 ^b	0.7 ± 0.1 ^a	8.9 ± 0.5 ^b
T5—BioCWB + K12 + K2	48.3 ± 3.7 ^a	14.3 ± 0.5 ^a	10.0 ± 0.5 ^a	0.7 ± 0.1 ^a	10.7 ± 0.7 ^a
CD (0.05)	17.1	3.2	0.3	0.9	0.3

Lowercase letters represent significant variations among the data at $p < 0.05$

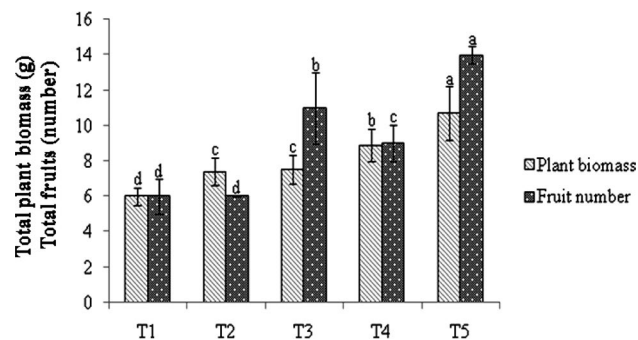


Fig. 6 Evaluation of *Bacillus* spp. at field level in *Capsicum* plant. The following treatments were applied T1—uninoculated control, T2—*B. subtilis* BioCWB, T3—*B. amyloliquefaciens* K12, T4—*B. luciferensis* K2, T5—*B. subtilis* BioCWB + *B. amyloliquefaciens* K12 + *B. luciferensis* K2

showed significantly higher plant biomass (7.3 g per plant) as compared to *Bacillus* spp. The combined PGP traits viz., phytohormone production and nutrient solubilization abilities observed in *Bacillus* spp. (K2, K12 and BioCWB) might have improved plant growth promotion in rice. The present results are corroborated with the earlier reports of Govindasamy et al. (2010) and Posada et al. (2018), who have described plant growth promoting potentials of *Bacillus* spp. Further, the evaluation of *Bacillus* spp. (K2, K12 and BioCWB) in *Capsicum* plants under field conditions in Sikkim (Table 7 and Fig. 6) clearly shows their plant growth promoting abilities and suitability other than cereals. In

Capsicum plants, *B. luciferensis* K2 recorded significantly higher root dry weight, plant dry biomass and fruit number, followed by *B. amyloliquefaciens* K12 and *B. subtilis* BioCWB. Further, the combination of three strains was also carried in *Capsicum* plant and was found superior in enhancing plant height (48.3 cm), dry shoot (10.0 g), root weight (0.7 g), plant biomass (10.7 g) and fruit number (14.3) as compared to individual strains. In general, application of *Bacillus* spp. either individually or in combination significantly increased plant growth parameters as compared to uninoculated control. Many previous studies have proved that, species of *Bacillus* spp. like *B. amyloliquefaciens*, *B. subtilis*, *B. thuringiensis* and *B. megaterium* (Jamal et al. 2018; Kour et al. 2018) possess PGP traits and enhance plant growth in several agricultural and horticultural crops.

Conclusion

The following native bacterial strains viz., *B. subtilis* BioCWB, *B. luciferensis* K2 and *B. amyloliquefaciens* K12 could be used as bio-inoculants for enhancement of growth and yield of *Capsicum* under organic cultivation in Sikkim, India. Further, in the present study, the documented anti-fungal and insecticidal properties of bacterial strains demonstrated possibilities of application as biocontrol agents for organic cultivation of agricultural crops. For the first time,

the novelties of multifunctional plant growth promotion and biocontrol potential associated with *B. luciferensis* K2 were documented from organic soils of Sikkim, India.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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