Research Article

Ariyan Manikandan, Iruthayasamy Johnson[#], Nanjundan Jaivel[#], Ramasamy Krishnamoorthy, Murugaiyan SenthilKumar, Rajasekaran Raghu, Nellaiappan Olaganathan Gopal, Prasun K. Mukherjee, Rangasamy Anandham^{*}

Gamma-induced mutants of *Bacillus* and *Streptomyces* display enhanced antagonistic activities and suppression of the root rot and wilt diseases in pulses

<https://doi.org/10.1515/bmc-2022-0004>

received December 30, 2021; accepted February 7, 2022

Abstract: This study aims to increase *Bacillus* and *Streptomyces* antagonistic activity against the root rot and wilt diseases of pulses caused by *Macrophomina phaseolina* and *Fusarium oxysporum* f. sp. *udum*, respectively. To increase antagonistic action, *Bacillus subtilis* BRBac4, *Bacillus siamensis* BRBac21, and *Streptomyces cavourensis* BRACB10 were subjected to random mutagenesis using varying doses of gamma irradiation (0.5–3.0 kGy). Following the irradiation, 250 bacterial colonies were chosen at random for each antagonistic strain and their effects against pathogens were evaluated in a plate assay. The ERIC, BOX, and random amplified polymorphic studies demonstrated a clear distinction between

mutant and wild-type strains. When mutants were compared to wild-type strains, they showed improved plant growth-promoting characteristics and hydrolytic enzyme activity. The disease suppression potential of the selected mutants, *B. subtilis* BRBac4-M6, *B. siamensis* BRBac21-M10, and *S. cavourensis* BRACB10-M2, was tested in green gram, black gram, and red gram. The combined inoculation of *B. siamensis* BRBac21-M10 and *S. cavourensis* BRACB10-M2 reduced the incidence of root rot and wilt disease. The same treatment also increased the activity of the defensive enzymes peroxidase, polyphenol oxidase, and phenylalanine ammonia-lyase. These findings suggested that gamma-induced mutation can be exploited effectively to improve the biocontrol characteristics of *Bacillus* and *Streptomyces*. Following the field testing, a combined bio-formulation of these two bacteria may be utilised to address wilt and root-rot pathogens in pulses.

Keywords: *Bacillus*, *Streptomyces*, gamma irradiation, PGPR, plant defense enzyme, disease control

[#] Equally contributed.

*** Corresponding author: Rangasamy Anandham**, Department of Agricultural Microbiology, Tamil Nadu Agricultural University (TNAU), Coimbatore 641003, Tamil Nadu, India, e-mail: anandhamranga@gmail.com

Ariyan Manikandan, Nanjundan Jaivel, Rajasekaran Raghu, Nellaiappan Olaganathan Gopal: Department of Agricultural Microbiology, Tamil Nadu Agricultural University (TNAU), Coimbatore 641003, Tamil Nadu, India

Iruthayasamy Johnson: Department of Plant Pathology, Tamil Nadu Agricultural University (TNAU), Coimbatore, Tamil Nadu, India

Ramasamy Krishnamoorthy: Department of Crop Management, Vanavarayar Institute of Agriculture, Pollachi, Tamil Nadu, India

Murugaiyan SenthilKumar: Department of Crop Management, Agricultural College and Research Institute, Tamil Nadu Agricultural University (TNAU), Eachangkottai, Tamil Nadu, India

Prasun K. Mukherjee: Environmental Biotechnology Section Nuclear Agriculture and Biotechnology Division, Bhabha Atomic Research Centre, Trombay, Mumbai 400085, India

Introduction

Pulses are India's most important protein dietary source, with minimal fat and high fibre and nutrient content [1]. India cultivates roughly 34% of the worldwide cultivable land for pulses and contributes 24% of the global pulses production [2]. However, productivity of pulses in India is extremely low (652 kg ha⁻¹) (FAOStat, 2013). It is estimated that wilt and rot diseases cause 40–45% of the yield loss in pulses [3,4]. *Fusarium oxysporum* f. sp. *udum* and *Macrophomina phaseolina* are the primary pathogens responsible for wilt and root rot diseases in pulses, respectively. Traditional methods for controlling soil-borne fungal infections include fungicide treatment,

fumigation, and crop rotation [5,6]. However, the use of fungicides has greater drawbacks, such as environmental pollution, negative impact on soil-beneficial bacteria and insects, and development of disease resistance to the specific fungicide [7,8]. As a result, alternate sustainable and environmentally beneficial solutions are required.

The antagonistic microorganisms are used as biocontrol agents, which is a cost-effective and environmentally beneficial technique of controlling phytopathogens [9,10]. Certain antagonistic rhizobacteria utilised as biocontrol agents also have plant growth-promoting properties [11]. *Bacillus* sp. [12–16], *Pseudomonas* sp. [12,17], *Trichoderma* spp. [18–20], and *Streptomyces* [21–25] were utilised to suppress *Macrophomina* root rot and *Fusarium* wilt in diverse crops. Various modes of action in disease control have been identified in these antagonistic bacteria, including the formation of antifungal metabolites, lytic enzymes [24,26–28], volatile chemicals [15], and the establishment of systemic resistance [8,27,29].

Natural microbial strains produce only trace levels of antifungal compounds. This is because they require these secondary metabolites for competitive advantage and do not overproduce them. Microorganisms have evolved regulatory systems that allow a strain to prevent overproduction of its metabolites. Hence, strain enhancement programmes are essential for commercial application [30]. The use of random mutagenesis to modify strains aids in the improvement of biocontrol abilities and/or the synthesis of antifungal metabolites. Chemical or physical mutagens (ultraviolet or gamma irradiation) are employed [31]. Gamma irradiation boosted *Bacillus subtilis* secondary metabolites and disease control abilities [32,33], *Trichoderma harzianum* protease activity [34–36], *Streptomyces* antibiotic synthesis [37–39], and *Bacillus thuringiensis* chitinase production [40].

B. subtilis BRBac4, *Bacillus siamensis* BRBac21, and *Streptomyces cavourensis* BRACB10 isolated from the pulses rhizosphere previously demonstrated numerous plant growth-promoting features as well as antagonistic activity against both the fungal diseases, *F. oxysporum* f. sp. *udum* and *M. phaseolina* (unpublished data). Random mutagenesis was performed in *B. subtilis* BRBac4, *B. siamensis* BRBac21, and *S. cavourensis* BRACB10 using gamma irradiation and the mutation was validated by ERIC, BOX, and random amplified polymorphic DNA (RAPD) studies. We then tested these effective mutants against *F. oxysporum* f. sp. *udum* and *M. phaseolina* *in vitro* and in pot culture in pulses.

Materials and methods

Microorganisms and growth conditions

B. subtilis BRBac4 (GenBank: MN165562), *B. siamensis* BRBac21 (GenBank: MN165655), and *S. cavourensis* BRACB10 (GenBank: OK427229) were isolated from pulses rhizosphere soils and used for this study. *Bacillus* spp. and *Streptomyces* were grown in an incubator shaker at $28 \pm 2^\circ\text{C}$ in liquid nutrient and tryptic soya media, respectively. *F. oxysporum* sp. *udum* NAIMCC-F-01125 was obtained from the Indian Council of Agricultural Research, Maunath Bhanjan, Uttar Pradesh, India, and *M. phaseolina* BRMP1 (GenBank: MN165654) was previously isolated from disease-infected pulses and obtained from the Department of Agricultural Microbiology, Tamil Nadu Agricultural University, Coimbatore, India. At $28 \pm 2^\circ\text{C}$, the fungal pathogens were cultured in potato dextrose agar medium.

Gamma-induced mutation

The mutation was carried out at the Gamma Chamber 5000 at the Board of Radiation and Institute of Technology, Department of Atomic Energy (DAE), in Mumbai, India. The mutation was carried out at the Gamma Chamber 5000 at the Board of Radiation and Institute of Technology, Department of Atomic Energy (DAE), in Mumbai, India as described by Afsharmanesha *et al.* [32]. Bacterial strains previously cultivated in liquid media were placed in cryovials in triplicate and directly exposed to various gamma irradiation dosages, namely 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 kGy. C60 had an efficiency of 2.2 kGy. Following irradiation, the bacteria were serially diluted up to 10^{-6} in 0.8% saline and plated on their respective growth medium. Mutant colonies were chosen based on the physical differences between them and their wild-type counterparts. All the *Bacillus* and *Streptomyces* colonies that were chosen as putative mutants were kept in their respective growth medium for further testing.

Screening of antifungal activity

Using the dual plate approach previously reported by Khan *et al.* [41], all of the selected colonies were tested for antifungal activity against the pathogens *M. phaseolina* and *F. oxysporum* sp. *udum*. On one side of the potato dextrose agar Petri plates, a 6 mm fungal disc was inserted

and the bacterial stain was streaked immediately opposite the mycelial disc. As a control, a fungal disc with no bacterial inoculation was used. All of the plates were incubated for 7 days at $28 \pm 2^\circ\text{C}$. The following formula was used to compute the percentage of fungal growth:

$$[(C - T)/C] \times 100,$$

where C – mycelial growth in control, T – mycelial growth in the treatment plates.

DNA isolation, rep-polymerase chain reaction (PCR), and RAPD

The Hi-GenoMB Miniprep kit (Hi Media, India) was used to isolate DNA from wild and putative mutant *Bacillus* and *Streptomyces* strains. Extracted DNA was electrophoresed on 0.8% agarose gel and visualised in the Gel documentation unit (Vilber, Germany). For mutation confirmation, RAPD, ERIC, and BOX amplifications were performed [34]. The PCR mixture contains two PCR master kits (Sigma, India), 10 pmol of each primer (Table S1) (BioKart, Bengaluru), and 10 ng of genomic DNA. Initial denaturation at 95°C for 5 min was followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 45°C (ERIC), 46°C (BOX), and 33°C (RAPD) for 1 min, and final extension at 72°C for 1 min. In 1% agarose gel, amplicons were electrophoresed.

PCR amplification of the antibiotic resistance gene

Two *Bacillus* and mutant colonies with significant anti-fungal activity were examined for the presence of lipopeptide genes (surfactin and iturin) [42]. Lipopeptide gene PCR amplification began with a 5 min denaturation at 95°C , followed by 30 cycles of denaturation (1 min at 94°C), annealing (1 min at 55°C), extension (1 min at 72°C), and final extension for 10 min at 72°C . Table S1 lists the primers utilised for the various lipopeptide genes.

Plant growth-promoting (PGP) traits

Indole acetic acid (IAA)

The synthesis of IAA was quantified using the previously reported method [43]. Overnight developed cultures were inoculated in 5 mL of nutrient broth containing 0.1%

tryptophan and cultured for 72 h at $28 \pm 2^\circ\text{C}$. The supernatant was then collected for 10 min at 12,000 rpm. Around 500 μL of supernatant was mixed with 50 μL of 0.1 mM ortho phosphoric acid and 2 mL of Salkowski reagents and incubated in the dark for 30 min to generate red colour. IAA was measured using a spectrophotometer at 530 nm (Systronics®, Spectrophotometer 166) and represented as $\mu\text{g mL}^{-1}$.

Bacterial motility assays

The swimming and swarming motility of the bacterial strains *B. subtilis* BRBac4, *B. siamensis* BRBac21, and their mutations was measured using the method published previously [44]. For the swimming and swarming motility assays, Luria Bertani (LB) plates were altered with 0.3 and 0.7% agar, respectively. The plates were incubated for 18 h at $28 \pm 2^\circ\text{C}$ and the colony diameter was measured.

Phosphate solubilisation

In a plate assay, the selected *Bacillus*, *Streptomyces*, and their mutants were examined for phosphate solubilisation efficiency. Overnight grown bacterial strains were inoculated (1×10^7 CFU mL^{-1}) into Pikovskaya's agar medium and incubated for 48 h at $28 \pm 2^\circ\text{C}$. Phosphate solubilisation was thought to be positive in the clear zone surrounding the bacterial colonies.

Hydrolytic activity

Chitinase activity

Chitinase activity was measured using the previously reported method [45]. The bacterial strains were cultivated in liquid nutrient medium enriched with 0.3% colloidal chitin and shaken for 24 h at $28 \pm 2^\circ\text{C}$ (100 rpm). The crude enzyme was then collected by centrifugation at 10,000 rpm for 20 min at 40°C . The reaction solutions were incubated at 35°C for 25 min with 0.3% colloidal chitin (0.1 mL), a crude enzyme solution (0.1 mL), and 0.1 M McIlvaine buffer of pH 6.0 (0.2 mL). The reaction was stopped by adding 2.0 mL of 1.5 mmol potassium ferricyanide reagent. A spectrophotometer (Systronics®, Spectrophotometer 166) was used to measure the final absorbance at 420 nm. The enzyme activity was measured in micromoles of *N*-acetyl glucosamine per minute.

Protease and cellulase activity

The skim milk agar medium was used to test the protease activity of *Bacillus*, *Streptomyces*, and their mutants. Overnight developed bacterial cultures were spotted (1×10^7 CFU mL⁻¹) on skim milk agar medium and incubated for 72 h at $28 \pm 2^\circ\text{C}$. The presence of a clear zone around the colonies suggested the presence of protease activity. To assess cellulase activity, nutrient agar medium was supplemented with 1% carboxy methyl cellulose instead of glucose, and $10 \mu\text{L}$ (1×10^7 CFU mL⁻¹) of bacterial strains were spot inoculated. The plates were incubated for 72 h at $28 \pm 2^\circ\text{C}$. Following the incubation period, the hydrolysis zone was seen by flooding the plates with 0.1% iodine solution for 15–20 min, after which the plates were rinsed with distilled water and destained with 1 M NaCl. The presence of translucent hydrolytic zones around the colonies showed the presence of positive cellulase activity [46].

Analysis of the volatile compounds

The volatile organic compounds (VOCs) of mutant *Bacillus* and *Streptomyces* strains were investigated using gas chromatography – mass spectrometry (GC-MS) and a thermal desorber (TD). In a 500 mL Erlenmeyer flask, the bacteria were inoculated into liquid nutrient medium. With the help of a rubber cork, a tenax-coated stainless-steel column was fitted with the flask. The VOCs collected by the column were analysed using TD-GC-MS on a TurboMatrix 150 (Perkin Elmer, USA). For separation, a non-polar capillary column (30 m, 250 μm) coated with 5% phenyl methyl siloxane was utilised. The following temperature regime resulted in volatile separation: the initial oven temperature of 50°C was increased to 250°C at a rate of 10°C per min; the injection port was kept in split mode with a 1:10 split ratio; and the carrier gas was helium at a constant flow rate of 0.7 mL min^{-1} . The MS settings were as follows: full scan mode; scan range of 0.2 s at intervals of 0.1 s; electron impact spectra of 70 eV; and positive ion mode. By comparing the collected mass spectra to the NIST/EPA/NIH Mass Spectral Library, NIST 14, the VOC was identified. The approach described by Passari *et al.* [47] was used for VOC detection and analysis.

Effect of antagonistic mutants on suppression of root rot and wilt pathogens

Under pot culture conditions, the best-performing *Bacillus* and *Streptomyces* mutants were tested against the wilt and

root rot pathogens. Red gram cv., Co (Rg)-7, Green gram cv., Co (Gg)-7, and Black gram cv., Vamban 8 seeds were received from the Department of Pulses at Tamil Nadu Agricultural University in Coimbatore, India. The seeds were surface cleaned with 0.1% HgCl_2 and washed four times with sterile distilled water before soaking for 30 min in *Bacillus* (10^8 CFU mL⁻¹) and *Streptomyces* (10^8 CFU mL⁻¹) mutants produced in their respective media. For pot culture study, soil and sand were taken in the ratio of 3:1 and 2 kg of this mixture were filled in a pot (18 cm outer diameter and 20 cm height) and 5 seeds were sown in each pot. In total, five pots were maintained for each treatment and each treatment was replicated four times. Treatment details in this study were T1 – *B. subtilis* BRBac4-M6, T2 – *B. siamensis* BRBac21-M10, T3 – *S. cavourensis* BRACB10-M2, T4 – *B. subtilis* BRBac4-M6 + *B. siamensis* BRBac21-M10, T5 – *B. subtilis* BRBac4-M6 + *S. cavourensis* BRACB10-M2, T6 – *B. siamensis* BRBac21-M10 + *S. cavourensis* BRACB10-M2, T7 – *B. subtilis* BRBac4-M6 + *B. siamensis* BRBac21-M10 and *S. cavourensis* BRACB10-M2, T8 – standard bio-control agent (*Trichoderma viride*), T9 – chemical fungicide Thiram, T10 – control (pathogen inoculated). Oatmeal medium was used to produce *M. phaseolina* inoculum. Mycelial discs were injected into the sterile oat meal medium and grown at 30°C . Before sowing the seeds of black gram and green gram, the inoculum (0.1% w/w) was combined. Inoculum of *F. oxysporum* f. sp. *udum* was made by adding 150 mL (2×10^8 spores mL⁻¹) of broth per kilogram of soil and incubating for 7 days for spore germination. This inoculum was combined before to the sowing of red gram [48]. The plant defence enzyme was analysed 28 days after the seeds were sown (DAS). Plant samples were uprooted and taken to the laboratory for biometric measures after 35 DAS.

Enzyme analysis

After collecting 28 DAS leaf samples, they were pulverised with liquid nitrogen in a pre-chilled pestle and mortar. One gram of powdered leaf sample was combined with 2 mL of 0.1 M sodium phosphate buffer (pH 7.0), and the supernatant was collected after 15 min of centrifugation at 14,000 rpm at 4 $^\circ\text{C}$. The enzyme activity of peroxidase (PO), polyphenol oxidase (PPO), and phenylalanine ammonia-lyase (PAL) were determined using the supernatant.

PO, PPO, and PAL assay

PO activity was measured using the method described earlier [49]. The reaction mixture was composed of 0.5 mL of enzyme extract, 1.5 mL of 0.05 M pyrogallol, and 0.5 mL

of 1% H_2O_2 . The reaction mixture was incubated at $28 \pm 2^\circ\text{C}$ for 3 min, and the change in absorbance was recorded at 420 nm every 30 s. The boiled enzyme was added to the test mixture as a blank. PO activity was measured as a change in OD per minute per gram fresh weight. PPO activity was assessed using the method described by Park et al. [50]. Around 200 μL of enzyme extract and 1.5 mL of 0.1 M sodium phosphate buffer (pH 6.5) were used in the test. The reaction was started by adding 200 μL of 0.01 M catechol and the absorbance at 495 nm was measured. PPO activity was measured as a change in OD per minute per gram fresh weight. The PAL assay was carried out exactly as stated by Lee et al. [51]. The assay mixture was made up of 100 μL of enzyme extract, 500 μL of 50 mM tris-HCl (pH 8.8), and 600 μL of 1 mM L-phenylalanine, and it was incubated for 1 h. To stop the process, 2 N HCl was added. The mixture was then vortexed and centrifuged after 1.5 mL of toluene was added (1,000 rpm, 5 min). After separating the toluene phase, the *trans* cinnamic acid level was measured at 290 nm with a spectrophotometer (Systronics®, Spectrophotometer166). A standard was prepared by using cinnamic acid in toluene. PAL activity is expressed as *trans* cinnamic acid per hour per gram fresh weight.

Statistical analysis

Analysis of variance was used to analyse all the data and the mean values were compared using Tukey's honest significant difference test. The figures were created with the statistical software R and Origin pro. The PRIMER 7 programme was used to create the dendrograms.

Results

Screening of mutants

After gamma irradiation, 250 colonies were randomly picked for each antagonistic bacterial strain based on colony morphology (Figure 1). As a result, in a dual plate assay, all of the selected colonies were tested for anti-fungal activity against *M. phaseolina* and *F. oxysporum* sp. *udum*. The results revealed that mutant strains had stronger inhibitory action than their wild-type counterparts. The rate of inhibitory activity was found to be higher at 2,500 and 3,000 Gray radiation doses. *M. phaseolina* was inhibited by *B. siamensis* BRBac 21-M10 to a maximum of 69%. *F. oxysporum* sp. *udum* was inhibited by *B. siamensis* BRBac 21-M15 to a maximum of 56%. Also in *S. cavourensis* BRACB10 M2 mutant showed the increased inhibition activities against the tested pathogens (Table 2; Figures S1 and S2).

Analysis of rep-PCR, RAPD fingerprint patterns, and lipopeptide genes

Six primers were utilised to amplify the selected mutants and their wild types for fingerprinting analysis. The banding pattern of each primer differed from one another. In the mutants of *B. subtilis* BRBac4 and *B. siamensis* BRBac21, the BOX1AR showed four different types of bands ranging from 800 to ~2,500 bp (Figure 2). The identical BOX1AR primer, however, produced nine distinct bands in the *S.*

Figure 1: Comparison of colony morphology of wild and mutant strains. (a) – *B. subtilis* BRBac4 (wild); (b, c, and d) – mutants of *B. subtilis* BRBac4 (b-M4, c-M6, d-M8), (f) – *B. siamensis* BRBac21 (wild); (g, h, and i) mutants of *B. siamensis* BRBac21 (g – M2, h – M10, i – M12), (e) – *S. cavourensis* BRACB10 (wild); (j) – mutant of *S. cavourensis* BRACB10.

Figure 2: ERIC, BOX fingerprinting pattern in wild and mutants strains of *B. subtilis* BRBac4, *B. siamensis* BRBac21, and *S. cavourensis* BRACB10. BOX (i), ERIC (ii) of *B. subtilis* BRBac4 and their respective mutants. Lanes 1–11 (1-M1, 2-M2, 3-M3, 4-M4, 5-M5, 6-M6, 7-M7, 8-M8, 9-M9, 10-M10, and 11-M11). BOX (iii), ERIC (iv) of *B. siamensis* BRBac21 and their respective mutants. Lanes 1–11 (1-M1, 2-M2, 3-M3, 4-M4, 5-M5, 6-M6, 7-M7, 8-M8, 9-M9, 10-M10, and 11-M11). BOX (v), ERIC (vi) of *S. cavourensis* BRACB10 and their respective mutants. Lanes 1–14 (1-M1, 2-M2, 3-M3, 4-M4, 5-M5, 6-M6, 7-M7, 8-M8, 9-M9, 10-M10, and 11-M11); Lane W – Wild strains, L – 100 bp DNA Ladder.).

cavourensis BRACB10 mutants, with amplicons spanning from ~80 to 3,500 bp (Figure 2). For BRBac4 and BRBac21 mutants, the ERIC 1R primer produced 6–7 bands with amplicons ranging from 300 to ~3,000. In addition, 11 bands ranging from 180 to ~2,500 were detected in BRACB10 mutants (Figure 2). RAPD primers OPA9, OPA 10, OPA 18, and OPA 19 were used in this study. OPA 9 amplified 10 different bands ranging between 200 bp and ~3,000 bp in BRBac4, BRBac21, and their mutants (Figure S3). OPA 9 exhibited 17 different amplicons which ranged from 100 to ~2,500 bp in the BRACB10 (Figure S4). OPA 10 amplified

only 4 clear bands with a range of 700–1,500 bp for BRBac4 and BRBac21. OPA 10 showed 4–10 different bands in BRACB10 with the amplicon size of 350 to ~2,500 bp. The other RAPD primer OPA 18 and OPA 19 results are also given in Figures S3 and S4.

In this study, all of the primers examined exhibited a considerable amount of polymorphism in all of the strains tested. Furthermore, the presence and disappearance of bands was revealed to be a clear difference between the RAPD and rep-PCR fingerprint profiles of wild and mutant colonies. For example, the BOXA1R

primer showed only four bands in BRBac4 except the M8 mutant, it showed the additional 5 bands in ~1,000 to ~1,500 bp. Similarly, in the BRBac21, two mutants showed additional bands in ~150, ~250, and ~450 bp. Likewise in the BRACB10, two mutants showed a higher intensity of few extra bands compared to its wild types. The ERIC 1R primer, bands were observed with the molecular weight of 200, ~550, 600, 1,000, 1,500, and one additional band at ~2,500 bp which was absent in mutants M10 and M11 of BRBac4. In BRBac21, ~2,500 bp amplicon was absent in mutants while it was noticed in wild type. Also in BRACB10, ERIC 1R primer showed the 11 bands with some additional bands at ~650 and ~1,200 bp were also noticed in mutants which were not observed in wild-type strains. The band with a molecular weight of 700 bp, on the other hand, was missing in BRACB10 mutants. Amplification of lipopeptide genes such as iturin A (~650 bp) and surfactin (~440 bp) from BRBac4 and BRBac21 isolates was confirmed.

Cluster analysis of the rep-PCR and RAPD fingerprints

To separate the wild-type strains and their mutants, cluster analyses were done using data from rep-PCR and RAPD primers. The resulting dendrogram revealed a 91% similarity level between the mutants of *B. subtilis* BRBac4 and their wild type (Figure 3). Cluster I contained 50% of the isolates, together with the wild type; the remaining mutants belonged to Cluster II. Dendrogram results for *B. siamensis* BRBac21 and its mutants demonstrated 92% similarity. Cluster I included just two mutants of BRBac21, M11 and M12. Cluster II contained the remaining mutants and wild types. Similarly, the dendrogram of *S. cavourensis* BRACB10 and its mutants showed 73% similarity, with two clusters. Cluster I accounted for 41.66% of the mutants among the two clusters. Cluster II contained the remaining 58.3% mutants and wild-type strains (Figure 3).

In vitro screening for plant growth-promoting traits

Among the mutants studied, *B. subtilis* BRBac4-M8 had the highest phosphate solubilisation efficiency (182.45%), followed by BRBac4-M6 (179.12%) and BRBac21-M10 had the lowest (153.25%) (Table 1). All *Bacillus* and

Streptomyces mutants had an orange clear zone around the colonies, indicating that they were producing siderophores. When compared to wild types, mutants produced significantly less IAA. The highest level of IAA was found in *B. subtilis* BRBac 4 ($16.4 \mu\text{g mL}^{-1}$), while the lowest amount was found in *S. cavourensis* BRACB10-M4 ($3.1 \mu\text{g mL}^{-1}$) (Table 1). In 0.3% agar containing LB medium, all wild and mutant *Bacillus* showed greater rates of swimming motility; however, in swarming motility *B. subtilis* BRBac4-M6 only exhibited the minimal range of swarming motility (Figure S5).

Screening for hydrolytic enzymatic activity

B. subtilis BRBac4-M8 had the highest chitinase activity of $2822.89 \mu\text{M min}^{-1} \text{mL}^{-1}$, whereas *S. cavourensis* BRACB10-M4 had the lowest at $837.25 \mu\text{M min}^{-1} \text{mL}^{-1}$ (Table 1). The maximal proteolytic activity was identified in *B. siamensis* BRBac21-M1 (128.57%) with a small quantity of proteolytic activity found in other *Streptomyces* and its mutants. *Bacillus* and *Streptomyces* mutants all had positive cellulase activity. Cellulase levels were highest (148%) and lowest (113%) in *S. cavourensis* BRACB10-M3 and *B. siamensis* BRBac21, respectively (Table 1).

Analysis of the volatile compounds

The TD-GC-MS analysis of VOCs from chosen mutants of *B. subtilis* BRBac4, *B. siamensis* BRBac21, and *S. cavourensis* BRACB10 revealed a total of 26 compounds (Table S2). Cyclotrisiloxane (6.7%), hexane (24.1%), carbon disulphide (1.9%), 1,4-pentadiene (6.8%), and cyclopropane were the most abundant volatiles generated by the BRBac21 mutant (6.1%). Furan 2 methyl (28.9%), 1,4-pentadiene (6.9%), carbon disulphide (0.2%), and tetrahydroxybutyl imidazole are all present in BRBac4 (7.6%). Similarly, furan 2 methyl occupied the most amount (38.7%) in BRACB10, followed by disulphide, dimethyl (10.3%), carbon disulphide (1.8%), 1-pentanol (10.3%), and dodecane (0.7%). Carbon disulphide, dimethyl disulphide, benzoic acid, xylene, phenol, dodecane, and benzothiazole are among the recognised volatile chemicals that play an important role in biocontrol and plant growth-promoting qualities. The row Z score values for the VOCs were acquired from the heat map (Figure S6). The Z score on the heat map ranged from 0 to 1. Cyclopropane, butane 2 methyl, cyclohexane,

Figure 3: Dendrogram of *B. subtilis* BRBac4, *B. siamensis* BRBac21, *S. cavourensis* BRACB10 and their mutants from fingerprint data of ERIC, BOX, and RAPD.

Table 1: Pathogen inhibition, production of hydrolytic enzymes and PGP traits of mutants and wild strains of *Bacillus* and *Streptomyces*

Isolates	Inhibition in dual plate assay (%)		Chitinase activity ($\mu\text{M min}^{-1} \text{mL}^{-1}$)	Protease (%)	IAA production ($\mu\text{g mL}^{-1}$)	Phosphate solubilisation (%)	Cellulase activity (%)
	<i>M. phaseolina</i>	<i>F. oxysporum</i> f. <i>sp. udum</i>					
BRBac 4 (wild)	59.4 ± 2.3 ^{efgh}	49.1 ± 1.5 ^{efg}	2634.00 ± 31.0 ^b	66.67 ± 0.2 ^g	16.4 ± 0.15 ^a	176.6 ± 6.2 ^{bc}	118 ± 2.99 ^{gh}
BRBac 4-M1	61 ± 0.8 ^{def}	53 ± 1.0 ^{bcd}	2489.56 ± 37.3 ^c	80.00 ± 1.5 ^f	12.1 ± 0.34 ^g	156 ± 4.56 ^f	124 ± 3.54 ^{efg}
BRBac 4-M4	59 ± 0.9 ^{gh}	52 ± 1.5 ^{cde}	2567.33 ± 48.9 ^{bc}	98.18 ± 2.2 ^c	10.6 ± 0.29 ⁱ	148 ± 0.20 ^h	136 ± 0.09 ^{bcd}
BRBac 4-M6	67 ± 0.2 ^{ab}	59 ± 1.8 ^a	2822.89 ± 42.3 ^a	103.00 ± 2.2 ^b	11.2 ± 0.25 ^h	179 ± 4.99 ^{ab}	121 ± 3.70 ^{gh}
BRBac 4-M8	63 ± 0.6 ^{cde}	43 ± 1.2 ^j	2300.67 ± 20.4	85.00 ± 2.5 ^{de}	9.7 ± 0.01 ⁱ	182 ± 4.5 ^a	128 ± 1.74 ^{def}
BRBac 21 (wild)	62.9 ± 1.2 ^{cde}	45.6 ± 0.2 ^{ghi}	1778.44 ± 7.3 ^e	96.67 ± 1.1 ^c	13.4 ± 0.33 ^{de}	159 ± 1.11 ^{ef}	113 ± 1.46 ^h
BRBac 21 M1	65 ± 0.9 ^{bc}	48 ± 0.4 ^{fgh}	1656.22 ± 22.5 ^f	128.57 ± 3.8 ^a	12.8 ± 0.31 ^f	172 ± 2.81 ^{cd}	124 ± 2.45 ^{efg}
BRBac 21 M2	59 ± 0.2 ^{fgh}	51 ± 1.2 ^{cdef}	1208.07 ± 23.0 ^h	83.33 ± 0.3 ^{ef}	13.1 ± 0.02 ^{ef}	168 ± 1.03 ^e	132 ± 1.08 ^{cde}
BRBac 21 M10	69 ± 1.9 ^a	53 ± 1.2 ^{bcd}	745.11 ± 21.8 ^k	87.50 ± 0.7 ^d	15.6 ± 0.21 ^b	153 ± 2.19 ^g	117 ± 2.87 ^{gh}
BRBac 21 M12	64 ± 0.7 ^{bcd}	50 ± 0.4 ^{def}	1411.78 ± 35.5 ^g	80.00 ± 2.3 ^d	14.5 ± 0.15 ^c	172 ± 4.21 ^{cd}	121 ± 3.05 ^{fgh}
BRBac 21 M15	60 ± 0.7 ^{efg}	56 ± 0.7 ^{ab}	1334.00 ± 11.8 ^g	37.50 ± 0.7 ^h	13.7 ± 0.23 ^d	175 ± 2.62 ^{bc}	126 ± 0.17 ^{efg}
BRACB10 (wild)	54 ± 0.6 ^{ijk}	51 ± 1.0 ^{cdef}	834.00 ± 13.6 ^{jk}	0.36 ± 0.01 ⁱ	3.8 ± 0.05 ^m	ND	137 ± 3.73 ^{bc}
BRACB10-M2	57 ± 1.2 ^{ghi}	48 ± 1.2 ^{fgh}	896.96 ± 22.6 ^{ij}	0.36 ± 0.02 ^j	4.8 ± 0.13 ^l	ND	141 ± 0.58 ^{ad}
BRACB10-M2	56 ± 0.5 ^{hij}	52 ± 0.4 ^{cde}	912.45 ± 7.0 ^{ij}	0.47 ± 0.01 ⁱ	5.8 ± 0.06 ^k	ND	125 ± 2.58 ^{efg}
BRACB10-M3	51 ± 1.1 ^k	45 ± 0.4 ^{hi}	1010.25 ± 3.4 ⁱ	0.81 ± 0.03 ^j	4.9 ± 0.11 ^l	ND	148 ± 0.50 ^a
BRACB10-M4	53 ± 1.0 ^{jk}	50 ± 1.6 ^{def}	837.25 ± 7.4 ^{jk}	0.68 ± 0.01 ⁱ	3.1 ± 0.07 ⁿ	ND	142 ± 2.51 ^{ab}
BRACB10-M5	55 ± 0.9 ^{ijk}	54 ± 0.3 ^{bc}	925.24 ± 24.6 ^{ij}	0.72 ± 0.02 ^j	5.7 ± 0.12 ^k	ND	135 ± 0.55 ^{bcd}

Values are mean of three replicates ± standard errors. Means sharing common letter(s) in each column do not differ significantly at $P > 0.05$. ND – not detected. BRBac4 – *B. subtilis*, BRBac 21 – *B. siamensis*, and BRACB10 – *S. cavourensis*. M stands for respective mutants.

methylene chloride, and hexane all showed Z scores greater than one in BRBac4 VOCs (Figure S6). Furan 2 methyl, benzothiazole, formic acid, and cyclopropane all had multiple Z scores in BRBac21. Further, the Z scores of dimethyl trisulphide, disulphide dimethyl, xylene, phenol, benzothiazole, and carbon disulphide are greater than one in BRBac10.

Antagonistic effects on wilt and root rot disease

The disease symptoms were discovered after the plant had germinated. The control (T9) treatment had a higher percentage of disease occurrence, 89 and 92% in the black and green gram, respectively, followed by T1 (*B. subtilis* [57 and 53%]) and T2 (BRBac4-M6 *B. siamensis* BRBac21-M10 [49 and 51%]), while T6 (*B. siamensis* BRBac21-M10 + *S. cavourensis* BRACB10-M2). showed the minimum of 19 % root rot incidence in black gram and 19.5% in green gram (Figure 4). Red gram treated with *F. oxysporum* f. sp. *udum* exhibited wilt symptoms. Compared to root rot, the maximum wilt incidence (90.2%) was noticed in pathogen inoculated control, followed by T1 (53%) and T3 (45%). Minimum wilt disease incidence of 14.2 and 17.5%, was observed in T9 – fungicide (Thiram @ 4 g kg⁻¹) and T6 – (*B. siamensis* BRBac21-M10 + *S. cavourensis* BRACB10-M2), respectively (Figure 4).

In leaf samples collected on 28 DAS, various defensive enzymes were examined. T5 (*B. subtilis* BRBac4-M6 + *S. cavourensis* BRACB10-M2) had the highest PAL activity (149 µg cinnamic acid h⁻¹ g⁻¹ fresh wt) in green gram, followed by T4 (*B. subtilis* BRBac4-M6 + *B. siamensis* BRBac21-M10). T10 had the lowest amount of PAL activation (pathogen alone). Similar patterns were seen in red gram and black gram (Table 2). T6 (*B. siamensis* BRBac21-M10 + *S. cavourensis* BRACB10-M2) had a higher degree of PO activity (0.42 changes in OD min⁻¹ g⁻¹ fresh wt) in black gram, followed by T5 – *B. subtilis* BRBac4-M6 + *S. cavourensis* BRACB10-M2 (0.39 changes in OD min⁻¹ g⁻¹ fresh weight). The T10 treatment (pathogen alone) exhibited the minimum level of PO activity compared to all other treatments. In the case of PPO, the higher activity (0.0164 changes in OD min⁻¹ g⁻¹ fresh wt) was noticed in T6, followed by T5 (0.37 changes in OD min⁻¹ g⁻¹ fresh wt) in red gram. The least PPO activity was noticed (0.074 changes in OD min⁻¹ g⁻¹ fresh wt) in T10 and the same trends were noticed in green gram and black gram (Table 2).

At 35 DAS, root and shoot lengths were measured. T6 (*B. siamensis* BRBac21-M10 + *S. cavourensis* BRACB10-M2) had the longest root and shoot lengths, followed by

Figure 4: Root rot disease incidence (a) on black gram and green gram and wilt incidence (b) on the red gram. T1 – *B. subtilis* BRBac4-M6, T2 – *B. siamensis* BRBac21-M10, T3 – *S. cavourensis* BRACB10-M2, T4 – *B. subtilis* BRBac4-M6 + *B. siamensis* BRBac21-M10, T5 – *B. subtilis* BRBac4-M6 + *S. cavourensis* BRACB10-M2, T6 – *B. siamensis* BRBac21-M10 + *S. cavourensis* BRACB10-M2, T7 – *B. subtilis* BRBac4-M6 + *B. siamensis* BRBac21-M10, and *S. cavourensis* BRACB10-M2, T8-Standard biocontrol agent (*T. viride*), T9-Chemical fungicide Thiram, T10 – Control (pathogen inoculated).

T7 (*B. subtilis* BRBac4-M6 + *B. siamensis* BRBac21-M10 + *S. cavourensis* BRACB10-M2). When compared with individual inoculation, combined inoculation resulted in longer root and shoot lengths (Figure S7). Nonetheless, for all the studied crops, the treatments T10 (pathogen infected control) had the shortest shoot and root lengths (Figure S7).

Discussion

Strain improvement is essential during the natural product commercialisation process, as the low titer of wild-

Table 2: Induction of defense enzyme in pulses by mutant *Bacillus* and *Streptomyces* against *F. udum* and *M. pahseolina*

Treatments	Phenylalanine ammonia-lyase (cinnamic acid h ⁻¹ g ⁻¹ fresh wt)			PPO (Δ changes in absorbance min ⁻¹ g ⁻¹ fresh wt)			PO (Δ changes in absorbance min ⁻¹ g ⁻¹ fresh wt)		
	Red gram	Green gram	Black gram	Red gram	Green gram	Black gram	Red gram	Green gram	Black gram
T1	110 ± 0.19 ^{gh}	120 ± 2.05 ^{ef}	117 ± 2.55 ^{gh}	0.098 ± 0.0018 ^f	0.102 ± 0.0002 ^b	0.112 ± 0.0009 ^f	0.29 ± 0.0068 ^d	0.29 ± 0.0021 ^e	0.22 ± 0.0017 ^f
T2	128 ± 3.83 ^{cde}	128 ± 1.74 ^{cde}	128 ± 1.65 ^{def}	0.112 ± 0.0034 ^{de}	0.098 ± 0.0027 ^{bc}	0.121 ± 0.0006 ^{ef}	0.34 ± 0.0032 ^b	0.31 ± 0.0059 ^{cd}	0.27 ± 0.0033 ^{de}
T3	131 ± 1.96 ^{bcd}	131 ± 4.28 ^{cd}	139 ± 0.47 ^{bc}	0.109 ± 0.0015 ^{de}	0.102 ± 0.0004 ^b	0.134 ± 0.0005 ^{cd}	0.38 ± 0.0018 ^a	0.34 ± 0.0093 ^b	0.25 ± 0.0043 ^e
T4	138 ± 4.41 ^b	141 ± 0.96 ^{ab}	145 ± 4.64 ^{ab}	0.135 ± 0.0021 ^b	0.112 ± 0.0037 ^a	0.167 ± 0.0016 ^a	0.37 ± 0.0028 ^a	0.38 ± 0.0109 ^a	0.31 ± 0.0082 ^c
T5	152 ± 4.65 ^a	143 ± 3.60 ^a	149 ± 1.62 ^{aa}	0.137 ± 0.0022 ^b	0.117 ± 0.0006 ^a	0.171 ± 0.0006 ^a	0.34 ± 0.0067 ^b	0.34 ± 0.0106 ^b	0.39 ± 0.0048 ^b
T6	134 ± 2.92 ^{bc}	130 ± 1.95 ^{cd}	128 ± 3.92 ^{de}	0.164 ± 0.0047 ^a	0.114 ± 0.0019 ^a	0.162 ± 0.0042 ^{ab}	0.39 ± 0.0011 ^a	0.38 ± 0.0103 ^a	0.42 ± 0.0111 ^a
T7	118 ± 0.64 ^{fg}	134 ± 2.64 ^{bc}	132 ± 0.54 ^{cd}	0.125 ± 0.0027 ^c	0.098 ± 0.0017 ^{bc}	0.154 ± 0.0048 ^b	0.31 ± 0.0044 ^c	0.32 ± 0.0046 ^c	0.38 ± 0.0023 ^b
T8	124 ± 0.34 ^{def}	131 ± 3.39 ^{cd}	124 ± 4.05 ^{defg}	0.114 ± 0.0028 ^d	0.097 ± 0.0030 ^{bc}	0.142 ± 0.0016 ^c	0.28 ± 0.0011 ^d	0.30 ± 0.0037 ^{de}	0.28 ± 0.0051 ^d
T9	121 ± 0.58 ^{ef}	124 ± 1.35 ^{de}	120 ± 0.24 ^{eg}	0.102 ± 0.0017 ^f	0.094 ± 0.0001 ^c	0.124 ± 0.0023 ^{de}	0.28 ± 0.0021 ^d	0.29 ± 0.0032 ^e	0.26 ± 0.0050 ^{de}
T10	107 ± 0.15 ^h	114 ± 0.39 ^f	108 ± 3.16 ^h	0.074 ± 0.0005 ^g	0.065 ± 0.0004 ^d	0.091 ± 0.0007 ^g	0.25 ± 0.0032 ^e	0.22 ± 0.0067 ^f	0.21 ± 0.0027 ^f

Values are mean of three replicates ± standard errors, means sharing common letter(s) in each column do not differ significantly at $P > 0.05$. T1 – *B. subtilis* BRBac4-M6, T2 – *B. siamensis* BRBac21-M10, T3 – *S. cavourensis* BRACB10-M2, T4 – *B. subtilis* BRBac4-M6 + *B. siamensis* BRBac21-M10, T5 – *B. subtilis* BRBac4-M6 + *S. cavourensis* BRACB10-M2, T6 – *B. siamensis* BRBac21-M10 + *S. cavourensis* BRACB10-M2, T7 – *B. subtilis* BRBac4-M6 + *B. siamensis* BRBac21-M10 and *S. cavourensis* BRACB10-M2, T8 – standard bio-control agent (*T. viride*), T9 – chemical fungicide Thiram, T10 – control (pathogen inoculated).

type strains can never match the criteria of industrial development and sales [52]. The “random mutation and screening” technique has proven itself numerous times over the last century, making it the best option for strain enhancement today and in the foreseeable future. Mutation is a commonly utilised method in beneficial microorganisms for strain development in order to increase secondary metabolite production and antagonistic activity against diverse pathogens [53]. Ethyl methanesulphonate, hydroxylamine, methyl methanesulphonate, and *N*-methyl-*N*-nitro-*N*-nitroso guanidine are the most commonly used chemical mutagens. Physical approaches, like UV and gamma irradiation, can also be used to induce mutations [54]. The most popular way for obtaining high yielding mutants is to treat a population with a mutagenic agent until a specific “desired” kill is attained, plate out the survivors, and then test each resulting colony or a randomly selected group of colonies in flasks for product creation [30].

Random mutation was conducted in this study using gamma irradiation at different doses ranging from 0.5 to 3 kGy for both *Bacillus* and *Streptomyces*. The results showed that greater gamma-irradiated doses, such as 2.5 and 3 kGy, had a higher level of antifungal action. At dosages of 2, 2.5, and 3 kGy, antagonistic mutants of *B. subtilis* BRBac4-M4, BRBac4-M6, BRBac4-M8, *B. siamensis* BRBac21-2, BRBac21-10, BRBac21-15, and *S. cavourensis* BRACB10-M2 were produced. These findings are consistent with those of Afsharmanesh et al. [33], who discovered that gamma-irradiated *Bacillus subtilis* UTB1 mutants increased biosurfactant synthesis and control of *Aspergillus flavus*. *Trichoderma virens* treated with gamma rays produced two to three times as many secondary metabolites [55]. Ion beam implantation resulted in increased surfactin synthesis in *B. subtilis* E8 [56]. Gamma irradiation was also used to boost antibiotic paromomycin synthesis in *S. rimosus* subsp. *paromomycinus*. In addition, when compared with natural strains, this mutant produced 1.4–2 times the amount of paromomycin [38]. Through the DNA repair systems within cells, gamma ray irradiation may cause certain mutations in cell genes [57,58]. Following 250 Gy of gamma irradiation, C/G base repair substitutions were the most common form of gamma ray induced mutations in *Escherichia coli* and spontaneous mutational hot spots around positions 620–632 in the *lacI* gene were reduced [59]. Gamma irradiation may have also boosted the expression of genes responsible for lipopeptides, antifungal metabolites, and plant growth-boosting characteristics in this study. However, it will need to be verified in the future using whole genome sequencing and transcriptome analysis.

Genomic rep-PCR fingerprinting is a technique for identifying and classifying bacteria. This technique is highly reliable, reproducible, and fast [60], and it is utilised to disclose the molecular diversity in the bacterial genome. As a result, this approach was used to determine the genome modification after gamma irradiation in contrast to wild strains. Afsharmanesh et al. [32] used rep-PCR fingerprinting to compare the genomic diversity and polymorphic bands of *Bacillus subtilis* UTB1 gamma-irradiated mutants with wild-type strains. Abbasi et al. [34], like us, used rep-PCR and RAPD techniques to identify variance in natural and gamma-induced mutant strains of *T. harzianum*. Several investigations have found that gamma-irradiation can change enzyme activity and secondary metabolites in microorganisms [61,62]. Soufi et al. [35] used rep-PCR (ERIC and BOX) to distinguish the gamma mutation from wild *Trichoderma aureoviride* strains. *T. aureoviride* mutants Tv3, Tv2, Tv6, Tv4, and Tv23 showed no genetic resemblance to wild and significantly improved biocontrol abilities in plate experiment. Gomaa and El-Mahdy [40] used gamma mutation to enhance chitinase output in *B. thuringiensis* NM101-19 and the mutants were screened using the RAPD and inter simple sequence repeat techniques. Rep-PCR (ERIC, BOX) and RAPD primers were examined for *B. subtilis* BRBac4, *B. siamensis* BRBac21, and *S. cavourensis* BRACB10 and its mutants, resulting in significant differences in the wild type and mutant genomes. In *B. subtilis* BRBac4-M8, box primer produced bands at 450, 1,000, 1,200, and 1,300 bp, separating this mutant from its wild type (Figure 2). *B. siamensis* BRBac21-M4 exhibited extra bands of 150, 250, and 400 bp. These bands are most likely associated to the genes that control the expression of mycolytic enzymes, although more research is needed to prove this. *Bacillus* and *Pseudomonas* species produce lipopeptides which are antibacterial substances [63,64]. Surfactin, fengycin, and iturin are the three primary lipopeptides secreted by *Bacillus* spp. [65,66]. Surfactin and iturin genes were found in both *B. subtilis* BRBac4 and *B. siamensis* BRBac21 in this investigation. This is consistent with the findings of Gond et al. [42], who discovered the iturin gene as well as additional lipopeptide genes in *B. subtilis*.

In this study, pot culture data demonstrated that biocontrol agents inoculated treatments had longer root and shoot lengths than controls. Plant development was considerably increased by plant growth promoting rhizobacteria (PGPR) characteristics through both direct and indirect routes. According to Mehmood et al. [67], the use of biocontrol chemicals improved seed germination, root and shoot lengths, and dry biomass. Siderophore producing *B. subtilis* reduced the frequency of *F. oxysporum*

wilt in cotton and also demonstrated numerous PGPR features such as IAA, 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase, and phosphate solubilisation [68]. Several studies found that *Bacillus* spp. and *Streptomyces* isolated from the rhizosphere demonstrated phosphate solubilisation and antagonism against *Fusarium* spp. and other phytopathogens [69–71]. In chickpea and lentil, a mutant of *T. virens* outperformed natural strains in terms of antagonistic capacity against *Sclerotium rolfsii*. Mukherjee et al. and Yandigeri et al. [55,72] revealed that numerous strains of *Streptomyces* produced more IAA, which boosted wheat crop growth and yield. *Bacillus amyloliquefaciens* isolated from potato rhizosphere soil showed increased IAA and phosphate solubilisation efficiency, as well as antagonistic activity against *Rhizoctonia solani* and *F. solani*. Various *Bacillus* spp. exhibited ACC deaminase activity, which has a significant impact on plant development and biochemical properties [73,74]. Many actinobacterial taxa with ACC deaminase activity have been identified, including *Streptomyces*, *Amycolatopsis*, *Mycobacterium*, *Rhodococcus*, and *Nocardia* [75]. Hydrolytic enzymes (such as cellulase, protease, chitinases, amylase, lipase, and pectinase) have enhanced the PGPR properties associated with the control of plant fungal infections via cell wall disintegration [76]. Our strains also tested positive for a variety of hydrolytic enzymes. Several investigations have found that *Bacillus* spp. [8,77,78] and *Streptomyces* [79,80] produce mycolytic enzymes that efficiently suppress fungal infections.

Several previous studies [81,82] found that VOC might inhibit fungal infections. *B. subtilis* and *Actinomyces* produce VOCs such as alcohols, ketones, aldehydes, acids, hydrocarbons, and S-containing chemicals [62,80]. In the *B. subtilis* BRBac4-M6, *B. siamensis* BRBac21-M10, and *S. cavourensis* BRACB10-M2 mutants, approximately 26 compounds were discovered. VOC chemicals generated by mutants demonstrated similarity to their wild-type counterparts (unpublished data). *B. siamensis* BRBac21 wild strain generated 31.4% Furan, 2-methyl, which was also observed in the BRBac21-10 mutant (28%). Similarly, 4.8 and 6% of 1-pentadiene were found in BRBac21 and its mutant BRBac21-10, respectively. When mutants are compared with wild strains, certain novel chemicals are discovered (Table S2). When compared to the control, the activity of plant defence enzymes was relatively elevated in all treatments. Similar to these findings, other studies have found enhanced production of defence enzymes such as PAL, PO, and PPO in bacterial inoculation treatments compared with controls [8,83,84]. Many studies have found that the PGPR aids in plant defence response to pathogen invasion by possibly increasing chitinase activity, siderophore synthesis, and protease activity [85–87]. In this

study, highly effective mutants due to gamma irradiation, *B. siamensis* BRBac21-M10 and *S. cavourensis* BRACB10-M2, were chosen for investigation based on their enhanced anti-fungal activity against *M. phaseolina* and *F. oxysporum* f. sp. *udum*.

Conclusion

Mutant *Bacillus* and *Streptomyces* strains displayed significantly better antagonistic activity against *M. phaseolina* and *F. oxysporum* f. sp. *udum* compared with wild-type bacteria, owing to increased synthesis of lipopeptides, hydrolytic enzymes, and swarming and swimming motilities. Analyses of DNA fingerprinting revealed significant polymorphisms between wild type and mutant strains. The combined inoculation of *B. siamensis* BRBac21-M10 + *S. cavourensis* BRACB10-M2 resulted in the lowest incidence of root rot and wilt disease. In addition, the same treatment increased the activity of the defence enzymes PO, PPO, and PAL in pot culture conditions, indicating that, in addition to direct antagonism, these bacteria generated systemic resistance in pulses. These findings suggested that gamma-induced mutation can be used effectively to improve the biocontrol characteristics of *Bacillus* and *Streptomyces*. In conclusion, after field testing in multiple places, a combination bio-formulation of these two rhizobacteria may be used to address wilt and root rot pathogens in pulses.

Acknowledgements: The authors are grateful to the Department of Agricultural Microbiology, Tamil Nadu Agricultural University for infrastructure assistance.

Funding information: This study was supported by Government of India, Board of research in nuclear science (GoI-BRNS), and Department of Atomic energy (DAE. 35/14/17/2018-BRNS/10395).

Author contributions: RA conceptualised the research, designed the experiments, and corrected the manuscript, AM, NJ, and IJ conducted experiments, RK drafted the manuscript, PKM designed the experiments and corrected the manuscript, NOG and MS analysed metabolites in GC-MS.

Conflict of interest: Authors state no conflict of interest.

Data availability statement: The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

References

- Varshney RK, Song C, Saxena RK, Azam S, Yu S, Sharpe AG, et al. Draft genome sequence of chickpea (*Cicer arietinum*) provides a resource for trait improvement. *Nat Biotechnol.* 2013;31:240–6.
- Barik AK. Pulse cultivation-a venture for food, nutrition and livelihood. *Int J Bio-Resource Stress Manag.* 2021;12:i–ii. doi: 10.23910/1.2021.2192b.
- Iqbal U, Mukhtar T. Evaluation of biocontrol potential of seven indigenous *Trichoderma* species against charcoal rot causing fungus, *Macrophomina phaseolina*. *Gesunde Pflanz.* 2020;72:195–202.
- Ramyabharathi SA, Meena B, Raguchander T. Induction of chitinase and β -1, 3-glucanase PR proteins in tomato through liquid formulated *Bacillus subtilis* EPCO 16 against *Fusarium* wilt. *J Today's Biol Sci: Res Rev India.* 2012;1:50–60.
- Wang L, Li X. Steering soil microbiome to enhance soil system resilience. *Crit Rev Microbiol.* 2019;45:743–53.
- Tang L, Xia Y, Fan C, Kou J, Wu F, Li W, et al. Control of *Fusarium* wilt by wheat straw is associated with microbial network changes in watermelon rhizosphere. *Sci Rep.* 2020;10:1–14.
- Budi SW, van Tuinen D, Arnould C, Dumas-Gaudot E, Gianinazzi-Pearson V, Gianinazzi S. Hydrolytic enzyme activity of *Paenibacillus* sp. strain B2 and effects of the antagonistic bacterium on cell integrity of two soil-borne pathogenic fungi. *Appl Soil Ecol.* 2000;15:191–9.
- Dukare A, Paul S. Biological control of *Fusarium* wilt and growth promotion in pigeon pea (*Cajanus cajan*) by antagonistic rhizobacteria, displaying multiple modes of pathogen inhibition. *Rhizosphere.* 2021;17:100278.
- Sharma N, Sharma KP, Gaur RK, Gupta VK. Role of chitinase in plant defense. *Asian J Biochem.* 2011;6:29–37.
- Hartl L, Zach S, Seidl-Seiboth V. Fungal chitinases: diversity, mechanistic properties and biotechnological potential. *Appl Microbiol Biotechnol.* 2012;93:533–43.
- Glick BR. The enhancement of plant growth by free-living bacteria. *Can J Microbiol.* 1995;41:109–17.
- Dukare A, Paul S, Arambam A. Isolation and efficacy of native chitinolytic rhizobacteria for biocontrol activities against *Fusarium* wilt and plant growth promotion in pigeon pea (*Cajanus cajan* L.). *Egypt J Biol Pest Control.* 2020;30:1–12.
- Castaldi S, Petrillo C, Donadio G, Piaz FD, Cimmino A, Masi M, et al. Plant growth promotion function of *Bacillus* sp. strains isolated from salt-pan rhizosphere and their biocontrol potential against *Macrophomina phaseolina*. *Int J Mol Sci.* 2021;22:3324.
- Li H, Wang X, Han M, Zhao Z, Wang M, Tang Q, et al. Endophytic *Bacillus subtilis* ZZ120 and its potential application in control of replant diseases. *Afr J Biotechnol.* 2012;11:231–42.
- Ehling-Schulz M, Lereclus D, Koehler TM. The *Bacillus cereus* group: *Bacillus* species with pathogenic potential. *Microbiol Spectr.* 2019;7:7–3.
- Xu J-X, Li Z-Y, Lv X, Yan H, Zhou G-Y, Cao L-X, et al. Isolation and characterization of *Bacillus subtilis* strain 1-L-29, an endophytic bacteria from *Camellia oleifera* with antimicrobial activity and efficient plant-root colonization. *PLoS One.* 2020;15:e0232096.
- Islam MA, Nain Z, Alam MK, Banu NA, Islam MR. *In vitro* study of biocontrol potential of rhizospheric *Pseudomonas aeruginosa* against *Fusarium oxysporum* f. sp. *cucumerinum*. *Egyptian journal of Biological. Pest Control.* 2018;28:1–11.
- Fernández Gamarra MA, Maidana Ojeda M, Enciso, Maldonado GA. Molecular identification and growth rate of native strains of *Trichoderma* spp. isolated from the Northern Region of Paraguay. *Invest Agraria.* 2017;19:127–32.

- 19 Matarese F, Sarrocco S, Gruber S, Seidl-Seiboth V, Vannacci G. Biocontrol of Fusarium head blight: interactions between Trichoderma and mycotoxigenic Fusarium. *Microbiology*. 2012;158:98–106.
- 20 Larralde-Corona CP, Santiago-Mena MR, Sifuentes-Rincon AM, Rodríguez-Luna IC, Rodríguez-Perez MA, Shirai K, et al. Biocontrol potential and polyphasic characterization of novel native Trichoderma strains against *Macrophomina phaseolina* isolated from sorghum and common bean. *Appl Microbiol Biotechnol*. 2008;80:167–77.
- 21 Yadav AK, Yandigeri MS, Vardhan S, Sivakumar G, Rangeshwaran R, Tripathi CPM. *Streptomyces* sp. S160: a potential antagonist against chickpea charcoal root rot caused by *Macrophomina phaseolina* (Tassi) Goid. *Ann Microbiol*. 2014;64:1113–22.
- 22 Anitha A, Rabeeth M. Control of *Fusarium* wilt of tomato by bioformulation of *Streptomyces griseus* in green house condition. *Afr J Basic Appl Sci*. 2009;1:9–14.
- 23 Kim JD, Han JW, Lee SC, Lee D, Hwang IC, Kim BS. Disease control effect of streptomyces produced by *Streptomyces psammotus* against tomato *Fusarium* wilt. *J Agric Food Chem*. 2011;59:1893–9.
- 24 Getha K, Vikineswary S. Antagonistic effects of *Streptomyces violaceusniger* strain G10 on *Fusarium oxysporum* f. sp. cubense race 4: indirect evidence for the role of antibiosis in the antagonistic process. *J Ind Microbiol Biotechnol*. 2002;28:303–10.
- 25 Shrivastava P, Kumar R, Yandigeri MS. *In vitro* biocontrol activity of halotolerant *Streptomyces aureofaciens* K20: a potent antagonist against *Macrophomina phaseolina* (Tassi) Goid. *Saudi J Biol Sci*. 2017;24:192–9.
- 26 Gajbhiye A, Rai AR, Meshram SU, Dongre AB. Isolation, evaluation and characterization of *Bacillus subtilis* from cotton rhizospheric soil with biocontrol activity against *Fusarium oxysporum*. *World J Microbiol Biotechnol*. 2010;26:1187–94.
- 27 Zhao L, Jha BK, Wu A, Elliott R, Ziebuhr J, Gorbalenya AE, et al. Antagonism of the interferon-induced OAS-RNase L pathway by murine coronavirus ns2 protein is required for virus replication and liver pathology. *Cell Host Microbe*. 2012;11:607–16.
- 28 Wang B, Yuan J, Zhang J, Shen Z, Zhang M, Li R, et al. Effects of novel bioorganic fertilizer produced by *Bacillus amyloliquefaciens* W19 on antagonism of *Fusarium* wilt of banana. *Biol Fertil Soils*. 2013;49:435–46.
- 29 Faheem M, Raza W, Zhong W, Nan Z, Shen Q, Xu Y. Evaluation of the biocontrol potential of *Streptomyces goshikiensis* YCXU against *Fusarium oxysporum* f. sp. niveum. *Biol Control*. 2015;81:101–10.
- 30 Adrio JL, Arnold LD. Genetic improvement of processes yielding microbial products. *FEMS Microbiol Rev*. 2006;30(2):187–214.
- 31 Liu J, Liu M, Wang J, Yao JM, Pan RR, Yu ZL. Enhancement of the *Gibberella zeae* growth inhibitory lipopeptides from a *Bacillus subtilis* mutant by ion beam implantation. *Appl Microbiol Biotechnol*. 2005;69:223–8.
- 32 Afsharmanesh H, Ahmadzadeh M, Majdabadi A, Motamedi F, Behboudi K, Javan-Nikkhah M. Enhancement of biosurfactants and biofilm production after gamma irradiation-induced mutagenesis of *Bacillus subtilis* UTB1, a biocontrol agent of *Aspergillus flavus*. *Arch Phytopathol Plant Prot*. 2013;46:1874–84.
- 33 Afsharmanesh H, Ahmadzadeh M, Javan-Nikkhah M, Behboudi K. Improvement in biocontrol activity of *Bacillus subtilis* UTB1 against *Aspergillus flavus* using gamma-irradiation. *Crop Prot*. 2014;60:83–92.
- 34 Abbasi S, Safaie N, Shams-Bakhsh M, Shahbazi S. Biocontrol activities of gamma induced mutants of *Trichoderma harzianum* against some soilborne fungal pathogens and their DNA fingerprinting. *Iran J Biotechnol*. 2016;14:260.
- 35 Soufi E, Safaie N, Shahbazi S, Mojerlou S. Gamma irradiation induces genetic variation and boosting antagonism in *Trichoderma aureoviride*. *Arch Phytopathol Plant Prot*. 2021;54:1649–74. doi: 10.1080/03235408.2021.1936377.
- 36 Mukherjee PK, Mendoza-Mendoza A, Zeilinger S, Horwitz BA. Mycoparasitism as a mechanism of *Trichoderma*-mediated suppression of plant diseases. *Fungal Biol Rev*. 2022;39:15–33.
- 37 Khaliq S, Akhtar K, Ghauri MA, Iqbal R, Khalid AM, Muddassar M. Change in colony morphology and kinetics of tylosin production after UV and gamma irradiation mutagenesis of *Streptomyces fradiae* NRRL-2702. *Microbiol Res*. 2009;164:469–77.
- 38 Ibrahim AA, Aboshanab KM, Yassien MA, Hassouna NA. Improvement of paromomycin production by *Streptomyces rimosus* subsp *paromomycinus* NRRL 2455 using gamma irradiation mutagenesis. *Arch Pharm Sci Ain Shams Univ*. 2017;1:26–30.
- 39 Lazim H, Slama N, Mankai H, Barkallah I, Limam F. Enhancement of oxytetracycline production after gamma irradiation-induced mutagenesis of *Streptomyces rimosus* CN08 strain. *World J Microbiol Biotechnol*. 2010;26:1317–22.
- 40 Gomaa EZ, El-Mahdy OM. Improvement of chitinase production by *Bacillus thuringiensis* NM101-19 for antifungal biocontrol through physical mutation. *Microbiology*. 2018;87:472–85.
- 41 Khan N, Martínez-Hidalgo P, Ice TA, Maymon M, Humm EA, Nejat N, Sanders ER, Kaplan D, Hirsch AM. Antifungal Activity of *Bacillus* Species Against *Fusarium* and Analysis of the Potential Mechanisms Used in Biocontrol. *Front. Microbiol*. 2018;9:2363. doi: 10.3389/fmicb.2018.02363Q20.
- 42 Gond SK, Bergen MS, Torres MS, White Jr JF. Endophytic *Bacillus* spp. produce antifungal lipopeptides and induce host defence gene expression in maize. *Microbiol Res*. 2015;172:79–87.
- 43 Gordon SA, Weber RP. Colorimetric estimation of indoleacetic acid. *Plant Physiol*. 1951;26:192.
- 44 Bindel Connelly M, Young GM, Sloma A. Extracellular proteolytic activity plays a central role in swarming motility in *Bacillus subtilis*. *J Bacteriol*. 2004;186:4159–67.
- 45 Wiwat C, Thaithanun S, Pantuwatana S, Bhumiratanana A. Toxicity of chitinase-producing *Bacillus thuringiensis* ssp. *kurstaki* HD-1 (G) toward *Plutella xylostella*. *J Invertebrate Pathol*. 2000;76:270–7.
- 46 Blumer-Schuette SE, Kataeva I, Westpheling J, Adams MWW, Kelly RM. Extremely thermophilic microorganisms for biomass conversion: status and prospects. *Curr Opin Biotechnol*. 2008;19:210–7.
- 47 Passari AK, Leo VV, Chandra P, Kumar B, Nayak C, Hashem A, et al. Bioprospection of actinobacteria derived from freshwater sediments for their potential to produce antimicrobial compounds. *Microb Cell Factories*. 2018;17:1–14.
- 48 Pandey VN, Dubey NK. Antifungal potential of leaves and essential oils from higher plants against soil phytopathogens. *Soil Biol Biochem*. 1994;26:1417–21.
- 49 Fu J, Huang B. Involvement of antioxidants and lipid peroxidation in the adaptation of two cool-season grasses to localized drought stress. *Environ Exp Bot*. 2001;45:105–14.

- 50 Park JM, Vinuselvi P, Lee SK. The mechanism of sugar-mediated catabolite repression of the propionate catabolic genes in *Escherichia coli*. *Gene*. 2012;504:116–21.
- 51 Lee H-J, Park K-H, Shim J-H, Park R-D, Kim Y-W, Cho J-Y, et al. Quantitative changes of plant defense enzymes in biocontrol of pepper (*Capsicum annuum* L.) late blight by antagonistic *Bacillus subtilis*. *J Microbiol Biotechnol*. 2005;15:1073–9.
- 52 Parekh S, Vinci VA, Strobel RJ. Improvement of microbial strains and fermentation processes. *Appl Microbiol Biotechnol*. 2000;54(3):287–301.
- 53 Spadaro D, Gullino ML. Improving the efficacy of biocontrol agents against soilborne pathogens. *Crop Prot*. 2005;24:601–13.
- 54 Baltz RH. Genetic methods and strategies for secondary metabolite yield improvement in actinomycetes. *Antonie Van Leeuwenhoek*. 2001;79:251–9.
- 55 Mukherjee PK, Mehrete ST, Sherkhane PD, Muthukathan G, Ghosh A, Kotasthane AS, et al. A novel seed-dressing formulation based on an improved mutant strain of *Trichoderma virens*, and its field evaluation. *Front Microbiol*. 2019;10:1910.
- 56 Gong G, Zheng Z, Chen H, Yuan C, Wang P, Yao L, et al. Enhanced production of surfactin by *Bacillus subtilis* E8 mutant obtained by ion beam implantation. *Food Technol Biotechnol*. 2009;47:27–31.
- 57 Ellaiah P, Prabhakar T, Ramakrishna B, Taleb AT, Adinarayana K. Strain improvement of *Aspergillus niger* for the production of lipase. *Indian J Microbiol*. 2002;42(2):151–4.
- 58 Iftikhar TM, Niaz SQ, Abbas Z, Muhammad A, Ashraf I, Lee KJ, et al. Mutation induced enhanced biosynthesis of lipases by *Rhizopus oligosporus* var. *microsporus*. *Pak J Bot*. 2010;42(2):1235–49.
- 59 Wijker CA, Lafleur MVM. “Influence of the UV-activated SOS response on the γ -radiation-induced mutation spectrum in the *lacI* gene.”. *Mutat Res/DNA Repair*. 1998;408(3):195–201.
- 60 Versalovic J, Schneider M, De Bruijn FJ, Lupski JR. Genomic fingerprinting of bacteria using repetitive sequence-based polymerase chain reaction. *Methods Mol Cell Biol*. 1994;5:25–40.
- 61 Haggag WM, Mohamed HAA. Enhancement of antifungal metabolite production from gamma-ray induced mutants of some *Trichoderma* species for control onion white disease. *Plant Pathol Bull*. 2002;11:45–56.
- 62 Farag MA, Ryu C-M, Sumner LW, Paré PW. GC-MS SPME profiling of rhizobacterial volatiles reveals prospective inducers of growth promotion and induced systemic resistance in plants. *Phytochemistry*. 2006;67:2262–8.
- 63 Peypoux F, Guinand M, Michel G, Delcambe L, Das BC, Lederer E. Structure of iturine A, a peptidolipid antibiotic from *Bacillus subtilis*. *Biochemistry*. 1978;17:3992–6.
- 64 Cai X, Li H, Xue Y, Liu C. Study of endophytic *Bacillus amyloliquefaciens* CC09 and its antifungal cyclic lipopeptides. *J Appl Biol Biotechnol*. 2013;1:1–5.
- 65 Kakinuma A, Hori M, Isono M, Tamura G, Arima K. Determination of amino acid sequence in surfactin, a crystalline peptidolipid surfactant produced by *Bacillus subtilis*. *Agric Biol Chem*. 1969;33:971–2.
- 66 Vanittanakom N, Loeffler W, Koch U, Jung G. Fengycin—a novel antifungal lipopeptide antibiotic produced by *Bacillus subtilis* F-29-3. *J Antibiotics*. 1986;39:888–901.
- 67 Mehmood S, Muneer MA, Tahir M, Javed MT, Mahmood T, Afridi MS, et al. Deciphering distinct biological control and growth promoting potential of multi-stress tolerant *Bacillus subtilis* PM32 for potato stem canker. *Physiol Mol Biol Plants*. 2021;27:2101–14.
- 68 Lin Y, Du D, Si C, Zhao Q, Li Z, Li P. Potential biocontrol *Bacillus* sp. strains isolated by an improved method from vinegar waste compost exhibit antibiosis against fungal pathogens and promote growth of cucumbers. *Biol Control*. 2014;71:7–15.
- 69 Calvo P, Ormeño-Orrillo E, Martínez-Romero E, Zúñiga D. Characterization of *Bacillus* isolates of potato rhizosphere from andean soils of Peru and their potential PGPR characteristics. *Braz J Microbiol*. 2010;41:899–906.
- 70 Akgül DS, Mirik M. Biocontrol of *Phytophthora capsici* on pepper plants by *Bacillus megaterium* strains. *J Plant Pathol*. 2008;90:29–34.
- 71 Aallam Y, Maliki BE, Dhiba D, Lemriss S, Souiri A, Haddioui A, et al. Multiple potential plant growth promotion activities of endemic *Streptomyces* spp. from moroccan sugar beet fields with their inhibitory activities against *Fusarium* spp. *Microorganisms*. 2021;9:1429.
- 72 Yandigeri MS, Meena KK, Singh D, Malviya N, Singh DP, Solanki MK, et al. Drought-tolerant endophytic actinobacteria promote growth of wheat (*Triticum aestivum*) under water stress conditions. *Plant Growth Regul*. 2012;68:411–20.
- 73 Singh SB, Gowtham HG, Murali M, Hariprasad P, Lakshmeesha TR, Murthy KN, et al. Plant growth promoting ability of ACC deaminase producing rhizobacteria native to Sunflower (*Helianthus annuus* L.). *Biocatalysis Agric Biotechnol*. 2019;18:101089.
- 74 Misra S, Chauhan PS. ACC deaminase-producing rhizosphere competent *Bacillus* spp. mitigate salt stress and promote Zea mays growth by modulating ethylene metabolism. *3 Biotech*. 2020;10:1–14.
- 75 Nascimento FX, Rossi MJ, Glick BR. Role of ACC deaminase in stress control of leguminous plants. *Plant growth promoting actinobacteria*. Singapore: Springer; 2016. p. 179–92.
- 76 Dhar Purkayastha G, Mangar P, Saha A, Saha D. Evaluation of the biocontrol efficacy of a *Serratia marcescens* strain indigenous to tea rhizosphere for the management of root rot disease in tea. *PLoS One*. 2018;13:e0191761.
- 77 Brzezinska MS, Kalwasińska A, Świątczak J, Żero K, Jankiewicz U. Exploring the properties of chitinolytic *Bacillus* isolates for the pathogens biological control. *Microb Pathogenesis*. 2020;148:104462.
- 78 Abaidoo RC, Killani AS, Akintokun AK, Abiala MA. Antagonistic effect of indigenous *Bacillus subtilis* on root-/soil-borne fungal pathogens of cowpea. *Researcher*. 2011;3:11–18.
- 79 Shekhar N, Bhattacharya D, Kumar D, Gupta RK. Biocontrol of wood-rotting fungi with *Streptomyces violaceusniger* XL-2. *Can J Microbiol*. 2006;52:805–8.
- 80 Palaniyandi SA, Yang SH, Zhang L, Suh J-W. Effects of actinobacteria on plant disease suppression and growth promotion. *Appl Microbiol Biotechnol*. 2013;97:9621–36.
- 81 Ryu C-M, Farag MA, Hu C-H, Reddy MS, Kloepper JW, Paré PW. Bacterial volatiles induce systemic resistance in *Arabidopsis*. *Plant Physiol*. 2004;134:1017–26.
- 82 Gutiérrez-Luna FM, López-Bucio J, Altamirano-Hernández J, Valencia-Cantero E, de la Cruz HR, Macías-Rodríguez L. Plant growth-promoting rhizobacteria modulate root-

- system architecture in *Arabidopsis thaliana* through volatile organic compound emission. *Symbiosis*. 2010;51:75–83.
- 83 Podile AR, Laxmi VDV. Seed Bacterization with *Bacillus subtilis* AF 1 Increases Phenylalanine Ammonia-lyase and Reduces the Incidence of Fusarial Wilt in Pigeonpea. *J Phytopathol*. 1998;146:255–9.
- 84 Kloepper JW, Ryu C-M, Zhang S. Induced systemic resistance and promotion of plant growth by *Bacillus* spp. *Phytopathol*. 2004;94:1259–66.
- 85 Rais A, Jabeen Z, Shair F, Hafeez FY, Hassan MN. *Bacillus* spp., a bio-control agent enhances the activity of antioxidant defense enzymes in rice against *Pyricularia oryzae*. *PLoS One*. 2017;12:e0187412.
- 86 Senthilraja G, Anand T, Kennedy JS, Raguchander T, Samiyappan R. Plant growth promoting rhizobacteria (PGPR) and entomopathogenic fungus bioformulation enhance the expression of defense enzymes and pathogenesis-related proteins in groundnut plants against leafminer insect and collar rot pathogen. *Physiol Mol Plant Pathol*. 2013;82:10–9.
- 87 Bano A, Muqarab R. Plant defence induced by PGPR against *Spodoptera litura* in tomato (*Solanum lycopersicum* L.). *Plant Biol*. 2017;19:406–12.