Comparative expression analysis of defence-related genes in *Bacillus*-treated *Glycine max* upon challenge inoculation with selective fungal phytopathogens

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Activation of defence-related genes by the application of beneficial bacteria leads to prior protection against pathogens through induced systemic resistance. The present study was carried out to examine the qRT–PCR-based relative quantification of differently expressed defence-related genes in soybean (*Glycine max* L. Merrill) plants primed with *Bacillus* sp. strain SJ-5 against the fungal pathogen *Rhizoctonia solani* and *Fusarium oxysporum*. In this context, molecular characterization of plant growth promoting and biocontrol genes of SJ-5 was done by PCR followed by homology analysis. In the GC-MS analysis of SJ-5 volatile organic compounds, potent antifungal compound bis(2-ethylhexyl) phthalate and antioxidant compound butylated hydroxy toluene were reported with the highest peak area 47.96% and 21.82% respectively, along with other antifungal compounds in small proportion. Qualitative expression of different defence-related genes like lipoxygenase, phenylalanine ammonia-lyase 2, peroxidase, polyphenol oxidase, endo-1,3-beta-glucanase, catalase, defensin-like protein, vegetative storage protein and chitinase class I was found elicited in the plants primed with SJ-5 against the fungal pathogens. In the qPCR analysis, the highest upregulation was observed in the transcript profile of *ppojh2* in the treatments T_5 and T_6 with 4.12- and 4.06-fold increase respectively.

Keywords: Defence-related genes, induced systemic resistance, plant growth promoting bacteria, volatile organic compounds.

TO combat stress, plants develop an innate or induced defence system. In the present study, we deploy plant growth promoting bacteria (PGPB) to ameliorate soybean growth under biotic stress. Being a nutrient-rich plant, soybean is a soft target for a wide range of pathogens, among which *Rhizoctonia solani* and *Fusarium oxysporum* severely affect it by inciting 'rhizoctonia root rot' and 'fusarium wilt and necrosis of root and lower stem' respectively[1]. Use of PGPB as biocontrol agent to fight against agricultural phytopathogens provides a strong alternative and eco-friendly approach for controlling plant disease. PGPB can elicit prior induced systemic resistance (ISR) in plants as well as promote growth also through growth hormone production, nutrients recycling and acquisition from soil[2]. PGPB activate jasmonic acid/ethylene (JA/ET) pathway which leads to activation of nonexpressor of pathogenesis related protein-1 (NPR-1) gene. NPR-1, an ankyrin repeat protein, elicits plant defence-related genes and provides high level of resistance in the form of ISR[3,4]. Phenylalanine ammonia-lyase encoded by *Pal1* gene is one of the main factors of the phenylpropanoid pathway having important role in defence through cell-wall reinforcements by lignin formation, and is also found to be induced by JA[5]. Plant defensins proteins play an important role in defence as they possess antifungal, antibacterial, proteinase inhibitory and insect amylase inhibitory activity. Enhanced chitinase activity against fungal pathogens directed towards chitin which is a major cell-wall component of fungi, shows its functionality in defence response[6].

In addition, PGPB-produced volatile organic compounds (VOCs) have also been reported to elicit plant growth promotion and induce systemic resistance, which further provides a new insight into PGPB–plant interactions. A broad range of bacterial VOCs have been reported till now having a vital role in plant defence that include dodecane, 2-undecanone, 2-tridecanone, 2-tridecanol, tetramethylpyrazine, 2,3-butanediol and 3-hydroxy-2-butanone (acetoin), etc. Of these, 2,3-butanediol and 3-hydroxy-2-butanone are the most important ones which are found to be consistently released by two bacterial strains, namely *B. subtilis* GB03 and *B. amyloliquefaciens* IN937a. Further, *Arabidopsis thaliana* plants treated with these strains have shown significant resistance against the challenge inoculation of *Erwinia carotovora* subsp. *carotovora* SCC1 (refs 7, 8). In the present study, we have evaluated the role of rhizobacterial isolate *Bacillus* sp. strain SJ-5 (MCC-2607) in the protection of soybean plants against rhizoctonia root rot and fusarium wilt through characterization of the VOCs. Besides, molecular characterization of plant growth promoting activities of SJ-5 was employed along with validation of defence-related gene expression in plants using quantitative real-time analysis.

Plant growth promoting bacterial strain *Bacillus* sp. strain SJ-5 (MCC-2609), isolated from the rhizospheric soil was used for the study. Pure cultures of fungal phytopathogens *Rhizoctonia solani* (MTCC-9666) and *Fusarium oxysporum* (MTCC-8780) were purchased from MTCC and maintained on potato dextrose agar (PDA) plates. JS-9560 variety of soybean was used for the present study.

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Table 1. List of GSPs with their respective genes and plant growth promoting properties

Activity	Gene	Primer sequence (5′–3′)	Amplicon size (bp)	Annealing temperature (°C)
Phosphate solubilization	*Glucose-1-dehydrogenase*	ATGCAGGGATAGAAAACGCG CCCAGCTTGAAACGATGGAT	503	55
IAA production	*Tryptophan 2-monooxygenase*	GCAGAGTGTATACGATTCGGG GCCTCATCTGCCCACGTATA	946	62
N_2-fixation	*Ferredoxin-nitrite reductase*	GGTTATCGGCTCGTCCATATTT CGTCCTACCGGCACATTAAA	319	62
Ethylene reduction	*ACC deaminase*	CGCAAACGACTGAACTGAAATC CTTGGTGGGCCGACTATTTAT	490	62
Iron sequestering	*Aerobactin synthase*	GATGCACAAGAGGATGCAATTC TCCGCAAGTACGAATGCTAAT	443	62
Lytic enzyme	*Chitinase*	GAATATCCTGGCGTTGAAACGAT GCCACGTCCGTAAAAGGGT	402	59
Lytic enzyme	*β-1,3-Glucanase*	TGGCACACCATACGAAAGAA AGATGCTTGTCCATCACCTAAC	750	62
Antibiotic	*Zwittermicin*	ATGTGCACTTGTATGGGCAG TAAAGCTCGTCCCTCTTCAG	952	61

Genomic DNA was isolated from the overnight-grown bacterium *Bacillus* sp. strain SJ-5 using the method developed by Bollet *et al.*[9]. Plant growth promoting and biocontrol genes were amplified in Thermal cycler (Kyratec, Australia) using reaction mixture consisting of 2 µl of 10× *Taq* polymerase buffer, 2 µl of 2.5 mM dNTPs each, 2 µl of 25 mM $MgCl_2$, 1 µl of gene specific primer (GSP), 0.4 µl of *Taq* DNA polymerase (3U/µl; Merck Genei) and 2 µl of DNA (Table 1). Further, all genes were assessed by agarose gel electrophoresis followed by observation in UVITECH gel doc system. Sequencing of all the purified gene products was done at the National Chemical Laboratory (NCL), Pune using ABI 3730xl DNA analyzer (Applied Biosystems, USA). Phylogenetic and molecular evolutionary analysis of all the genes was performed using MEGA 6 software[10]. Upon confirmation of the genes, sequences were submitted to NCBI GenBank and accession numbers were assigned to them.

For the extraction of VOCs from bacteria, tripartite plate or Y-plate method with activated charcoal as volatile trapper was used. To check the VOC capturing activity of activated charcoal, bacterial strain SJ-5 along with either fungal pathogen was inoculated on separate compartments of the Y-plate on NA and PDA respectively, while the third compartment was filled with autoclaved activated charcoal. Plates inoculated with the same formulation but without activated charcoal acted as positive control. Plates inoculated with only fungal pathogen without bacterial strain but with activated charcoal were mentioned as control for comparative analysis, while one of the plates was inoculated with only SJ-5 along with activated charcoal for further VOC extraction. All the plates were properly sealed with parafilm and incubated for 28° ± 2°C till fungus grew fully on the control plates. After completion of incubation, charcoal from the bacterial plates was collected and washed with 5 ml of dichloromethane to extract all trapped volatile compounds, which were downstream analysed by GC-MS using GCMS-QP2010 Plus (Shimadzu, Kyoto, Japan) system equipped with a DB-5 (Optima-5) capillary column (length 30 m/diameter 0.25 mm and packing 5% diphenyl/95% dimethyl polysiloxane), at the Advanced Instrumentation Research Facility, Jawarlal Nehru University, New Delhi.

Elicitation of defence-related genes in soybean (JS-9560) plants was performed by application of *Bacillus* sp. strain SJ-5 as described by Jain *et al.*[11] (Figure 1). Bacterized and non-bacterized seeds (surface-sterilized) were used for plant experimental design. Six types of treatment were prepared: T_1 (control plants without any treatment), T_2 (SJ-5 treated), T_3 (*R. solani* treated), T_4 (*F. oxysporum* treated), T_5 (SJ-5 treated with challenge inoculation of *R. solani*), T_6 (SJ-5 treated with challenge inoculation of *F. oxysporum*). The experiment for each treatment was performed three times.

For the defence-related gene expression study, root samples of the different plants were collected on the eighth day after challenge inoculation based on the results of our previous study[11]. The collected root samples were immediately ground in liquid nitrogen and total RNA was extracted using TRIzol reagent (Merck, GeNei) according to the manufacturer's instruction. First-strand cDNA synthesis from the DNAase-I treated (Thermo Scientific) RNA was done using Verso cDNA synthesis kit (Thermo Scientific) according to the manufacturer's instruction.

Quantitative real-time PCR (qPCR) was performed using Applied Biosystems Real-time PCR system (Life Technologies). For each experimental sample, in a final volume of 20 µl, 2 µl of ten-fold diluted first-strand cDNA, 1 µl of each gene-specific primer (10 pM) (Table 2), and 10 µl Maxima SYBR Green/ROX qPCR Master Mix (2X, Thermo Scientific) were used. The reaction was

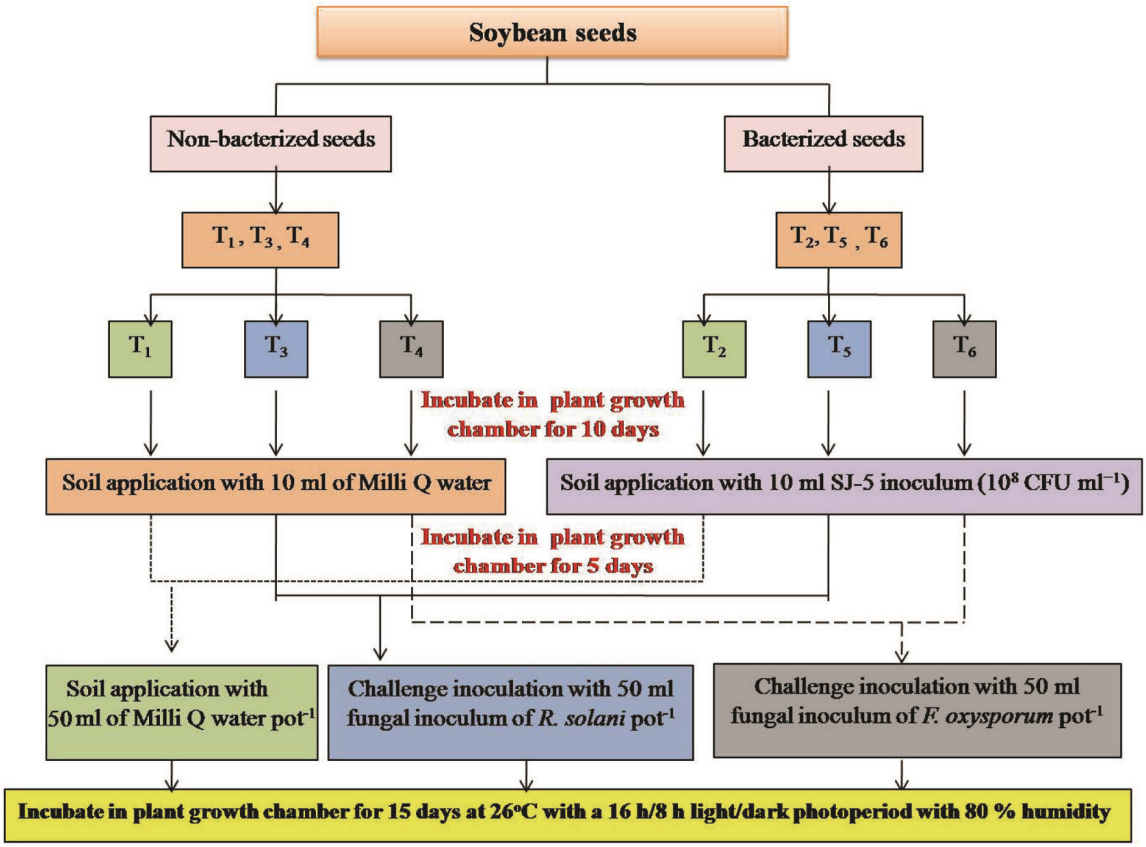

Figure 1. Schematic representation of plant experimental design and elicitation of ISR in soybean plants.

Table 2. List of GSPs with their respective defence-related genes and sequences

Defence-related genes	Primer sequence (5′–3′)	Amplicon size (bp)
Lipoxygenase (lox)	TGGAAAAGGAAAAGTTGGAAAGG TCCTTGGTTTGAAATGGCTT	205
Phenylalanine ammonia-lyase 2 (pal2)	CACCGGAGAACCAAACAGGG CAGAAGCTTTGTGATTGCCTCC	204
Peroxidase (pod)	ACAGAGGGTATGGTTACCTCA CGGGGGTCTGATTTAGAAACG	206
Polyphenol oxidase (ppojh2)	GGATTGTTCATGGGAAGCC CCACAGTCGATCCGAGTTC	201
Endo-1,3-beta-glucanase (gluc)	TCAATCTCTCACCAACGCTG GACACCTTCATTTGGCCTTGT	204
Catalase (cat1)	TCCGGTGCTCCCATCTGGA CGGCACATGTGAGGTGAG	202
Defensin-like protein(def)	GTGGTGCAAACTGAGGCAAAG TGCATGCATCTTCTTCACGTTG	200
Vegetative storage protein (vsp)	CAATGGAGCGATCTCCTCGG ACATGACTTGTTGACACACAGC	199
Chitinase class I (chia1)	GACTGCACAGGGAAACAAGC CATCTGGCAGTACCTTCGGT	199
Ubiquitin (ubiq)	TCTCCCTTCAAGATGCAGA GAGGTGAAGAGTACTCTCCTT	219

then subjected to an initial denaturation of 95°C for 5 min followed by 40 cycles of 95°C for 15 sec, 55°C for 30 sec and 72°C for 30 sec. To confirm that the signals were the result of a single amplified product, melting curve analysis was performed after the run by increasing the temperature at 0.5°C per cycle from 65°C to 95°C. For each repetition of samples in every run, the level of gene expression was normalized to that of reference gene

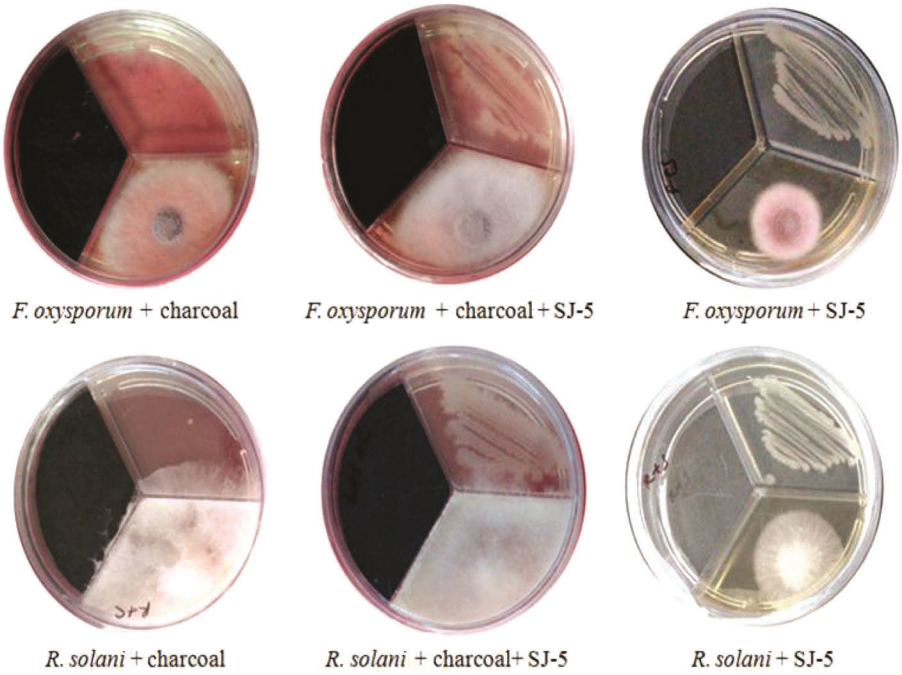

Figure 2. Estimation of VOCs capturing capacity of activated charcoal in tripartite plate assay.

Ubiquitin that provides ΔCt (cycle threshold) value. Comparison of relative gene expression among all six treatments of soybean plants for the nine defence-related genes was done using the delta–delta CT method in terms of fold changes according to the Livak method.

All the observations were taken in triplicate and data were analysed using analysis of variance technique (ANOVA) ($P < 0.05$), and the means were separated by Duncan's multiple range test using SPSS (version 16.0).

The P-solubilizing activity of bacteria is traditionally associated with the production of low-molecular-weight organic acids, mainly gluconic and keto-gluconic acids[12]. In bacteria, membrane-bound glucose dehydrogenase (GDH) catalyses the periplasmic oxidation of glucose to produce gluconic acid[13]. In the present study, gene amplification using GSP showed sharp bands of the specific genes which are responsible for plant growth promoting and biocontrol activities, close to the desired amplicon size. Upon submission to GenBank, accession numbers assigned were as follows: *Glucose-1-dehydrogenase* (KU366709), *ACC deaminase* (KU176881), *Tryptophan 2-monooxygenase* (KU183549), *Ferredoxin-nitrite reductase* (KU176883), *Aerobactin synthase* (KU176882), *β-1,3-glucanase* (KU183550), *Chitinase* (KT263585) and *Zwittermicin A* (KT263584). Earlier studies have highlighted the role of amplified genes in plant growth promotion. Tryptophan-2-monooxygenase (IaaM) converts tryptophan to indole-3-acetamide (IAM) in the IAM pathway for the production of plant growth hormone indole acetic acid (IAA) in bacteria[14]. Sun *et al.*[15] have shown the role of ACC deaminase in plant growth promotion using ACC deaminase (*acdS*) gene mutant *Burkholderia phytofirmans* YS2 that had lost ACC deaminase activity as well as the ability to promote elongation of the roots of canola seedlings.

On the other hand, study of the genes responsible to encode cell-wall degrading enzymes has also been done in recent years. Xu *et al.*[16] isolated and cloned the *β*-1, 3-1, 4-glucanase gene from *B. velezensis* ZJ20 followed by expression in *E. coli* BL21. They found that the purified *β*-1, 3-1, 4-glucanase destroyed the mycelial morphology of three pathogenic fungi, viz. *Cryphonectria parasitica*, *Helicobasidium purpureum* and *Cylindrocladium quinqueseptatum*, which causes devastating damage to *Castanea mollissima, Populus* spp. and *Eucalyptus* spp. respectively. Zwittermicin A is a novel, broad-spectrum linear aminopolyol antibiotic produced by *B. cereus* and *B. thuringiensis*. Yang *et al.*[17] have isolated chitinolytic bacteria *B. subtilis* CHU26 from Taiwan potato fields and molecularly characterized its chitinase-producing activity by amplifying, cloning and expression in *E. coli* against *R. solani* with antifungal effect.

The demonstration of the role of volatiles 2,3-butanediol and acetoin produced by two *Bacillus* sp. on the growth enhancement of *Arabidopsis* by Ryu *et al.*[7] indicates that a physical interaction between the plant growth promoting rhizobacteria and the plant is not necessarily required. In the present study, VOCs assay on tripartite plates also shows antifungal activity of SJ-5 through VOCs. In an experiment conducted to check the VOC capturing potential of activated charcoal, significant inhibition in fungal growth was observed in the tripartite

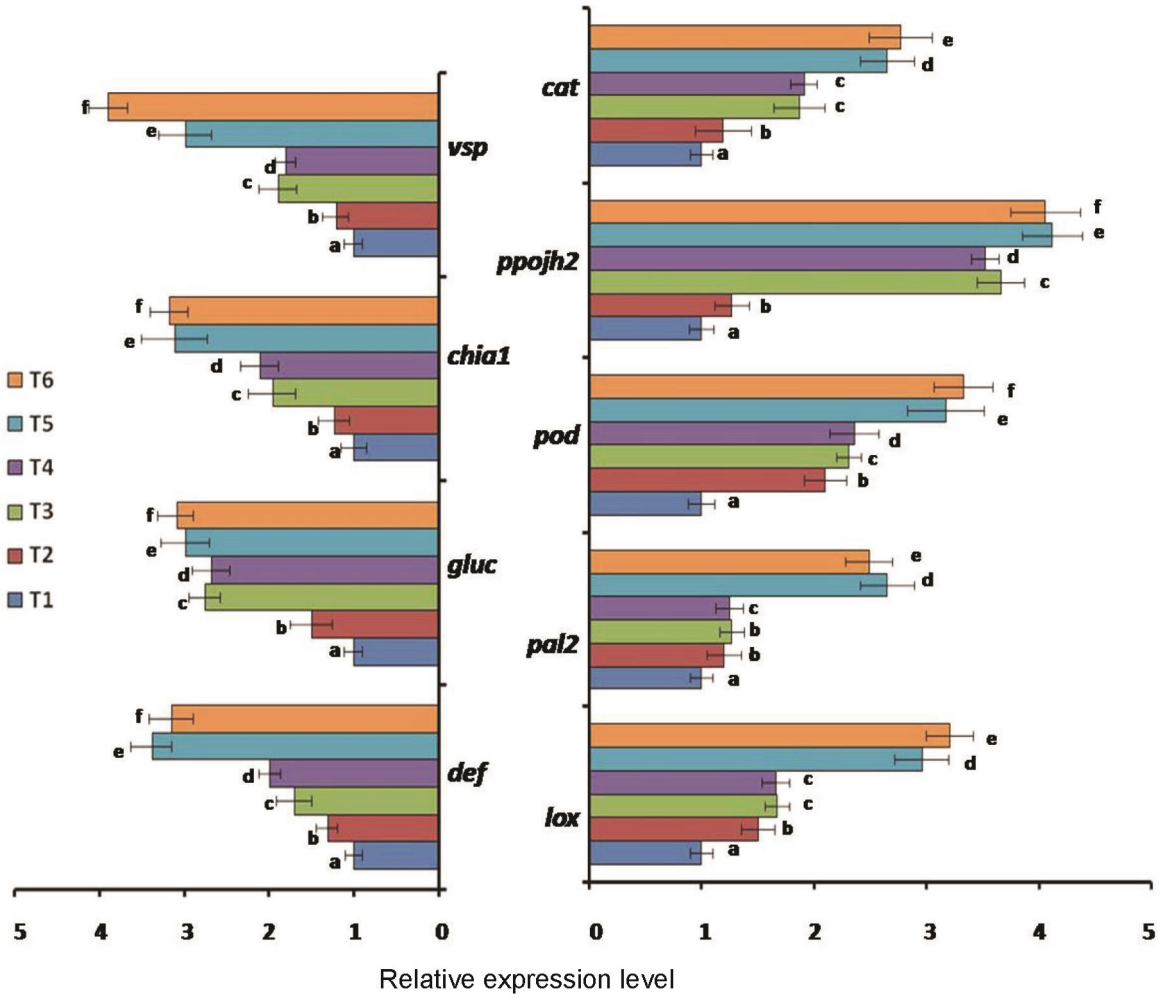

Figure 3. qPCR gene expression profile of nine defence-related genes of soybean. T_1, Control plants without any treatment; T_2, SJ-5 treated; T_3, *Rhizoctonia solani* treated; T_4, *Fusarium oxysporum* treated; T_5, SJ-5 treated with challenge inoculation of *Rhizoctonia solani*; T_6, SJ-5 treated with challenge inoculation of *Fusarium oxysporum*. Superscripted letters on each bar indicate significant differences using DMRT at $P = 0.05$.

plate incubated with either fungal culture along with bacterium SJ-5, but devoid of charcoal (Figure 2). No inhibitions were observed in the plate with the same combination, but having charcoal. This proves the VOC capturing potential of charcoal. Upon GC-MS profiling of SJ-5 VOCs, we found some promising compounds, reported earlier for the potent antifungal activity. Among these, two major compounds detected were bis(2-ethylhexyl) phthalate (BEP) and butylated hydroxy toluene (BHT) with peak area 47.96% and 21.82% respectively. BEP is the diester of phthalic acid and the branched-chain 2-ethylhexanol and reported for potent antifungal activity, BHT is a well-known antioxidant and reported earlier for plant growth by means of preventing the appearance of specific features of ageing and apoptosis in plants, hence, increasing the life span of plants[18–21]. Kavitha *et al.*[19] have purified bis-(5-ethylheptyl) phthalate VOC produced from the *Nocardia* MK-VL_113 and confirmed its antifungal activity. In earlier studies, BEP has been identified to be produced by different microbes, including *Streptomyces bangladeshiensis*[20], *Pseudomonas* sp. PBO1 (ref. 21), marine algae[22] and fungi[23]. Other than these, *n*-hexadecanoic acid (2.63%), octacosane (1.49%), geraniol (1.38%), di isobutyl phthalate (0.85%), eicosane (0.82%) and tetradecanoic acid (0.41%), were found to be present in lower proportion in bacterial SJ-5 volatiles and were also reported earlier for the potent antifungal activity[24–27]. So it can be inferred that SJ-5 inhibits soybean pathogen growth through a combined and complex mechanism that involves cell-wall-degrading enzymes and VOCs.

Defence-related proteins are responsible for providing heightened level of resistance to plants against invading pathogens. Desmond *et al.*[28] also reported upregulation in the eight genes, including a chitinase (*chi*) and peroxidase (*pod*) in wheat after pre-treatment with MeJA and acibenzolar-S-methyl. Ebadzad and Cravador[29] revealed through qPCR that transcript levels of QsRPc, QsCHI, QsCAD2 and QsPDI increased during the first 24 h

post-inoculation of *Phytophthora cinnamomi* in *Quercus suber* roots. Dos Santos *et al.*[30] showed induced expression of *acc2*, *hmg2*, *c4h*, *4cl*, *lox1*, *lox2*, *aos*, *PR-3*, *thi2.1* and *pdf1.2* upon defence-gene expression analysis of *A. thaliana* parasitized by *Orobanche ramose*. In the present study of quantitative gene expression analysis of defence-related genes in plants, we found the highest expression of all the genes in the root tissue of T_5 and T_6 plants followed by T_3 and T_4 plants. Gene expression was also found higher in the T_2 plants than the control T_1 plants, but lower compared to other treatments. Thus we may conclude that SJ-5 elicits the induction of defence-related genes in plants prior to pathogen attack. In case of pathogen attack, expression of defence-related genes significantly increased in the T5 and T6 plants compared to T3 and T4 plants. Although significant and similar pattern of expression was found for all the genes wherein the expression level was different in terms of fold changes (Figure 3). The highest upregulation was observed in the transcript profile of *ppojh2* in the treatments T_5 and T_6 with 4.12- and 4.06-fold increase respectively. The highest upregulation in the SJ-5-treated plants without any challenge inoculation (T_2) was observed for the *lox* and *gluc* genes with 1.5-fold increase for both, while for *R. solani* and *F. oxysporum*-treated plants without prior SJ-5 inoculation (T_3 and T_4) 3.66- and 3.52-fold upregulation was found for the *ppojh2* gene followed by 2.75- and 2.68-fold upregulation for the *gluc* gene respectively.

Conflict of interest: Authors declare no conflict of interest.

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Soil microbial characteristics in sub-tropical agro-ecosystems of North Western Himalaya

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Eight predominant land use systems, viz. agriculture (T_1), horticulture (T_2), agrisilviculture (T_3), silvopastoral (T_4), agrihorticulture (T_5), agrihortisilviculture (T_6), forest (T_7) and grassland (T_8) of subtropical parts of Himachal Pradesh were selected along two altitudinal ranges A_1 (365–635 m amsl) and A_2 (636–914 m amsl) to observe the variation in soil microbial activity and microbial characteristics. Agroforestry land uses and forest ecosystems displayed significantly higher microbial counts and microbial biomass carbon than agriculture and grasslands. The CO_2 evolution (soil microbial activity) was found higher in agrisilviculture, agrihortisilviculture, forest and grassland use systems at both altitudinal ranges. Soil biological properties (microbial count, microbial biomass and microbial activity) were maximum in forest land-use system. Among the agroforestry land-use systems, agrisilviculture had significantly higher microbial counts. The maximum microbial count (164.50×10^5 cfu g^{-1} soil) was recorded in forest and remained statistically at par with agrisilviculture (162.34×10^5 cfu g^{-1} soil). Minimum microbial count (80.66×10^5 cfu g^{-1} soil) was observed in agriculture land use. At both the altitudinal ranges, the CO_2 evolution was highest at 48 h time interval and decreased thereafter. The metabolic quotient (qCO_2) indicated that C-use efficiency is higher in grassland use and agriculture land use systems than other studied systems.

Keywords: Microbial biomass carbon, CO_2 evolution, metabolic quotient, land uses.

THE importance of microorganisms in ecosystem functioning has led to an increased interest in determining soil microbial biomass[1]. The soil microbial biomass is the active component of soil organic pool, responsible for organic matter decomposition, soil nutrient content and consequently primary productivity in most biogeochemical processes in terrestrial ecosystems[2–4]. Therefore, measuring microbial biomass is a valuable tool for understanding and predicting long-term effects on changes in land use and associated soil conditions[5]. Land use, climate change, habitat destruction as well as other human perturbations strongly alter natural ecosystems and understanding these responses is crucial to forecast sustainability of environmental services. Climatic, edaphic, topographic and biotic factors influence litter fall production in tree-based land-use systems and have direct or indirect effect on mitigation rate and soil carbon production.

Many studies have been carried out globally and in India on vegetation and soil carbon biomass of varying land use systems. However, studies on microbial biomass and CO_2 evolution have been rarely reported in different agroecosystems in the Indian Himalayan region. Due to its unique topographical conditions, Himalayan ecosystem offers outstanding potential to investigate microbial biomass variations in prevailing land uses along the altitudinal level. Microbial biomass being an important component of ecosystem carbon needs to be assessed and examined across different land-use systems and crop combinations and best land use from a mitigation perspective needs to be recommended to forest and farming dependent communities. In the present study, we report the effect of major land-use systems of subtropical ecosystems of North-Western Himalayas on bacterial counts, microbial biomass carbon, their activities and metabolic quotient.

The study area is located between 32°50′ and 30°22′N lat. and 76°18′ and 77°47′E long. at 365 to 914 m amsl

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