Full Length Research Paper

Screening and characterization of rhizobacterial strains of *Bacillus* spp. isolated from rhizosphere of cauliflower (*Brassica oleracea* var. *botrytis* L.)

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A total of 40 isolates were isolated from different rhizospheric soil and roots of cauliflower from the vicinity of three districts (Kangra, Hamirpur and Bilaspur) of Himachal Pradesh belonging to sub temperate Himalayan location. Thereafter, these isolates were screened and characterized for their plant growth promotion and biocontrol properties as per standard methods. Out of 40, only 5 isolates (MK₂, MK₄, MK₅, MK₇ and MK₉) showed maximum plant growth promoting attributes like P- solubilization, N- fixation etc. All the five isolates solubilized tricalcium phosphate under *in vitro* conditions however, MK₅ and MK₇ had highest phosphate solubilizing efficiency of 172.21 and 166.67% were recorded. Production of IAA, HCN and siderophore demonstrated in broth assay were other important plant growth promoting characters. The isolates produced diffusible and volatile compounds that inhibited the growth of three soil borne phytopathogens namely *Fusarium* sp., *Rhizoctonia solani* and *Pythium* sp. The carrier based formulations of these isolates resulted in increased plant growth in bioassays. The isolate MK₅ showed maximum homology (99%) with *Bacillus subtilis* (HQ594544) and isolate MK₇ showed homology (100%) with *Bacillus pumilus* (GU290547) by 16S rRNA analysis.

Key words: *Bacillus* sp., cauliflower, Plant growth promoting rhizobacteria (PGPR), P-solubilization, biocontrol.

INTRODUCTION

Plant growth promoting rhizobacteria (PGPR) were first defined by Kloepper and Schroth (1978) as beneficial rhizobacteria that colonize the roots and exert beneficial effects on plant growth and development. The growth enhancement may be direct by the production of phytohormones and/or by increasing the available nutrients through production of secondary metabolites to solubilize native P content, siderophore producers or indirect means as biocontrol agent that are able to protect plant from infections by phytopathogenic organisms through production of antibiotics, HCN and cell wall degrading enzymes. PGPR have been applied to various crops to enhance seed emergence, growth and crop yield (Cleyet-Marcel et al., 2001), but only a few isolate

have been commercialized (Dey et al., 2004). The most predominant rhizosphere colonizing bacteria belong to genus *Pseudomonas* and *Bacillus* because of their association with soil organic matter, nutritional diversity and rapid growth rate. The extent of benefits from PGPR depends on their number and efficiency which is governed by a large number of soil and environmental factors. PGPR are likely to be of great interest in sustainable crop production and growth promotion. Significant enhancement in plant growth or control of pathogens by PGPR has been demonstrated under laboratory and net house conditions. In the present study, two efficient rhizobacterial strains of *Bacillus subtilis* (MK₅) and *Bacillus pumilus* (MK₇) isolated from soil and root samples collected from rhizosphere of cauliflower of sub temperate locations in Himachal Pradesh has been characterized with special reference to their plant growth promoting abilities.

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MATERIALS AND METHODS

Soil samples (up to 15 cm depth) were collected from rhizosphere (with roots) of cauliflower from three (Kangra, Hamirpur and Bilaspur) naturally growing agro-climatic zones of Himachal Pradesh. The samples were placed in plastic bags and stored in Soil Microbiology Laboratory of Department of Soil Science and Water Management for further isolation and analysis work. The isolation of the microorganisms was carried out from eight sites at different locations in Bilaspur (Namhol and Jukhala), Hamirpur (Bhota and Nadaun) and Kangra (Palampur, Dharamshala, Jachh and Pong Dam) area of Himachal Pradesh.

Isolation from soil and root samples

Isolation from soil and root samples was done by modified replica plating technique. After incubation of 24 to 48 h, the isolated colonies that developed on enriched medium (master plate) were replica plated onto the selective media: Nitrogen free medium (Jensen, 1987) for nitrogen fixing activity, Pikovskaya medium (Pikovskaya, 1948) for phosphate solubilizing ability.

Maintenance of the cultures

The isolated cultures were purified by streak plate method and maintained on the slants of respective medium at 4°C in refrigerator. The culture of *Rhizoctonia solani*, *Pythium* sp. and *Fusarium* sp. were procured from Department of Mycology and Plant Pathology, University of Horticulture and Forestry, Nauni, Solan. Fungal cultures were maintained on malt extract agar at 4°C.

Screening and characterization of bacterial isolates

The screening of the bacterial isolates for various plant growth promoting activities were performed by adopting the standard methods. Estimation of P- solubilization in liquid PVK medium containing tri-calcium phosphate (TCP) was determined as described by Bray and Kurtz (1945). Phosphate solubilizing activity, nitrogen fixing ability, siderophore production, HCN production, IAA production and antagonistic activity was determined by Pikovskaya (1948), Jensen (1987), Schwyn and Neilands (1987), Bakker and Schippers (1987), Gordon and Paleg (1957) and Vincent (1970) respectively.

Molecular characterization of selected isolate

Primers specific for best isolate will be developed based on the 16S rRNA gene sequence and were evaluated by polymerase chain reaction (PCR) performed with genomic DNA from selected isolates.

RESULTS AND DISCUSSION

Isolation and enumeration of rhizospheric and endophytic rhizobacteria

Microbial population in rhizosphere soil

The rhizosphere soil had great variation in total microbial counts at different locations. Among various sites, Bhota had highest (291.0×10^4 cfu/g soil) and Nadaun had the

minimum (180.3×10^4 cfu/g soil) bacterial counts. The population on the Jensen's medium which is specific for N-fixation was highest (72.3×10^4 cfu/g soil) at Jachh and lowest (56.0×10^4 cfu/g soil) was found for Jukhala. The percentage of phosphate solubilizing microorganisms to total PVK count ranged from 61.30% (Jukhala, Bilaspur) to 75.60% (Nadaun, Hamirpur).

Microbial population in the roots of cauliflower plants

Cauliflower roots collected from different location/sites harboured variable microbial population. The highest (101.7×10^1 cfu/g root) total bacterial count was found at Nadaun however, the lowest (62.3×10^1 cfu/g root) was noted at Jukhala. The maximum (47.7×10^1 cfu/g root) endophytic bacterial population capable of N-fixation was recorded for Nadaun and the minimum (33.3×10^1 cfu/g root) was noted for Namhol. Percent phosphate solubilizers to the total population on PVK medium at different location/sites varied from 45.3% at Namhol (Bilaspur) to 61.3% at Nadaun (Hamirpur).

Screening and characterization of bacterial isolates

Qualitative and quantitative estimation of TCP solubilization by bacterial isolates

Phosphorus is one of the major nutrients, second only to nitrogen in requirement for plants. Most of phosphorus in soil is present in the form of insoluble phosphates and cannot be utilized by the plants. PGPR have been shown to solubilize precipitated phosphates and enhance phosphate availability to cauliflower that represents a possible mechanism of plant growth promotion under field conditions (Verma et al., 2001). In our experiments, all isolates were able to solubilize phosphate in the rhizosphere soil. It is important to note that several phosphate-solubilizing bacilli occur in soil (Skrary and Cameron, 1998) but their numbers are not usually high enough to compete with other bacteria commonly established in the rhizosphere (Lifshitz et al., 1987). All the five isolates (MK₂, MK₄, MK₅, MK₇ and MK₉) were found to solubilize TCP in PVK agar medium (Table 1).

Indole-3-acetic acid production by bacterial isolates

The data presented in Table 2 revealed that isolate MK₅ produced a significantly higher concentration of IAA ($29.67 \mu\text{g/ml}$) corresponding maximum (60.67×10^5 cfu/ml) viable count as compared to other tested isolates. It has been reported that IAA production by PGPR can vary among different species and strains, and it is also influenced by culture condition, growth stage and substrate availability (Mirza et al., 2001).

Table 1. Solubilization of Tri calcium Phosphate (TCP) by selected bacterial isolates in solid and liquid Pikovskaya's medium.

Bacterial isolates	P-solubilization in solid medium		P-solubilization in liquid medium (µg/ml)
	Phosphate solubilization index (PSI)	% P-solubilization efficiency (%SE)	
MK ₂	2.67	151.36 (12.27)*	444.33 (2.64)**
MK ₄	2.47	147.22 (11.90)	567.67 (2.73)
MK ₅	2.76	172.21 (13.06)	664.33 (2.82)
MK ₇	2.77	166.67 (12.87)	640.33 (2.80)
MK ₉	2.75	158.33 (12.54)	604.00 (2.77)
Isd	0.28	(0.78)	(0.06)

*Figures in parentheses are square root transformed values, ** Figures in parentheses are log transformed values.

Table 2. Indole-3-acetic acid production by selected bacterial isolates in Luria Bertani Broth.

Bacterial isolates	Viable count (10 ⁵ cfu/ml)	Final pH of supernatant	Indole-3-acetic acid (µg/ml)
MK ₂	47.67	5.10	24.83
MK ₄	58.33	5.20	24.67
MK ₅	60.67	5.13	29.67
MK ₇	56.67	5.18	28.33
MK ₉	58.67	5.22	25.50
Isd	3.19	0.09	3.26

Table 3. Qualitative and quantitative estimation of siderophore activity by selected bacterial isolates on CAS medium.

Bacterial isolates	Siderophore activity			
	Viable count (×10 ⁵ cfu/ml)	Final pH of supernatant	Qualitative estimation (Zone size, mm)	Quantitative estimation (% siderophore unit)
MK ₂	51.63	5.10	8.67	19.86 (26.37)
MK ₄	52.67	5.22	11.33	19.40 (26.01)
MK ₅	48.33	5.12	12.67	51.36 (45.78)
MK ₇	48.67	5.23	13.33	33.03 (35.03)
MK ₉	49.67	5.14	14.67	31.14 (33.90)
Isd	3.29	0.12	2.77	15.94

Siderophore production by selected bacterial isolates

In our study, all the five bacterial isolates produced a bright zone with yellowish colour around the bacterial colony on Chrome-azurol-S (CAS) medium. Maximum (14.67 mm) bright zone with yellowish colour was observed in case of MK₉. Quantitative estimation of siderophore using Chrome-azurol-S (CAS) liquid assay (Table 3) revealed that bacterial isolate MK₅ produced maximum (51.36% siderophore unit) at 72 h of incubation

and maximum (52.67 × 10⁵ cfu/ml) viable count after 72 h of incubation corresponded to isolate MK₄.

HCN production by bacterial isolates

All the bacterial isolates were screened for HCN production on King's B medium. Only the isolates MK₅ and MK₇ caused change of colour of filter paper from yellow to orange and to dark brown.

Table 4. Antifungal activity of selected bacterial isolates against fungal pathogens using dual culture technique.

Bacterial isolates	Antifungal activity (% growth inhibition)		
	<i>Rhizoctonia solani</i>	<i>Pythium</i> sp.	<i>Fusarium</i> sp.
MK ₂	81.81 (9.04)	83.72 (9.14)	83.33 (9.12)
MK ₄	78.40 (8.85)	81.39 (9.02)	90.38 (9.50)
MK ₅	84.09 (9.16)	86.04 (9.26)	89.28 (9.44)
MK ₇	77.27 (8.78)	81.39 (9.02)	85.71 (9.25)
MK ₉	81.81 (9.04)	77.90 (8.82)	88.09 (9.38)
Isd	(0.21)	(0.26)	(0.23)

Figures in parentheses are square root transformed values.

Characterization of the bacterial isolates for antifungal activity

The antifungal activity of individual isolates was compared using dual culture method (Table 4). MK₄ bacterial isolate, in general, showed maximum growth inhibition against *Fusarium* sp. (90.38%). Bacterial isolate MK₅ showed comparatively maximum growth of inhibition (84.09%) against *Rhizoctonia solani*. The MK₅ isolate showed maximum growth of inhibition (86.04%) against *Pythium* sp. A large body of evidence suggests that PGPR enhance the growth and crop yield, and contribute to the protection of plants against certain pathogens and pests (Minorsky, 2008).

Molecular characterization (16S rRNA gene sequencing) of efficient native PGPR isolates

An attempt was made to characterize the efficient bacteria isolated from the rhizosphere of cauliflower plants using 16S rRNA gene sequencing to identify and decipher their phylogenetic affiliation of these bacteria. Sequence data of 16S rRNA gene of two efficient strains obtained through automated sequencer using eubacterial universal primers revealed 1409 bp partial sequence in isolates MK₅ and MK₇ (Figure 1).

The 16S rRNA gene sequence is about 1,500 bp long and is composed of both variable and conserved regions. Universal primers are usually chosen as complementary to the conserved regions at the beginning of the gene or at either the 540 bp region or at the end of the whole sequence (about the 1,500 bp region), and in between sequence of the variable region is used for the comparative taxonomy (Relman, 1999). The gene is large enough, with sufficient interspecific polymorphisms, to provide distinguishing and statistically valid measurements. The 16S rRNA gene serve as molecular

chronometer, since it is the most conserved part during evolution (Clarridge 2004). Therefore, 16S rRNA gene sequencing is used and accepted worldwide for identification and phylogenetic analysis of the bacterium.

Nucleotide sequence analysis

Nucleotide sequence analysis of test isolates using clustalW program revealed that isolate MK₅ showed maximum homology (99%) with *B. subtilis* (HQ594544) and isolate MK₇ showed homology (100%) with *B. pumilus* (GU290547). The test bacterial isolates clustered with members of the genera *Bacillus*, thus differentiating the bacterial isolates on the genetic basis. Earlier workers have also reported the isolation of these genera from the rhizosphere of various crop plants (Chan et al., 1994).

The pair wise genetic distance of the two efficient isolates with other selected sequences from the national centre for biotechnology information (NCBI) is depicted in Table 5. The pair wise genetic distance of the isolates viz., MK₅ and MK₇ with other selected sequences ranged from 0.004 to 0.302. Dendrogram based on phylogenetic analysis presented in Plate 1 shows that the two isolates viz., MK₅ and MK₇ clustered with *Bacillus* a typical Firmicute.

Various other workers also used this technique for identification and phylogenetic analysis of the isolates (Catara et al., 2002). Therefore, to get reliable and accurate identification of bacterial isolates, molecular characterization (16S rRNA gene sequencing) is an important tool. Taken together, results suggest that PGPR are able to induce the production of IAA, solubilization of phosphorus, and resistance to pathogens and pests, thereby improving growth of plants. The use of PGPR as inoculants biofertilizers is an efficient approach to replace chemical fertilizers and pesticides for sustainable cauliflower cultivation in Himachal Pradesh.

1 AGTCGAGCGGACAGATGGGAGCTTGCTCCCTGAATAGTTAAGCAGGCGGACGGGTGAGTAACACGTGGGT 70
 71 AACCTGCCTGTAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATGGTTGTTTGAACCGCATG 140
 141 GTTCAAACATAAAAGGTGGCTTCGGCTACCACTTACAGATGGACCCGCGGCGCATAGCTAGTTGGTGAGG 210
 211 TAACGGCTCACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACAC 280
 281 GGCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGC 350
 351 CGCGTGAGTGATGAAGGTTTTTCGGATCGTAAAGCTCTGTTGTTAGGGAAGAACAAGTACCCTTCGAATAG 420
 421 GGCGGTACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTA 490
 491 GGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGGGCTCGCAGGCGGTTTCTTAAGTCTGATGTGAAA 560
 561 GCCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGGGGAACCTTGAGTGCAGAAGAGGAGAGTGGAATTC 630
 631 CACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCACTGGCGAAGGCGACTCTCTGGTCTGTAA 700
 701 CTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAACGA 770
 771 TGAGTGCTAAGTGTAGGGGGTTTCCGCCCTTAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGG 840
 841 AGTACGGTCGCAAGACTGAAACTCAAAGGAATTGACGGGGGCCCGCACCAGCGGTGGAGCATGTGGTTTA 910
 911 ATTCGAAGCAACGCGAAGAACCCTTACCAGGTCTTGACATCCTCTGAAATCCTAGAGATAGGACGTCCCCT 980
 981 TCGGGGCGCAGAGTGACAGGTGGTGCATGGTTGTCGTAGCTCGTGTGCTGAGATGTTGGGTAAAGTCCCG 1050
 1051 CAACGAGCGCAACCCCTTGATCTTAGTTGCCAGCATTAGTTGGGCACTCTAAGGTGACTGCCGGTGACAA 1120
 1121 ACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCTTATGACCTGGGCTACACACGTGCTACAAT 1190
 1191 GGACAGAACAAGGGCAGCGAAACCGCGAGGTTAAGCCAATCCACAAATCTGTTCTCAGTTCGGATCGC 1260
 1261 AGTCTGCAACTCGACTGCGTGAAGCTGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGT 1330
 1331 TCCCGGGCCTTGATACACACCGCCCGTCACACCACGAGAGTTTGTAAATCCGAAGTCGGTGAGGTAACCT 1400
 1401 CTTAGGAGC 1409

A

1 AGTCGGAGCGGACAGAAGGGAGCTTGCTCCCGGATAGTTAACAGGCGGACGGGTGAGTAACACGTGGGTA 70
 71 ACCTGCCTGTAAGACTGGGATAACTCCGGGAAACCGGAGCTAATACCGGATAGTTCCTTGAACCGCAGGT 140
 141 TCAAGGATGAAAGACGGTTTTTCGGCTGTCACTTACAGATGGACCCGCGGCGCATAGCTAGTTGGTGGGGTA 210
 211 ATGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGG 280
 281 CCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCG 350
 351 CGTGAGTGATGAAGGTTTTTCGGATCGTAAAGCTCTGTTGTTAGGGAAGAACAAGTGCGAGAGTAAGTCTGCT 420
 421 CGCACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGT 490
 491 GGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGGGCTCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCC 560
 561 CCCGGCTCAACCGGGGAGGGTCATTGGAAACTGGGAACTTGAGTGCAGAAGAGGAGAGTGGAATTCAC 630
 631 GTGTAGCGGTGAATTGCGTAGAGATGTGGAGGAACACCACTGGCGAAGGCGACTCTCTGGTCTGTAACTG 700
 701 ACGCTGAGGAGCGATAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAACGATGA 770
 771 GTGCTAAGTGTAGGGGGTTTTCCGCCCTTAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGT 840
 841 ACGGTCGCAAGACTGAAACTCAAAGGAATTGACGGGGGCCCGCACCAGCGGTGGAGCATGTGGTTTAAT 910
 911 CGAAGCAACGCGAAGAACCCTTACCAGGTCTTGACATCCTCTGACAACCCTAGAGATAGGGCTTTCCCTTC 980
 981 GGGGACAGAGTGACAGGTGGTGCATGGTTGTGTCAGCTCGTGTGCTGAGATGTTGGGTAAAGTCCCGCA 1050
 1051 ACGAGCGCAACCCCTTGATCTTAGTTGCCAGCATTAGTTGGGCACTCTAAGGTGACTGCCGGTGACAAAC 1120
 1121 CGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCTTATGACCTGGGCTACACACGTGCTACAATGG 1190
 1191 ACAGAACAAGGGCTGCAAGACCGCAAGGTTTAGCCAATCCCATAAATCTGTTCTCAGTTCGGATCGCAG 1260
 1261 TCTGCAACTCGACTGCGTGAAGCTGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTC 1330
 1331 CCGGGCCTTGATACACACCGCCCGTCACACCACGAGAGTTTTCAACATCCGAAGTCGGTGAGGTAACCTTT 1400
 1401 ATGGTAGCC 1409

B

Figure 1. Partial nucleotide sequence of 16S rRNA gene of efficient isolates: (A) *B. subtilis* (MK5), (B) *B. pumilis* (MK7).

Plate 1. Phylogenetic tree constructed by Neighbor-Joining method derived from analysis of the 16S rRNA gene sequences of native isolates and related sequences obtained from NCBI. Scale bar, 0.02 substitutions per nucleotide position.47

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