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Identification of Beneficial Endophytes Which Helps in Suppressing Black Rot Disease in Cauliflower

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ABSTRACT

Black rot is a destructive disease of cauliflower through out the world caused by Xanthomonas campestris pv. campestris (Pammel) Dowson. The black rot pathogen is seed-borne and spreads in the vascular system of the leaf and stem. In the present study endophytic microorganisms that colonize the internal tissues of plants enhance agricultural production through plant growth promoting mechanisms. Considering the enormous potential of the endophytic bacteria, a research programme was framed to study the role of these bacteria in plant growth promotion of crop plants belonging to vegetables. The endophytic bacteria were isolated from root and shoot of cauliflower plants. Forty one out of the eighty eight bacterial isolates selected were Gram positive belonging to Bacillus sp. while forty seven were Gram negative belonging to Citrobacter, Acinetobacter, Pseudomonas.

Root and shoot associated endophytic bacteria were isolated, selected and characterized for their biotechnological potential of growth promotion and biocontrol of plant pathogenic bacteria. Eighty eight strains were isolated and subjected to chemical tests, and the maximum producer of HCN as well as ammonia were PSBnR5 and PSBkS10. The 16S rRNA sequence of isolates PSBkS10 and PSBnR5 have been analyzed and submitted in GenBank with accession numbers MH257751 (Pseudomonas fluorescens) and MH257752 (Bacillus subtilis), respectively. Phylogenetic tree of 16S rRNA of both isolates was constructed using the neighbor- joining (NJ) algorithm with bootstrap analysis for 1000 replicates

Key words: *Endophytes, Biocontrol agent, Bacillus subtilis, Pseudomonas fluroscens,*

INTRODUCTION

Cauliflower is a vegetable that belongs to the species Brassica oleracea, in the family Brassicaceae. It is an annual plant that reproduces by seed. Typically, only the head (the white curd) of aborted floral meristems is eaten, while the stalk and surrounding thick, green leaves are either used in vegetable broth or discarded

Second to China, India has the largest area and production of cauliflower. About half of all cauliflower is raised in China and one fourth in India.

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In 2003, the world's largest exporters of cauliflower were Spain and France, which respectively accounted for 36% and 22% of the world exports[5]. But cauliflower is prone to many fungal, bacterial and viral diseases. Among these, black rot caused by *Xanthomonas campestris* pv. *campestris*, is one of the most destructive diseases of cauliflower, affecting the shoot region above the ground level throughout its growth stage.

In India, black rot disease of crucifers was first reported by Pathwardhan[15] on cabbage. Cauliflower. Black rot was first reported by Patel[14] and subsequently it was reported from Himachal Pradesh[17], from Varanasi in Uttar Pradesh[11], from Manipur[2], from Ranchi[10], from Nainital[4], from Sikkim[3], from Maharashtra[6] and from Karnataka[30].

Although the priming by non-pathogenic rhizobacteria is reasonably well understood, there is limited information on participation of endophytes in plant defense[20]. Endophytic bacteria settle in the plant tissue without harming the host[19]. A group of endophytes is likely to have established mutualistic relationship with the host by promoting plant growth on one hand and by inducing resistance against biotic and abiotic stressors on the other[16,20,31]. Endophytic bacteria are good candidates for being used as biofertilizers and biocontrol agents, as they are better protected from environmental stressors compared to rhizospheric bacteria, and, unlike rhizospheric bacteria, can be transferred between plant generations[19].

PGPR have been subjected to numerous investigations focusing on biotechnological applications in agriculture, horticulture, forestry and environmental protection[32]. Early studies in the 1950's began with a focus on nitrogen fixing bacteria. Since then, a large number of PGPR belonging to different bacterial classes and genera with multifunctional traits have been described[18]. PGPR strains are broadly distributed among many taxa including Actinobacteria, Bacteroidetes, Cyanobacteria, Firmicutes and Proteobacteria[29], such that PGPR have been applied to various crops to enhance growth, seed emergence as well as crop yield, and some have been commercialized[1]. Under salt stress, PGPR have shown positive effects in plants on such parameters as germination rate, tolerance to drought, weight of shoots and roots, yield, and plant growth[9]. Another major benefit of PGPR is the growth of the ability to produce antibacterial compounds that are effective against certain plant pathogens and pests.

Thus the aim of this study was to determine the effect of PGP endophytes strains that are compatible with cauliflower on controlling black rot disease caused by *Xanthomonas campestris pv campestris*

MATERIAL AND METHODS

The study was conducted at the Centre of Protected Cultivation Technology, ICAR-Indian Agricultural Research Institute (IARI), New Delhi-110012 (latitude $28^0$38' N, longitude $77^0$12'E and altitude 228.4m) in raised soil beds (15 m long and 1.25 m wide) at 18 to 36 inches space between rows (with three rows) and 12- 24 inches space between plants in a randomized block design with 3 replications, and each replication contained 20 plants. The three different varieties of cauliflower plant (Pusa sharad, Pusa snowball and Pusa deepali) were cultivated at different time because all the three cutivars requires different condition for proper growth.

Isolation of endophytes from cauliflower Plant

Sample collection:

The samples of the crops such as Pusa sharad, Pusa snowball and Pusa deepali were collected before flowering stage at 30 days of planting. These cultivars were chosen because of their recommended growth conditions in medium to low soil fertility and their wide suitability in indian climate conditions.

Bacterial isolation:

Five plants of each genotype were sampled at the flower bud phase and split into root and shoot. The plant tissues were surface-sterilized by immersion in 2% hypochlorite solution for 15 min and rinsed 3 times for 15 min with sterile distilled water. Five grams of each plant

tissue was ground in 1:10 (w/v) saline solution (0.9% NaCl) and serially diluted up to 10^{-10} One hundred microliters of the 10^{-10} dilutions was used to inoculate five different types of media i.e TSA- Trypticase Soya Agar- for overall bacterial diversity, NA- Bacillus sp., KMA- Kenknight and Munaier's Agar – for actinobacteria ,R2A- for oligotrophic organisms (mainly bacteria) , Kings B Agar- for *Pseudomonas* sp. for diversity purpose (in 3 replica vials for each dilution), The inoculated media were incubated at 28° C for 7 to 8 days, and then a bacterial count was performed using the most probable number (MPN) method or the colony-forming unit (CFU) method. Bacterial colonies and pellicles present in the higher dilutions were streaked on TSA medium for purification. Purified bacterial isolates were maintained in liquid glycerol:TSA [1:1 (v/v)] at-20° C until used for molecular and biochemical characterization.

Identification of isolates

The bacterial strain was studied for cultural, morphological and biochemical characteristics based on Bergey's Manual of Systematic Bacteriology.

Cultural characteristics

All the isolates were streaked on LB agar plates. After 3 days of incubation, different characteristics of colonies such as growth, form and colour.

Morphological characteristics

The suspected organisms were subjected to Gram's staining. The bacteria which retained the primary stain called gram + ve while those that lost the crystal violet and counter stained by safranin were referred as gram – ve.

Qualitative assay for ammonia production

Bacterial isolates were tested for the production of ammonia in peptone water. Freshly grown cultures were inoculated in 10 mL peptone water in each tube and incubated for 48–72 h at 28 ± 2°C. Nessler's reagent (0.5 mL) was added in each tube. Development of brown to yellow colour was a positive test for ammonia production.

HCN production by bacterial isolates

All the isolates were screened for the production of hydrogen cyanide by adapting the method of. Briefly, nutrient broth was amended with 4.4 g glycine/l and bacteria were streaked on modified agar plate. A Whatman filter paper no. 1 soaked in 2% sodium carbonate in 0.5% picric acid solution was placed in the top of the plate. Plates were sealed with parafilm and incubated at 28± 2°C for 4 days. Development of orange to red colour indicated HCN production.

Identification of the best Isolates:

DNA extraction and gel electrophoresis:

Genomic DNA of rhizobacterial isolates were extracted by using Quick-DNA Fungal/Bacterial Kit (Zymo Research, CA, USA) according to manufacturer's instruction. Extracted DNA were electrophoretically analyzed on 0.8% agarose gel containing 0.5 µg/ml ethidium bromide, using 1 x Tris-acetate- ethylenediamine tetraacetic acid (TAE) electrophoresis buffer and run for one hour at 90 volts to confirm the presence of genomic DNA. The purity and quantity were also determined with a NanoDrop 3300 spectrofluorometry (Waltham, MA, USA).

16S rRNA gene amplification and sequencing:

Bacterial 16s rRNA gene was amplified using Veriti®96 well thermal cycler Applied Biosystem (Waltham, MA, USA). Bacterial genomic DNA was amplified using 16S rDNA gene universal primers 9bfm (5'-GAGTTTGATYHTGGCTCAG-3') and 1512R (5'-ACGGHTACCTTGTTACGACTT-3') (Muhling et al. 2008). 25µL reaction volume contains 12.5µL RedTaq® ReadyMix (Sigma-Aldrich Biotechnology), 1µL Bovine serum albumin (10mg/L), 0.5µL of each forward and reverse primer from stock of 10µM, 10-15 ng DNA template. The PCR procedure included an initial denaturation step of 5 min at 95°C, followed by 35 cycles of amplification (30 s at 95°C, 30 s 55°C and 90 s at 72°C). The final extension step was performed at 72°C for 10 min. Amplification was electrophoretically analyzed on 1.2% agarose gel containing 0.5 µg/ml ethidium bromide, using 1 x Tris- acetate-ethylenediamine tetraacetic acid (TAE) electrophoresis buffer and run for one hour at

90 volts. PCR products were purified and concentrated using DNA Clean & Concentrator kit (Zymo Research, CA, USA) according to manufacturer's instruction. Sequencing of 16S rRNA performed at the KAD Bioresources Pvt.Ltd. (India) using the same primer set.

Phylogenetic analysis:

The DNA sequences were checked and edited carefully using DNASTAR SeqMan software (DNASTAR, Madison, WI, USA). 16S rRNA sequences were compared against nucleotide databases using the NCBI BLASTn and EzTaxon-e server to identify the closest known taxa[8]. All 16S rRNA along with their closest homology sequences were aligned using CLUSTAL W algorithm provided by MEGA 6.0 software by using default parameters. A phylogenetic tree was constructed by neighbor- joining (NJ) method using same software[28]. Bootstrap analysis was performed with 1000 replicate datasets in order to evaluate confidence limits of the branching.

GenBank accession numbers:

The complete sequences of both isolates were submitted in GenBank, that is, in the National Center for Biotechnology Information (NCBI).

Results and discussion

Isolation of PGPR strains from cauliflower root and shoot:

To isolate the PGPR strains from cauliflower field soil samples were collected and inoculated on TSA- Trypticase Soya Agar- for overall bacterial diversity

NA- *Bacillus* sp.

King's B-*Pseudomonas* sp.

KMA- Kenknight and Munaier's Agar – for actinobacteria

R2A- for oligotrophic organisms (mainly bacteria) agar media.

They were designated as initials of variety followed by media name initials in lower case and root as R and shoot as S and were subjected to cultural, morphological, biochemical characterization.

Cultural characteristics

Out of eighty eight, isolates screened from the roots and shoots of different varieties of cauliflower, a majority of them were rods, short rods and long rods whereas only a small proportion of them were cocci.

Isolation and characterization of endophytes

The diversity of the putative endophytic bacteria isolated from different tissues of the hosts was assessed using phenotypic characterization methods in the present study. Colony morphology gave an indication of the variation among the endophytes. The isolates studied were chosen for their dominance as well as uniqueness or differences with others in colony morphology.

Morphological characteristics

Eighty eight isolates were selected from the root and shoot of cauliflower plants and were named according to the host plant. 41 of these bacterial isolates were Gram positive while /rest of them were Gram negative[26]. In a similar study[33], reported a similar presence of Gram negative and Gram positive bacteria in soybean, sorghum, wheat and maize. Endophytic, bacteria can follow water fluxes for passive movement, and they need to move inside the plant as they tend to congregate at specific plant parts that may not always correspond to their entry point in the plant[27].

Qualitative assay for ammonia production.

Total Eighty eight bacterial isolates were screened for ammonia production in peptone broth after 4th day of incubation by using Nessler's reagent. The change in colour from yellow to brown indicated ammonia production. Results are represented in (Table 1). In present study Out of 47 bacterial isolates isolated from root of all three cultivars, 28 isolates showed ammonia production in peptone broth. Maximum ammonia (+++) production has been shown by 10 bacterial isolates viz. PSHtR2, PSHkR14, PSHrR18, PSBtR3, PSBnR5, PSBkR9, PSBkR10, PDtR4, PDnR6 and PDrR17 respectively. Rest of all the isolates showed less or moderate ammonia production. Likewise in shoot of all three cultivars, 23 isolates showed ammonia production, these strains could further be exploited for the fermentative production of ammonia (Table 2). Maximum ammonia (+++) production has been shown by 8

bacterial isolates viz. PSHmS6, PSHkS12, PSHrS14, PSBtS3, PSBkS10, PDrS1, PDnS7 and PDkS14 respectively. Whether these ammonia producing strains have any influence on the growth promotion and disease suppression is yet to be evaluated in details. Volatile compounds such as ammonia are known to be produced by a number of rhizobacteria. They are reported to play a significant role in bioantagonisms. It has also been found out that ammonia production indirectly influences the plant growth. Ammonia production may be as high as 95% of the isolates from the rhizosphere of rice. They significantly influence plant growth promotion[21,7]. found out that 95% of *Bacillus* isolates and 94.2% of *Pseudomonas* produced ammonia. Saraf *et al.*[22], tested 10 strains of *Pseudomonas* from chickpea rhizosphere. They detected that all the strains produced ammonia, its maximum being 46µg/ml by isolate M1P3 after 10 days of incubation[12]. also reported that *B. subtilis MA-2* and *Pseudomonas fluorescens* MA-4 were most efficient in ammonia production and significantly increased the biomass of medicinal plant *Geranium.*

HCN production by bacterial isolates.

In present study, 88 bacterial isolates from root and shoot of all three cultivars of cauliflower were tested for qualitative HCN production on nutrient agar plates supplemented with 0.14 % glycine. Out of 47 isolates, 33 showed HCN production (Table 3). 1 bacterial isolates showed maximum HCN production (++++), while minimum HCN production was shown by sixteen isolates along with standard strain *P.aeruginosa*. similar trends were followed in shoot, out of 41 isolates, 35 showed HCN production (Table 4).8 bacterial isolates showed maximum HCN production (+++), while minimum HCN production was shown by twenty three isolates along with standard strain *P.aeruginosa*. Siddiqui and Shakeel[25] reported HCN production by twenty-one *Pseudomonas* isolates from pigeon pea rhizosphere. Out of them three were potent producers of HCN, whereas eleven others were moderate producers[22]. isolated 10 strains of *Pseudomonas* from chickpea rhizosphere, out of which three produced HCN, the strongest producer being *Pseudomonas* M1P3. Isolates from rhizosphere of rice, mangrove, chick pea produced HCN[21] particularly in the effluent contaminated soil.

Phylogenetic tree and accession numbers:

The 16S rRNA sequence of isolates PSBkS10 and PSBnR5 have been analyzed and submitted in GenBank with accession numbers MH257751 (*Pseudomonas fluorescens*) and MH257752 (*Bacillus subtilis*), respectively. Phylogenetic tree of 16S rRNA of both isolates was constructed using the neighbor-joining (NJ) algorithm with bootstrap analysis for 1000 replicates (Fig1).

Thus the aim of this study was to determine the effect of PGP endophytes strains that are compatible with cauliflower on controlling black rot disease caused by *Xanthomonas campestris pv campestris*

Table 1. Ammonia production by Bacterial isolates isolated from root of all three cultivars of cauliflower.

Positive ammonia producer	Negative ammonia producer
PSHtR1 ++	PSHtR3
PSHtR2 +++	PSHnR6
PSHtR4 +	PSHnR8
PSHnR5 +	PSHmR9
PSHnR7 +	PSHmR10
PSHkR12 ++	PSHmR11
PSHkR13 +	PSHrR17
PSHkR14 +++	PSBtR2
PSHkR15 +	PSBmR6
PSHkR16 ++	PSBmR7
PSHrR18 +++	PSBrR11

PSBtR1	+	PDtR1
PSBtR3	+++	PDtR3
PSBnR4	+	PDnR7
PSBnR5	+++	PDmR8
PSBkR8	++	PDmR9
PSBkR9	+++	PDmR11
PSBkR10	+++	PDmR12
PSBrR12	++	PDrR16
PDtR2	++	
PDtR4	+++	
PDnR5	+	
PDnR6	+++	
PDmR10	++	
PDkR13	++	
PDkR14	+	
PDrR15	+	
PDrR17	+++	

+ = indicates light brown colour
++ = indicates dark brown colour
+++= indicates orange brown colour

Table 2. Ammonia production by Bacterial isolates isolated from Shoot of all three cultivars of cauliflower.

Positive ammonia producer		Negative ammonia producer
PSHtS1	+	PSHtS3
PSHtS2	++	PSHnS5
PSHnS4	++	PSHmS7
PSHmS6	+++	PSHmS9
PSHmS8	+	PSHrS13
PSHkS10	+	PSBtS2
PSHkS11	+	PSBnS4
PSHkS12	+++	PSBmS6
PSHrS14	+++	PSBmS7
PSBtS1	+	PSBrS11
PSBtS3	+++	PDtS3
PSBnS5	++	PDtS4
PSBkS8	+	PDnS5
PSBkS9	+	PDtS3
PSBkS10	+++	PDtS4
PDtS1	+++	PDnS5
PDtS2	++	PDmS8
PDnS6	++	PDmS9
PDnS7	+++	PDmS10
PDkS12	+	PDmS11
PDkS13	+	PDrS15
PDkS14	+++	
PDrS16	++	

+ = indicates light brown colour
++ = indicates dark brown colour
+++= indicates orange brown colour

Table 3. HCN production by Bacterial isolates isolated from root of all three cultivars of cauliflower.

Positive HCN producer	Negative HCN producer
PSHtR1 +	PSHtR3
PSHtR2 ++	PSHtR4
PSHnR5 +	PSHnR6
PSHnR7 +++	PSHnR8
PSHmR11 ++	PSHmR9
PSHkR12 +	PSHmR10
PSHkR13 +	PSBtR1
PSHkR14 ++++	PSBtR2
PSHkR15 +	PSBnR4
PSHkR16 +	PSBrR12
PSHrR17 ++	PDtR1
PSHrR18 +++	PDtR4
PSBtR3 +	PDmR10
PSBnR5 +++	PDrR15
PSBmR6 +	
PSBmR7 ++	
PSBkR8 +	
PSBkR9 +++	
PSBkR10 +	
PSBrR11 ++	
PDtR2 ++	
PDtR3 +	
PDnR5 ++	
PDnR6 +++	
PDnR7 +	
PDmR8 +	
PDmR9 +	
PDmR11 +	
PDmR12 +++	
PDkR13 +	
PDkR14 ++	
PDrR16 +++	
PDrR17 ++	

+ = indicates light brown colour, ++ = indicates dark brown colour,
+++= indicates orange brown colour.

Table 4. HCN production by Bacterial isolates isolated from shoot of all three cultivars of cauliflower.

Positive HCN producer	Negative HCN producer
PSHtS2 +	PSHtS1
PSHtS3 +	PSHnS5
PSHnS4 +	PSHmS8
PSHmS6 ++	PSHrS13
PSHmS7 +	PSBtS1
PSHmS9 +	PDtS3
PSHkS10 +	
PSHkS11 +	
PSHkS12 +++	
PSHrS14 ++	
PSBtS2 +	
PSBtS3 +++	
PSBnS4 +	
PSBnS5 +++	
PSBmS6 +	
PSBmS7 +	
PSBkS8 ++	
PSBkS9 +	
PSBkS10 +++	
PSBrS11 +	
PDtS1 +++	
PDtS2 +	
PDtS4 ++	
PDnS5 +	
PDnS6 +++	
PDnS7 +++	
PDmS8 +	
PDmS9 +	
PDmS10 +	
PDmS11 +	
PDkS12 +	
PDkS13 +	
PDkS14 +++	
PDrS15 +	
PDrS16 +	

+ = indicates light brown colour, ++ = indicates dark brown colour,
+++= indicates orange brown colour

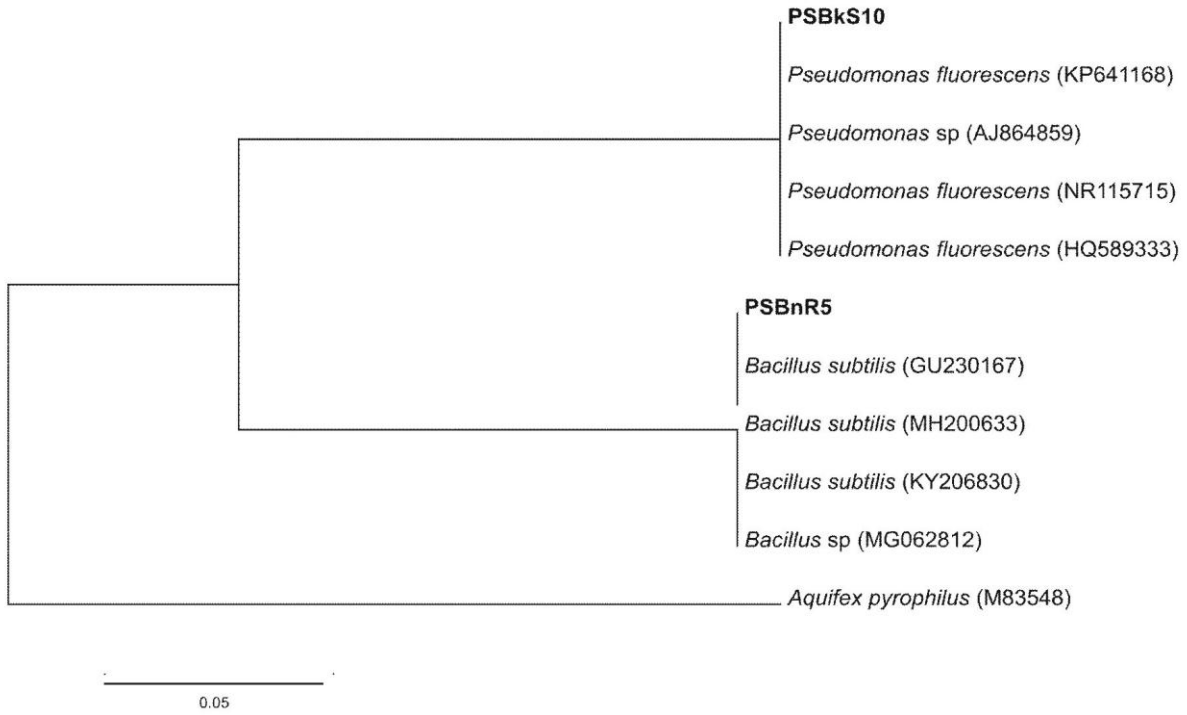

Fig. 1: Construction of phylogenetic tree based on 16S rRNA gene sequencing by neighbor-joining method

CONCLUSION

Tracing the relationships between the obtained data in vitro in analyses for Ammonia production and HCN production with plant growth promotion observed in the greenhouse, correlations were found with both features found in isolates. eight strains with high Ammonia production and HCN production, PSHkR14, PSHrR18, PSBnR5, PSBkR9, PDnR6, PSHkS12, PSBtS3 and PSBkS10

The present results are consistent with the possibility that a single ecological function can be shared by different bacterial species. For example, above mentioned PGP parameters was observed in two isolates PSBnR5 and PSBkS10, which were identified as *B. subtilis* and *Pseudomonas fluroresnens* respectively. Bacteria could promote the development of different parts of the host plant; roots (strain PSBnR5) and shoots (strain PSBkS10). The suggestion that there may be co-colonization resulting in complementary effects on plant development by strains PSBnR5 and PSBkS10, still remains to be explored.

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