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Screening of efficient rhizobacteria associated with cauliflower (*Brassica oleracea* var. *botrytis* L.) for plant growth promoting traits

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Abstract: In the current study, a total of 25 isolates were isolated from the rhizosphere and roots of cauliflower (*Brassica oleracea* var. *botrytis* L.) from the vicinity of Una district of Himachal Pradesh. The isolates were tested *in vitro* for their ability to solubilise phosphorous and produce siderophore, indole acetic acid (IAA), hydrogen cyanide (HCN) and antifungal metabolites against the soil borne pathogens. Results revealed that out of 25, only 4 rhizospheric isolates (SB_5, SB_{11}, SB_8 and SB_{10}) have maximum plant growth promoting attributes. The isolates were identified as *Bacillus* sp. on the basis of Bergey's manual of systematic bacteriology. The isolate SB_{11} recorded highest phosphate solubilizing efficiency in solid medium (109.09%) and in liquid medium (350µg/ml). Maximum production of IAA (51.96µg/ml), siderophore (91.41%) and HCN were also observed for the same isolate. Furthermore, the isolate SB_{11} produced highest antifungal metabolite production against *Rhizoctonia solani* (37.11%), *Sclerotinia sclerotiorum* (41.11%), and *Pythium* sp. (71.11%) causing root rot, stalk rot and damping off diseases in cauliflower, respectively. The selected isolate (SB_{11}) showed optimum growth at a pH of 7.0, 35°C temperature and 2% NaCl. On the basis of multifarious PGP-traits the SB_{11} isolate has tremendous potential to be used as a biofertilizer/bioprotectant for growth promotion and natural protection of cauliflower under low hill conditions of Himachal Pradesh.

Keywords: Biofertilizer, Cauliflower, HCN, P-solubilisation and Siderophore

INTRODUCTION

Plant microbe interactions are the key determinants of plant health and soil fertility. The interactions may be harmful, beneficial or neutral to the plants. Our focus should however be towards the exploitation of beneficial interactions of plants and microbes. With the identification of new bacterial strains with growth promoting traits, nowadays, the use of microbial technologies is expanding rapidly in agriculture (Garcia-Fraile *et al*., 2015). Studies have shown that the growth-promoting ability of some bacteria may be highly specific to certain plant species, cultivar, and genotype (Gupta *et al*., 2000). With the increasing problems associated with the use of synthetic chemicals in agriculture i.e. impacts on human health and the environment, development of resistance in plant pests, etc., there has been an ever increasing interest in the use of native and/or non native beneficial microorganisms to improve plant health and productivity while ensuring safety for human consumption and protection of the environment (Barea, 2015). Numerous soil bacteria which flourish in plant rhizosphere stimulate plant growth by a plethora of mechanisms and are collectively known as plant growth promoting rhizobacteria (PGPR). The direct mechanisms include atmospheric nitrogen fixation, phosphate solubilization, siderophore production and secretion of plant growth promoting hormones whereas; the indirect mechanisms include biological control of phytopathogens/deleterious microbes through antibiotic production, lytic enzymes, siderophore and HCN secretion (Vejan *et al*., 2016). These mechanisms remarkably improve plant health and promote growth in terms of increase in seedling emergence, vigour index and crop yield (Gholami *et al*., 2009). The present investigation mainly deals with isolation of PGPR from rhizosphere of cauliflower in low hills of Himachal Pradesh and screening with special reference to their plant growth promoting traits.

MATERIALS AND METHODS

Sample collection: The rhizospheric soil and root samples of cauliflower were collected from two blocks i.e. Una and Haroli block of Una district of Himachal Pradesh. Samples were aseptically stored in plastic bag at about 4°C for further isolation and characterization work.

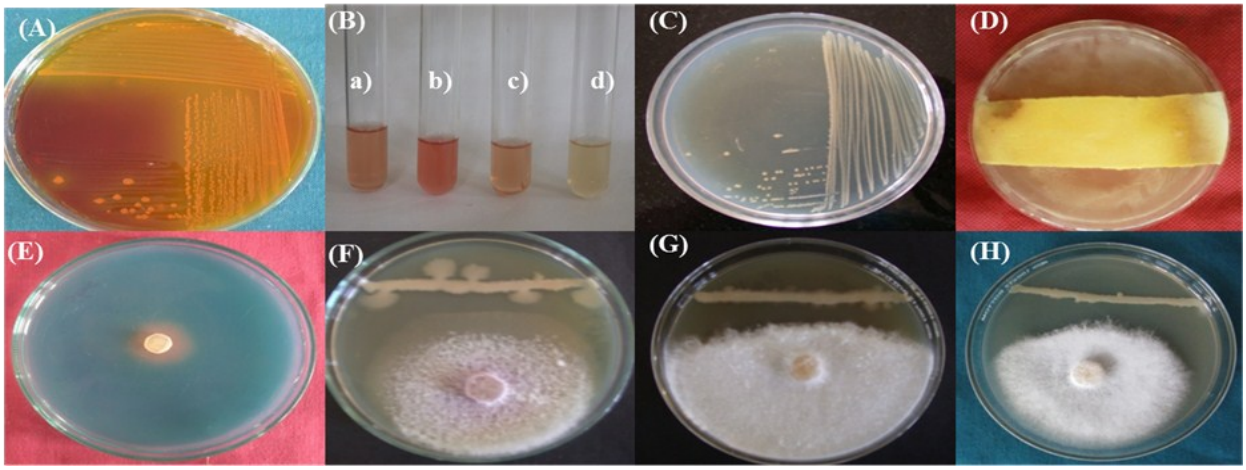

Fig. 1. *(A) Phosphate solubilisation on Pikovskaya's medium by SB_{11}, (B) IAA production on Luria bertani broth by a) SB_5, b) SB_{11}, c) SB_{10} and d) Control, (C) Growth of SB_{11} on N_2 free medium (D) HCN production on King's B medium by SB_{11}, (E) Siderophore production on CAS medium by SB_{11}, (F) Inhibition of Rhizoctonia solani by SB_{11}, G) Inhibition of Sclerotinia sclerotiorum by SB_{11}, H) Inhibition of Pythium sp. by SB_{11}.*

Isolation and enumeration of microbial population: Total bacterial population was determined on nutrient agar (NA) and Soil extract medium (SEM) by the standard serial dilution and plate count technique (Subba Rao, 1999). The isolated colonies that developed on enriched NA medium (master plate) after incubation of 24 to 48 h, were replica plated onto the selective media: Pikovskaya's agar (PVK) and Jensen medium (JM). The microbial count was expressed as colony forming unit per gram of soil (cfu g^{-1} soil).

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. Morphological and biochemical characterization of the isolates was performed as per the criteria of Bergey's Manual of Systematic Bacteriology (Claus and Berkley, 1986).

Screening for multifarious plant growth promoting traits

Mineral phosphate solubilisation: Phosphate solubilizing activity of each bacterial isolate was done on Pikovskaya's (PVK) agar plate as per the method of Pikovskaya (1948) and noted for clear yellow zone around the colony. Phosphate solubilization index (PSI) was measured using the formula as given by Edi-Premono *et al.* (1996). Further, quantitative estimation of P was done in PVK broth amended with 5.0 g/l tricalcium phosphate (TCP) by the vanadomolybdate method (Sundara Rao and Sinha, 1963).

IAA production: Each bacterial isolate was grown in Luria Bertani broth (amended with 5 mM L-tryptophan, 0.065% sodium dodecyl sulfate and 1% glycerol) for 72 h at 35±2°C under shake conditions. Quantitative estimations were done using Salkowski reagent spectrometrically (Glick, 2012).

Growth on N-free medium: Each of the purified isolate was streaked on Jensen's medium and was incubated for 72 to 120 h and the plates showing growth of bacteria in the form of bacterial colony were selected (Jensen, 1987).

Hydrogen cyanide production: Bacterial isolates were streaked on King's B agar medium with 4.4 g glycine/l. Whatman no. 1 filter paper, was cut into uniform strips, 8 cm long and 0.5 cm wide; saturated with an alkaline picrate solution (0.5% picric acid + 0.2% sodium carbonate; pH 13); and placed inside the lid of a petri dish. The plates were then sealed air tight with parafilm and incubated at 35±2°C for 48 h. Thereafter, a colour change in the sodium picrate present in the filter paper from yellowish to reddish brown was

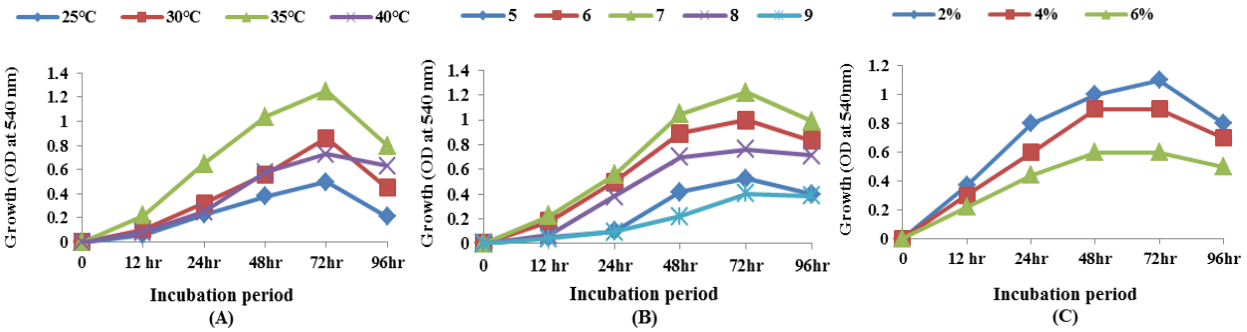

Fig 2. *(A) Effect of temperature on growth of SB_{11}, (B) Effect of pH on growth of SB_{11} and (C) Effect of salinity on growth of SB_{11}.*

Table 1. Enumeration of rhizospheric and endophytic bacterial population associated with cauliflower at Una district of Himachal Pradesh.

Districts	Blocks	Locations	Rhizospheric soil bacterial population ($\times10^4$ cfu/g soil)				Endophytic bacterial population ($\times10^1$ cfu/g root)			
			Nutrient Agar (NA)	PVK medium	Jenesn's medium (JM)	Soil extract medium (SEM)	Nutrient Agar (NA)	PVK medium	Jenesn's medium (JM)	Soil extract medium (SEM)
UNA	UNA	Lalsingi	139.7	81.3 (46.3)*	75.7	154.3	87.7	49.3 (28.7)*	45.7	69.3
	HAROLI	Basal	216.7	129.3 (74.0)	97.3	230.0	103.0	53.0 (25.3)	52.3	83.3
		Ghaluwal	225.7	119.0 (82.7)	108.0	240.0	68.0	43.0 (22.7)	39.7	52.3
		Bhadsali	164.0	81.7 (46.0)	71.0	175.7	97.3	56.7 (34.3)	47.0	68.0
$CD_{0.050}$			7.3	7.1	6.5	8.7	5.5	3.6	4.6	8.8

* Number of colonies forming halozone on PVK medium.

Table 2. Screening of selected bacterial isolates for multifarious plant growth promoting traits.

Isolates	P-solubilization in solid medium		P-solubilization in liquid medium		Growth on N free medium	IAA production (µg/ml)	HCN production	% siderophore production efficiency
	Phosphate solubilization index (PSI)	% P-solubilization efficiency (%SE)	P-solubilization in liquid medium (µg/ml)	Final pH of supernatant				
SB_5	2.00	100.00 (10.00)*	337.5 (2.53)**	4.12	+	15.33	+	79.66 (63.86)***
SB_{11}	2.09	109.09 (10.44)	350.0 (2.54)	3.99	+	51.96	+	91.41 (76.08)
SB_8	1.92	91.67 (9.57)	312.5 (2.49)	4.25	+	7.28	-	35.37 (36.48)
SB_{10}	1.80	80.00 (8.94)	250 (2.40)	4.42	+	12.83	+	59.88 (50.69)
$CD_{0.05}$	0.19	(0.33)	(0.01)	0.13	-	2.92	-	(12.73)

*Figures in parentheses are square root transformed values; ** Figures in parentheses are log transformed values; *** Figures in parentheses are arc sine transformed values; (+) indicates positivity of test; (-) indicates negativity of test.

considered to be an indication of HCN production (Bakker and Schippers, 1987).

Siderophore production: The ability of the isolates to produce siderophore was determined using blue agar plates containing chrome azurol S (Schwyn and Neilands, 1987). Each isolate was inoculated on to the plate and incubated at 35±2°C for 48 h. Orange halos around the isolate on the blue agar served as indicators of siderophores excretion.

Antifungal activity: A dual plate method was used for *in vitro* screening of bacterial strain against different fungal pathogens *viz., Rhizoctonia solani*, *Sclerotinia sclerotiorum* and *Pythium* sp. Percent growth inhibition was calculated using the formula proposed by Vincent (1947).

$$I = \frac{C - T}{C} \times 100$$

Where,

I is the percentage of growth inhibition,

C is the growth of fungus in control and

T is the growth of fungus in treatment.

Growth under different temperature, pH and salinity conditions: 3 ml of nutrient broth was taken in 5 ml test tubes and inoculated with 0.1 ml of 48 h old bacterial cell suspension (O.D. 1.0 at 540 nm). Growth curves were drawn by growing the culture at different temperature (25, 30, 35, 40 and 45 °C), pH (5, 6, 7, 8 and 9) and NaCl (2, 4, 6, 8 and 10%). The optimum temperature, pH and salinity suitable for the growth were selected on the basis of turbidity caused by the bacterial growth in test tube.

RESULTS AND DISCUSSION

Enumeration of microbial population: Significant variations in the population of both rhizospheric and endophytic microbes have been noted in the present investigation. The total viable microbial counts, in general, dominated in rhizosphere (139.7 to 225.7×10^4 cfu/g soil) as compared to endophytic (68.0 to 103.0×10^1 cfu/g root) population. A summary of microorganisms colonizing the rhizosphere and root of cauliflower at different locations of Una district of Himachal Pradesh is presented in Table 1. Ghaluwal had the highest rhizospheric microbial count (240.0×10^4 cfu/g soil) on Soil Extract Medium (SEM) whereas; Bhadsali had the lowest rhizospheric count (71×10^4 cfu/g soil) on the Jensen medium (JM). The maximum endorhizo bacterial population on nutrient agar was recorded from roots of cauliflower collected from Basal (103.0×10^1 cfu/g root) and minimum from Ghaluwal (39.7×10^1 cfu/g root). The variation in microbial population in the rhizosphere at all the sampling sites is dependent upon various attributes like location, age of plant, variety/cultivar type, and time of sampling, physico-chemical properties of soil and environment conditions of the locations as well as other

Table 3. Per cent growth inhibition of fungal pathogens by selected bacterial isolates.

Isolates	Per cent inhibition against		
	Rhizoctonia solani	***Sclerotinia sclerotiorum***	***Pythium* sp.**
SB_5	33.33 (35.25) *	38.88 (38.56) *	62.22 (52.06) *
SB_{11}	37.11 (37.51)	41.11 (39.86)	71.11 (57.47)
SB_8	28.89 (32.46)	36.67 (37.24)	33.33 (35.35)
SB_{10}	32.20 (34.45)	32.22 (34.55)	00.00 (00.00)
$CD_{0.05}$	(3.05)	(3.14)	(1.92)

*Figures in parentheses are arc sine transformed values

selection procedures. The results are in conformation with those of Kaushal *et al*. (2011) who has reported a great variation in rhizospheric and endophytic counts under natural conditions for cauliflower in mid hills of Himachal Pradesh.

Screening for multifarious plant growth promoting traits: On the basis of morphological and biochemical characteristics, all isolates were found to be endospore forming gram positive rods. All isolates were positive for glucose fermentation, production of gelatinase and catalase enzymes.

Mineral phosphate solubilisation: All the bacterial isolates exhibited variation for different plant growth promoting traits (Table 2 and Fig. 1). The selected four isolates (SB_5, SB_{11}, SB_8 and SB_{10}) were capable of hydrolyzing phosphorus in tricalcium phosphate (TCP) amended PVK broth as well as on solid PVK medium. The per cent P-solubilization efficiency shown by different isolates had great variation with the value ranging from 80.00 to 109.09 per cent. The maximum (109.09 %) P-solubilization was noted for SB_{11} isolate in solid PVK medium and 350 µg/ml in liquid PVK with drop in final pH from 7.0 to 3.99 after 72 hr of incubation at 35°C which was statistically at par with SB_5 isolate. Phosphorus (P) is second most essential master key element required for growth and development of the plants. Microorganisms play a fundamental role in mobilizing inorganic and organic P in the soil and simultaneously increases P uptake by the plant to enhance sustainable production. One of the major mechanisms of the mineral phosphate solubilization is the lowering of soil pH through production of microbial metabolites such as organic acids, chelation and/or exchange reaction (Mehta *et al*., 2015). Similar release of phosphate (182 µg/ml) with drop in pH to 4.91 by *Pseudomonas* sp. MP18 has been reported by Dinic *et al.* (2014).

IAA production: IAA is a phytohormone that regulates cell division, elongation, differentiation and pattern formation in plants and is considered to be the most important native auxin (Sahasrabudhe, 2011). All the four PGPR isolates produced auxin with the range from 7.28 to 51.96 µg/ml in Luria Bertani broth after 72 hr of incubation. Our results are in agreement with those of Mandyal *et al.* (2012) and Sharma *et al.* (2015) who have reported IAA production on Luria bertani broth by *Bacillus* sp. ranging from 12 to 25 µg/ml and 15 to 95 µg/ml, respectively.

Growth on N-free medium: All isolates were able to grow on N-free medium. Biological nitrogen fixation is considered as one of the major mechanisms by which plants get benefited from PGPR. Gupta *et al.* (2015) also reported ability of *Bacillus* sp. to grow on N-free medium.

HCN production: All the isolates except SB_8 were able to produce HCN and showed colour change on the edge of filter paper. Microbial production of HCN has been suggested as an important antifungal feature to control root fungi pathogen (Junaid *et al*., 2013). The host plants are generally not harmfully affected by inoculation with HCN producing bacteria and host specific rhizobacteria can operate as biological control agents (Saharan and Nehra, 2011).

Siderophore production: All the selected bacterial isolates were able to grow on CAS medium, showing their ability to produce siderophore with a value ranging from 35.37 to 91.41 per cent. The maximum siderophore production efficiency on solid CAS media (91.41%) was noted for SB_{11} isolate followed by SB_5 isolate. The potential to produce siderophores by microorganisms in improving iron availability to plants and sequestering it from pathogens has been reported by many workers (Wani *et al*., 2008; Bharucha *et al*., 2013; Ahemad and Kibret, 2014). Thus, it is considered as a potential mechanism involved in biocontrol activity. Our findings are in agreement with Kaushal and Kaushal (2013) who also have reported siderophore production in range of 19.40 to 51.36 per cent by *Bacillus* sp.

Antifungal activity: All four isolates (SB_5, SB_{11}, SB_8 and SB_{10}) showed inhibition against *Rhizoctonia solani* and *Sclerotinia sclerotiorum*. Only three isolates (SB_5, SB_{11} and SB_8) showed inhibition against *Pythium* sp. However, bacterial isolate SB_{11} showed maximum inhibition against all the test fungi i.e. *Rhizoctonia solani* (37.11%), *Sclerotinia sclerotiorum* (41.11%) and *Pythium* sp. (71.11%) followed by isolate SB_5 (Table 3.). The formation of zone may be due to secretion of antifungal substance that might have diffused in the medium and inhibited the fungal growth. Our results are in conformation with those of Chauhan *et al* (2014) who have reported per cent inhibition against *Rhizoctonia solani* in the range of 69.77-91.58 by *Bacillus* strain. Further, results are also in corrobation

with those of Mehta *et al.* (2014) and Chen *et al.* (2014) who have reported antifungal activities of *Bacillus methylotrophicus* CKAM against *Pythium aphanidermatum* (62.5%) and *Bacillus subtilis* EDR4 (42.9%) against *Sclerotinia sclerotiorum*, respectively.

Growth under different temperature, pH and salinity conditions: The isolates were grown at different temperature in the range of 25-45°C. The rate of growth increased with the increase in temperature from 25°C to 35°C, however, no growth was observed at 45°C [Fig 2. (A)]. On the basis of maximum O.D. at 540 nm, 35°C was found to be the optimum temperature for growth. The isolates were able to grow over a wide range of pH. All the selected isolates grew well from pH 5-9 but exhibited maximum growth at neutral pH 7 [Fig 2. (B)]. Kaushal *et al*. (2011) also reported the growth of *Bacillus* sp. in the temperature range of 25 to 40°C and pH 5-8.

Further, the isolates were able to grow over a wide range of salt concentration. The selected isolates were able to grow at 2-6 per cent salt concentration and none was in a position to grow at 8 and 10 per cent salinity, however, the rate of growth decreased with increase in salinity beyond 2 per cent concentration [Fig 2. (C)]. Thus, concentration of 2 per cent was considered optimum for the growth. Our results are in agreement with those of Mohanapriya *et al.* (2016) who also reported 2 per cent NaCl concentration to be optimum for the growth of *Bacillus subtilis* under *in vitro* conditions.

Conclusion

The screening of indigenous PGPR with multiple plant growth promoting traits for cauliflower in low hills is a pioneer work. The selected isolate SB_{11} can be used as biofertilizer and bioprotectant agent as it has the potential to supplement the chemical fertilizers and pesticides.

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