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In-vitro Screening of *B. cepacia; C. freundii* and *S. marcescens* for Antagonistic Efficacy

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Since rhizobacterial strains play vital role in plant growth, objective of this research was to isolate antagonistic rhizobacterial strains namely, *B. cepacia, C. freundii and S. marcescens* from mustard plant rhizospheric soil sample based on morphological, biochemical and molecular identification. The16S rRNA gene sequences of rhizobacterial isolates *Burkholderia cepacia, Citrobacter freundii and Serratia marcescens* were submitted to GenBank with accession numbers LC169488, LC169489, LC169490 respectively. Some biochemical test were performed like siderophore production test, HCN test, cellulase and lipase test to estimate different mechanisms adopted by our isolates to inhibit fungal and bacterial phytopathogens. The rhizobacterial isolates were investigated for the ability to perform as antagonistic biocontrol control agents to inhibit fungal pathogens namely *Rhizoctonia solani* and *Phytophthora infestans* that causes stem canker, black scurf in potato and late blight or potato blight disease respectively. The rhizobacterial isolates were also screened for their ability to inhibit *Staphylococcus aureus and Escherichia coli* that are common bacterial pathogens. Antibiotic susceptibility profile of our isolates was also observed. Present study thus reveal that our isolated strains *B.cepacia, C. freundii and S. marcescens* are efficient biocontrol agents and can be used as potential biofertilizers to wean our society off of its widespread use to agricultural chemicals.

Keywords: Rhizobacteria, antifungal activity, antibiotic susceptibility.

Quality and quantity of food are going to be important challenges to overcome in coming future. Exceeding population growth rate requires production of more agricultural products and to inevitably move towards increased production per unit area. Since fertilizer management is considered as one of the main factors of sustainable agriculture, gradual replacement of chemical fertilizers with biological fertilizers is quite inevitable since biofertilizers are environment friendly and cost-effective. Elucidating non-chemical control methods to reduce postharvest decay is becoming increasingly important (Conway *et al*. 2004). Naturally occurring antagonists on host surfaces are a promising component of biological crop protection (De Costa and Erabadupitiya 2005). The rhizosphere, the narrow zone of soil that surrounds and influences the plant roots, is home to a large number of microorganisms and is considered to be one that can have profound effects on the growth,

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nutrition and health of plants in agro-ecosystems (Berendsen *et al*. 2012). Bacteria able to colonize plant root systems and promote plant growth are referred to as plant growth promoting rhizobacteria (PGPR) (Kloepper and Schroth 1978). PGPR can affect plant growth by a wide range of mechanisms such as solubilization of inorganic phosphate, production of phytohormones, siderophore and organic acids, lowering of plant ethylene levels, nitrogen fixation and biocontrol of plant diseases (Rani *et al*. 2012). Along with the promotion of plant growth, PGPR also help in sustainable agricultural development, thereby protecting the environment and thus help in maintaining the ecological balance. Therefore, the use of such beneficial bacteria as biofertilizers and biocontrol agents has attracted increased interest worldwide in attempts to achieve sustainability in agriculture (Datta *et al*. 2011).

The Biocontrol bacterial species generally employ an array of mechanisms such as antibiosis, competition, production of hydrocyanic acid (HCN), siderophore and antifungal compounds to antagonize pathogens (Singh *et al*. 2010). Various rhizobacteria, including for example *Burkholderia cepacia*, *Staphylococcus epidermidis*, and strains of the *Bacillus subtilis* group, stimulate plant growth by the emission of volatile organic compounds such as hydrogen cyanide and ammonia (Bitas *et al*. 2013). Voisard *et al*. (1989) reported that HCN production by the rhizobacteria plays a significant role in the biological control of pathogens by acting as an inducer of plant resistance. It was also reported that HCN indirectly influences plant growth promotion (Kumar *et al*. 2012). Afsharmanesh *et al*. (2010) suggested that fungal growth is mainly inhibited by HCN production and siderophore production. This in turn can indirectly enhance the plant growth by keeping plant healthy and disease free (Glick and Pasternak 2003). Additionally, secretion of siderophore and production of chitinases, glucanases, cellulases, lipases and other lytic enzymes by PGPR can affect plant growth as these are also found to suppress the growth of fungal pathogens (Kloepper *et al*. 1980). Siderophores can be defined as small peptidic molecules containing side chains and functional groups that can provide a high-affinity set of ligands to coordinate ferric ions (Crosa and Walsh 2002). Siderophores are secreted to solubilize iron from their surrounding environments, forming a complex ferric- siderophore that can move by diffusion and be returned to the cell surface (Andrews *et al*. 2003). The potent siderophore, pyoverdin, for example, can inhibit the growth of bacteria and fungi that present less potent siderophores in iron-depleted media in vitro (Kloepper *et al*. 1980a). A pseudobactin siderophore produced by *P. putida* B10 strain was also able to suppress *Fusarium oxysporum* in soil deficient in iron; this suppression was lost when the soil was replenished with iron, a condition that represses the production of iron chelators by microorganisms (Kloepper *et al*. 1980b). *Burkholderia cepacia* has shown antagonistic activity against a broad range of plant pathogens (Cartwright and Benson 1995). Several studies suggested that pyrrolnitrin production by *B. cepacia* as well as other *Pseudomonas* spp, is closely associated with biocontrol of plant diseases (Hwang *et al*. 2002). *Serratia* species, usually found in diverse natural environments, have been found to reduce disease severity of various foliar and soilborne diseases**.** Among *Serratia* species, *S. marcescens* was reported as an important bacterium that has the ability to induce systemic resistance to various pathogens and to exhibit antifungal activity against several soil-borne fungi like *Rizoctonia solani* and *Phytophthora infestans* that causes stem canker, black scurf in potato and late blight or potato blight disease respectively.

The disease protection measures of medicinal plants are still restricted to the application of various chemical fungicides which strictly do not fit with the basic theory of usefulness of herbal drugs. Also, the residual effects of different chemicals eventually contaminate the purity of such plant drugs and are also of serious concern from environmental point of view (Sharma *et al*. 2004). Many researchers have reported that effective colonization by PGPR contributed to the successful suppression of plant pathogens and thus these can be considered as potential use of biocontrol agents as replacements for agrochemicals (Guo *et al*. 2007). In recent years, certain plant associated rhizobacteria belonging to various genera like *Pseudomonas, Burkholderia* and *Bacillus* have drawn attention because of their potential to suppress the soil borne pathogens causing diseases in different crops (Cazorla *et al*. 2007). Due to excessive use of various agrochemicals including

various pesticides of different chemical families in crop production, these beneficial microorganisms and their physiological activities important to soil fertility are adversely affected (Srinivas *et al*. 2008). Bacteria strains isolated from soils that have been contaminated with various biochemicals, including pesticides, are increasingly being used for bioremediation (reclaiming the soil by inoculating it with organisms able to degrade certain compounds which are detrimental to other life forms). In other words, the bacteria are using specific pesticides to meet their energy needs, i.e., using them as food (Hernández *et al*. 2008). To overcome the deleterious effects of pesticides on plants and of soil fumigants on soil fertility, the treatment of seeds with PGPR could be used as a bio-inoculant which displays a wide range of tolerance to pesticides and exhibit PGP activities under pesticide-stress (Ahemad and Khan 2010).

METHODOLOGY

Isolation of antagonistic rhizobacterial strains

When Indian mustard plant was found, a 6"X 2.5"X 5" soil sample was collected, the soil was serially diluted and appropriate dilutions (10^{-3}, 10^{-5}, 10^{-7}) were spread plated on nutrient agar medium. The plates were incubated at 38°C and developed colonies were identified on the basis of cultural, morphological characteristics as described in the Bergey's Manual of Determinative Bacteriology (Holt *et al.* 1994). Selective colonies were taken and streaked on selective media for individual strains and kept in incubator at 38°C for 24 h to obtain colonies.

Identification of specific bacterial species

The bacterial colonies on incubated plates were identified on the basis of cultural, morphological and biochemical characteristics as described in the Bergey's Manual of Determinative Bacteriology (Holt *et al.* 1994).

Cultural and Morphological Characteristics

The isolates were identified on the basis of different colony characteristics like diameter, consistency, colour, texture, elevation, margin etc. The organism was subjected to Gram's staining (Holt *et al.* 1994) for observing the morphological characteristics such as the shape and arrangement (clusters or chains) of cells, and gram-reaction.

Screening of Rhizobacteria for plant growth promoting traits

Siderophore production

For siderophore production, 1µl of overnight raised rhizobacterial culture in LB broth were spotted on Chrome Azurol S (CAS) agar plates and incubated at 37°C for 48 h. Plates were observed for the appearance of orange halo around the bacterial colony (Ali *et al*. 2013).

HCN Production

For qualitative estimation of HCN, isolate was streaked on nutrient agar plate supplemented with 4% glycine. A whatman filter paper soaked in a solution of 2% Na_2CO_3 and 0.5% picric acid was placed between base and lid of petriplate and incubated at 37°C in inverted position for 48 h and observed for color change from yellow to orange brown (Ali *et al.* 2013).

Chitinase test

For the Chitinase test spot inoculation of isolated strain was made on chitin agar plate amended with 2% phenol red and incubated for 48 h at 37°C. Presence of clear zone around the culture indicates the chitinase activity (Holt *et al.* 1994).

Protease production

The bacterial species were spot inoculated on nutrient agar containing casein and gelatin. The zone of hydrolysis was observed for the protease production (Pandey *et al.* 2013).

Lipase test

Lipolytic activity was carried out on trimethoprim plate and observation of halogen was considered as positive for lipase production.

Antibiotic sensitivity test

Isolate bacterial strains were tested for its resistance against standard antibiotics namely gentamicin (30 µg), ampicillin (10 µg), erythomycin (10 µg), kanamycin (5 µg), tetracyclin (10 µg), vancomycin (25 µg), and chloramphenicol (10 µg) by the antibiotic sensitivity assay. Briefly, the bacterial cultures were swabbed onto NA media plates. The standard antibiotic disc (6 mm) was placed over the media surface and the plates were incubated at 37 °C for 24 h. The experiment was done in triplicate. The results were interpreted on the basis of the diameter of inhibition zone using the zone size

Antagonistic test

Biocontrol ability of the isolate was evaluated by fungal mycelial inhibition by well diffusion method against the bacterial (*E.coli* and *S.aureus*) and fungal (*R.solani* and *P.infestans*) pathogens. After the solidification of potato dextrose agar (PDA) 100µl of fungal spore suspension in 0.85% sterile saline was spread on the solidified plate. Well diameter of 6mm was made by metallic borer and filled with overnight grown culture of isolate (1 × 108cfu) and kept for incubation at 28 °C for seven days.

Bacterial DNA Isolation

DNA was isolated using Phenol Chloroform Method. From the overnight grown liquid culture 1.5 ml of culture was taken in micro centrifuge tube and centrifuged at 8000 rpm for 5 mins and pellet the bacterial cells. Pellets were suspended in 900µl of the TE buffer. 1/10 volume of 10% SDS solution (900µl solution then add 100µl of solution into it) was then added and tubes were kept at 50-60ºC under water bath for approximately 2 hours. Mix 600µl of Phenol**:**Chloroform**:**Isoamyl alcohol(25:24:1) to it and shake gently by repeatedly inverting the tube. White precipitate appeared after mixing, this mixture was added to the tube and solution became creamy orange (white due to protein precipitation and orange due to phenol). Kept for 5 min and then centrifuged at 10000 rpm for 10 min. Upon centrifugation 3 layers were appeared. Upper transparent aqueous layer was collected in fresh new eppendorf tube and discard the lower layer. Kept the eppendorf tube in refrigerator to cool it for 10-15 min. Double volume of chilled propanol was added to the aqueous DNA solution drop by drop from its wall. After adding DNA precipitate out from the solution and which was kept again on 0ºC for 15 min. After precipitation, tubes were centrifuged at 10000rpm for 10 min and supernatant was discarded. DNA pellet was dried in air and dissolved in 50µl TE buffer and then run on gel with 1kb ladder (Ali *et al*. 2013).

16S rRNA amplification

For molecular characterization, bacterial genomic DNA was quantified at 260/280nm. The 16SrRNA gene was amplified by PCR using forward 5'-CCGAATTCGTCGACAACAG AGTTTGATCCTGGCTCAG-3' and reverse primer 5'-CCCGGGATCCAAGCTTACG GCTACCTTGTTACGACTT-3 primers under standard conditions (initial denaturation 94°C for 3 min, 35 cycles of denaturation at 94°C for 30 sec, annealing at 60°C for 30s, extension at 72°C for 60s, and final extension at 72°C for 7 min). The PCR product was found the size of 1424, 1487 and 1306 bp for *Burkholderiacepacia, Citrobacter feurendii* and *Serratia marcescens* respectively and was purified and sequenced (Applied Biosystems, New Delhi). The sequence of 16S rRNA genes of all three isolates were obtained and compared with the existing database of 16S rRNA gene and submitted to Gene Bank of NCBI.

RESULTS AND DISCUSSION

Isolation of rhizobacterial strains from soil samples

In the present study three rhizobacterial strains (i.e. *Burkholderia cepacia, Citrobacter freundii*, *Serratia marcescens*) were isolated by spread plate technique on selective media from 10^{-3} dilution of rhizospheric soil of mustard plant**.** Mustard plant rhizospheric soil was also used as sample for isolation of PGPR by (Lugtenberg and Kamilova 2009). Hiltner (1904) discovered that the

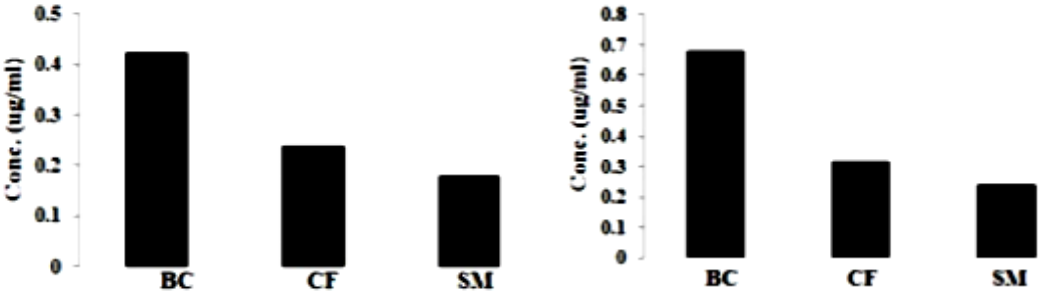

Fig. 1. Assay of (a) Indole acetic acid and (b) Gibberellic acid production for several rhizobia strains

rhizospheric soil is abundant in bacterial population as compared to the rest bulk soil.

Identification of rhizobacterial strains from soil samples

Colonies which were identified morphologically as *Burkholderia cepacia, Citrobacter freundii* and *Serratia marcescens* respectively were streaked again on selective media to obtain pure culture and then performed gram staining.The morphological characteristics were recorded (Table 1). Previous study reported that PGPR isolated from Indian mustard rhizospheric soil, possessed ACC deaminase enzyme and promoted root proliferation significantly (Belimov *et al.* 2001). Dubey and Maheshwari (2010) isolated genera of *Azatobacter, Bacillus, Burkholderia* from soil which supply nitrogen to legume plants.

Screening of Rhizobacteria for plant growth promoting traits

Screening of bacterial isolates for their ability to promote plant growth. Rhizobacteria exhibit several plant growth promoting characteristics like phosphate solubilization, Nitrogen fixation, production of siderophore, IAA, Ninhydrin and ACC deaminase (Fig.1,2,3) that was supported by study of Bhattacharyya and Jha, 2012 on *B. cepacia, S. marcescens* isolates. The bacteria included in PGPR category are distributed across different taxa comprising of *Firmicutes, Acinetobacter, Cyanobacteria, Bacteriodes* and *Proteobacteria* (Tilak *et al.* 2005). The proteobacteria falls in the genera *Bacillus, Azatobacter, Arthrobacter, Klebsiella, Azospirillum, Enterobacter, Serratia, Burkholderia, Pseudomonas, Alcaligenes,* (Somasegara and Hoben 1994).

Indole acetic acid production test

Indole acetic acid test was conducted (Fig. 1a) that showed 4.22 µg/ml concentration of *Burkholderia cepacia,* while lesser concentration values of 2.42 and 1.86 µg/ml (Fig.1a) were recorded for *Citrobacter freundii*, *Serratia marcescens* respectively. Results of Ali et al., 2013 showed 41-47 µg/ml concentration of IAA with species of *Pseudomonas.* In another research *V. paradoxus* 5C-2 was seen positive for indoles production *in vitro* (Jiang *et al.* 2012).

IAA production varies with bacterial isolates and concentration of tryptophan, although intrinsic IAA production ability of rhizospheric microorganism is regulated by the availability of precursors and uptake of rhizospheric IAA by plant (Arshad and Frankenberger 1993). (Belimov *et al.* 2009) reported that *V. paradoxus* 5C-2 strain elevated xylem abscisic acid concentrations in pea plant under drought conditions**.** Sharma et al. (2013) concluded in his study that all rhizobacterial isolates produced IAA, *Bacillus* produced (11.2-22.8 µg/ml) and *Pseudomona*s isolates (4.4-21.6 µg/ml)**.**

Gibberellic acid production test

All three isolated bacterial strains were tested for gibberellic acid production where *Burkholderia cepacia* showed concentration i.e. 0.68µg/ml, while *Serratia marcescens* and *Citrobacter freundii* showed 0.24 and 0.32 µg/ml (Fig.1b) concentration respectively. From the graph (Fig.1b) it can be observed that *Burkholderia cepacia* showed more concentration compare to other two isolates. Our results support the concept that the growth promotion in plants induced by *Azospirillum* infection may occur by a combination

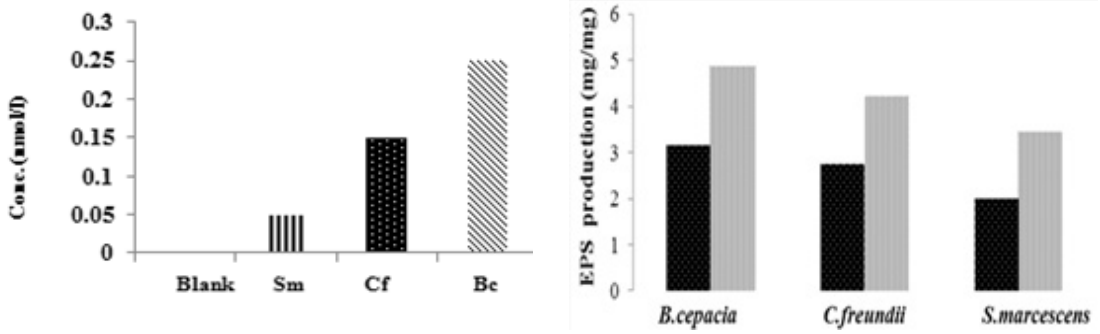

Fig. 2. Comparison of ACC Deaminase conc. for (a) Ninhydrin assay and (b) Exoploysaccharide production test of isolates

of both gibberellin production and gibberellin glucoside or glucosyl ester de-conjugation by the bacterium (Piccoli *et al*. 1997). Tien *et al*. (1979) detected gibberellins-like substances in supernatants from *A. brasilense* cultures, at an estimated concentration of 0.05µg/ml GA3 equivalent. A quantitative estimation was done by the dwarf rice cv. Tan-ginbozu microdrop bioassay, showing that 20-40 pg/ml were produced (Bottini *et al*. 1989). The same gibberellins were found in similar amounts in cultures of *A. brasilense* (Janzen *et al*. 1992). The GAs pools in roots and nodules were of similar size, indicating that *Rhizobium* does not make a major contribution to GAs content in infected tissues (Atzorn *et al.* 1988).

ACC determination by colorimetric Ninhydrin assay

Ethylene glycol is used as solvent stabilizing ninhydrin reagent and for colour development. Ascorbic acid addition into ninhydrin reagent prevents oxidation of hydrindantin. Values for absorbance of ACC solutions were found in the rage 0.015 to 0.3 mmol/1 at 570 nm after the reaction was highly correlated with ACC

Table 1. Cultural, morphological and biochemical characteristics of *B. cepacia, C. freundii* and *S. marcescens* isolate

Cultural characteristics	Colour of colony	opaque light yellow colonies	Light gray translucent to opaque colonies with glossy surface	Pink to magenta opaque iridescent colonies
	Shape of colony	Staright rods with rounded ends	Straight rods occurring singly or in pairs	Straight rods with rounded ends
	Elevation	convex	convex	convex
Morphological characteristics	Gram's reaction	Gram negative	Gram negative	Gram negative
	Spore formation	Non-spore forming	Non-spore forming	Non-spore forming
	Arrangement of cells	Cells occur singly, rarely in pairs	Cells form clusters	Cells form clusters
Biochemical	Indole test	Negative	Negative	Negative
	Methyl red test	Negative	Positive	Positive
	VP test	Negative	Negative	Positive
	Catalase test	Positive	Positive	Positive
	Citrate utilization test	Negative	Positive	Positive
	Nitrate reduction test	Positive	Positive	Positive
	Hydrogen sulphide test	Positive	Positive	Negative
	Oxidase test	Negative	Negative	Negative
	Urease test	Positive	Positive	Negative
	Starch hydrolysis	Negative	Negative	Negative
	ONPG	Positive	Positive	Positive
	PPA	Positive	Positive	Positive
Carbohydrate Fermentation	D-Glucose	Positive	Positive	Positive
	Sucrose	Positive	Positive	Positive
	Maltose	Positive	Positive	Positive
	Lactose	Negative	Positive	Negative
	Xylose	Positive	Positive	Negative
	D-Mannitol	Negative	Positive	Positive
	Raffinose	Positive	Positive	Positive
	L-Rhamnose	Positive	Positive	Negative
	L-Arabinose	Positive	Positive	Negative
	Esculin	Negative	Positive	Positive
	L-Arginine	Negative	Positive	Negative
	L-Ornithine	Positive	Positive	Positive
	L-Lysine	Negative	Negative	Positive

concentrations ($R^2 = 0.999$) and resulted in linear calibration curves by standard ninhydrin assay. Conc. of 0.05, 0.15 and 0.25 mmol/l was recorded for *S. marcescens, C. freundii and B. cepacia* respectively. The absorbance value of each ACC working concentration at wavelength of 570 nm in the standard assay was related to previous results of *B. cepacia* (Li *et al.*, 2011) (Fig.2a). Research of Li *et al*. 2011 adds more functionality to the ninhydrin assay, although it does not accurately determine

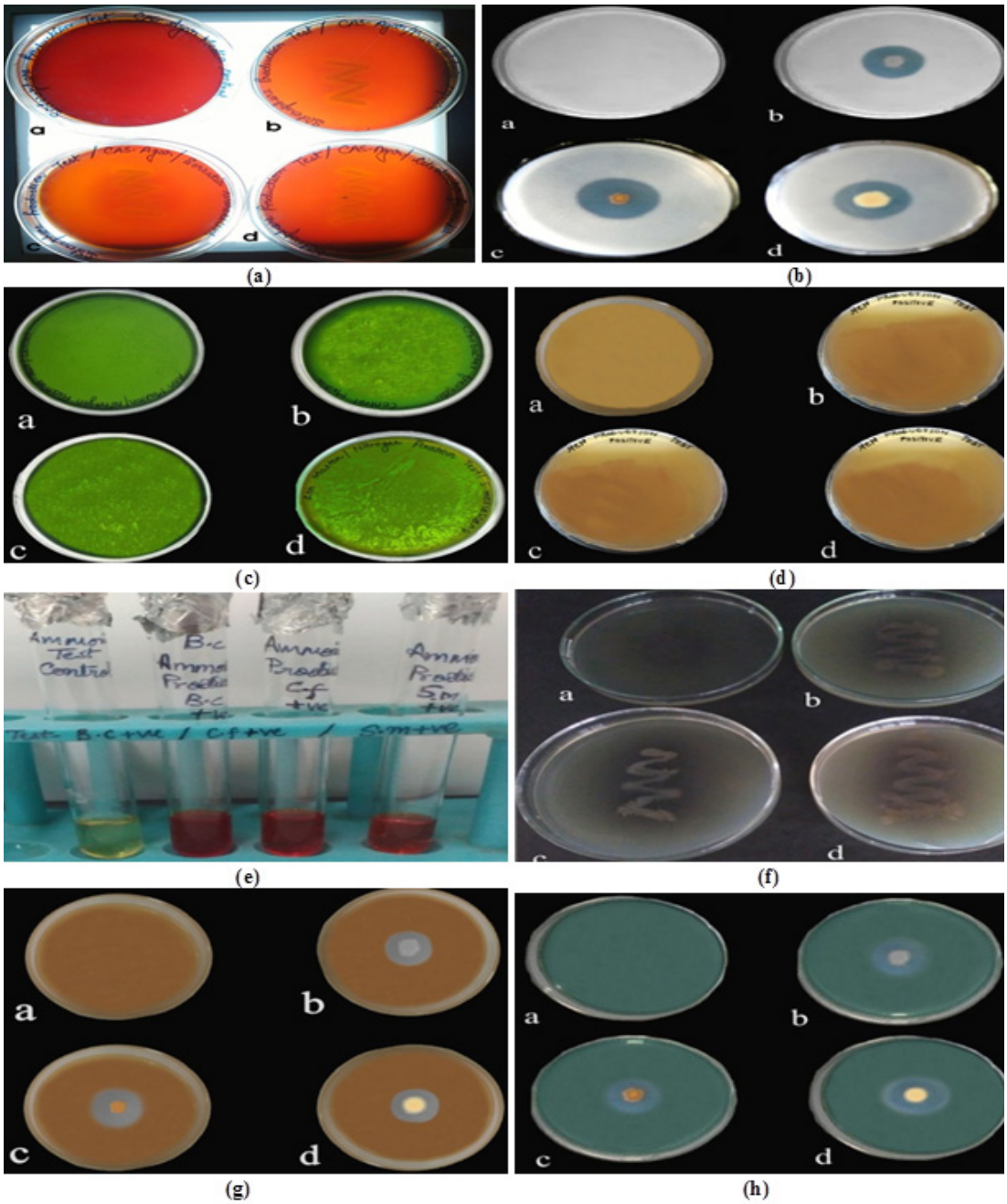

Fig. 3. Plant growth promoting (a) Siderophore production (b) Phosphate solubilisation (c) Nitrogen fixation (d) HCN production (e) Ammonia production (f) Cellulase production (g) Chitinase production and (h) Lipase production test for (a) Control (b) *B. cepacia* (c) *S. marcescens* (d) *C. freundii*

PGPR ACC deaminase activity, but provides a screening method with a relatively short turnaround time. Determination of ACC concentrations in rhizobacterial isolate after certain incubation period in the DF-ACC medium, by both assays were statistically identical at the 0.05 confidence level.

Exopolysaccharide production test

Isolates screened for EPS production under both no stressed conditions as well as under minimum water potential ("0.30 MPa). The strain *B.cepacia* produced maximum amount of EPS (3.18 ± 0.02 mg/mg protein) under non-stressed condition, while and *S.marcescens* produced lesser amount of EPS (2.76 ± 0.04 and 2.01± 0.3 mg/mg protein respectively). Under drought stress, *B. cepacia* was best isolate since produce (4.893±0.06 mg/mg protein) of EPS followed by *C.feurendii* (4.23 ± 0.03 mg/mg protein) and *S.marcescens* (3.46±0.05 mg/mg protein) (Fig.2b).

Ali *et al*. (2014) conducted study on *Pseudomonas* isolates and found maximum 3.22 mg/mg protein EPS production (from Rdgp10 strain) while another strain (BriP15) produced 2.18 mg/mg protein EPS. Hartel and Alexander (1986) observed a significant correlation between the amount of EPS produced by cowpea *Bradyrhizobium* strains and desiccation tolerance. Glick *et al*. (1998) established that PGPB that have ACC deaminase activity help plants to withstand stress (biotic or abiotic) by reducing the level of the stress hormone ethylene through the activity of enzyme ACC deaminase, which hydrolyzes ACC into á-ketobutyrate and ammonia instead of ethylene.

Antibiotic and Bio control efficiency of selected rhizobacterial isolates

Different antibiotics discs were used to check the efficiency of selected isolates (Fig. 3) and found resistant against all except two for *C. freundii*. Zone were measured against selected antibiotics and recorded as gentamicin (12, 12, 7mm), ampicillin (9, 13, 11mm), erythomycin (10, 10, 3mm), kanamycin (14, 9, 11mm), tetracyclin (8, 10, 0mm), vancomycin (7, 18, 13 mm), and chloramphenicol (11, 11, 0) for *B. cepacia*, *S. marcascens* and *C. freundii* respectively.

Screening of our rhizobacterial isolates was performed to estimate their efficacy to inhibit bacterial strains viz. *E.coli* (26, 25, 27mm) and *S.aureus* (27, 26, 13mm) while for fungal pathogen i.e. *R.solani* (17, 15, 14mm) and *P.infestans* (25, 14, 9mm) against *B. cepacia*, *S. marcascens* and *C. freundii* respectively (Fig.4-6).

Isolation and sequence characterization of Rhizobacterial 16S rRNA gene

Rhizobacterial genomic DNA was extracted and run on agarose gel with 1kb ladder. All three bacterial cultures showed genomic DNA size greater than 10kb size (Fig.7). Spectrophotometric analysis of samples was done to quantify rhizobacterial genomic DNA concentration and found 1.53 µg/ml for *Burkholderia cepacia***;** 1.38 µg/ml for *Serratia marcescens*; 1.35 µg/ml for *Citrobacter freundii* . DNA from the above mentioned 03 Rhizobia strains were PCR amplified and found the size of 1424, 1487 and 1306 bp for *Burkholderia cepacia*; *Citrobacter freundii*; *Serratia marcescens* respectively (Fig.7). All three amplified products were then sequenced for 16S rRNA at Applied Biosystems using the oligonucleotide primers i.e. 16S forward primer: 5'-CCGAATTCGTCGACAA CAGAGTTTGATCCTGGCTCAG-3' and 16S reverse primer: 5'-CCCGGGATCCAAGCTTACG GCTACCTTGTTACGACTT-3. After sequencing of all three bacterial isolates, sequences were submitted to DNA Database Bank of Japan (DDBJ), a collaborator of NCBI and obtained **accession number i.e. LC169488 , LC169489 and LC169490** for *Burkholderia cepacia*; *Serratia marcescens*; *Citrobacter freundii* respectively for all three isolates. Analysis of these three sequences by comparison with the sequences of GenBank database indicated that 26 strains were *Rhizobium leguminasarum* with > 99% identities with the type strain (i.e. 1398/1402 bp).

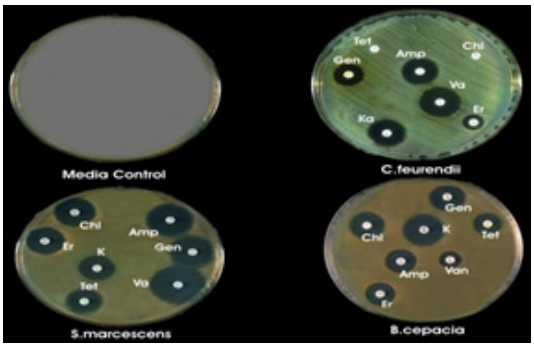

Fig. 4. Antibiotic sensitivity test (a) Control (b) *C. freundii* (c) *S. marcescens* (d) *B. cepacia*

PGPR that exhibit ACC deaminase enzymatic activity support plants to survive under stress (biotic or abiotic) by lowering stress ethylene concentration by hydrolyzing ACC (immediate precursor of ethylene) into α-ketobutyrate and ammonia (Arshad *et al*. 2007). Variations in ACC deaminase enzymatic activity potential of the strains were noted in drought stress. In a previous research secretion of enzyme ACC deaminase by *Pseudomonas* strain SorgP4 was confirmed by *acdS* gene amplification using the same primer as reported earlier obtained a partial *acdS* gene from *Pseudomonas* isolate SorgP4 (Farajzadeh *et al*. 2010). *acdS* gene that codes for enzyme ACC deaminase has been earlier isolated from some rhizobacteria from rhizosphere of plant with or without abiotic stresses (Jha *et al*. 2009). Rhizobacteria capable of producing ACC deaminase are also known to posses potential to elevate growth of plants especially under stressed conditions such as flooding, heavy metals, high salinity and drought, thus *acdS* gene is a useful candidate gene for developing bio-inoculants to manage abiotic stress in plants. (Ali *et al*. 2013)

Bacterial 16S rDNA sequences are generally considered as highly conserved (Woese 1987). Evolutionary or taxonomic relationships among bacteria and rhizobacteria are usually estimated through 16S rDNA sequence comparisons. According to the 16S rRNA sequences of our three rhizobacteria strains which exhibit ACC deaminase enzymatic activity, they are identified to be *Burkholderia cepacia*; *Serratia marcescens*; *Citrobacter freundii*.

Similar sequences generally code for similar structure; similar structure are considered to be associated in similar function. Therefore BLAST2 was performed to evaluate the association of ribosomal genes and growth promotion (Table 2) among these three species. In contrast to our notion of similar sequence to similar function, the sequences were found relatively divergent. Therefore, to search for more similar homologs of individual sequences, BLASTN was performed with an output value of 10.

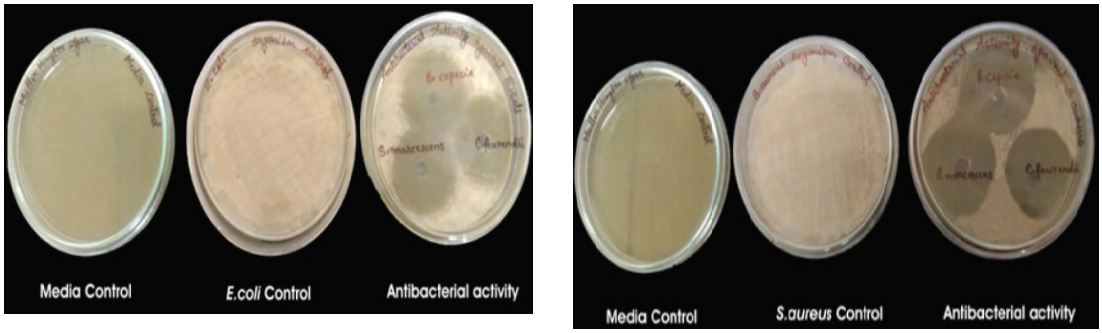

Fig. 5. Antibacterial efficiency of rhizobacterial isolates against (a) *E. coli* and (b) *S.aureus*

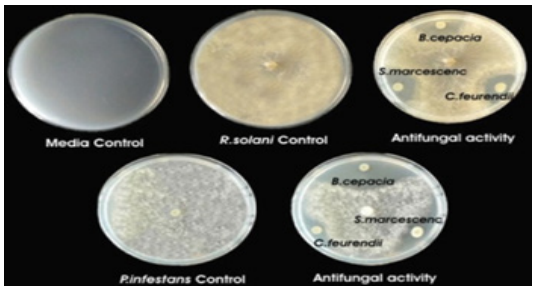

Fig. 6. Antifungal efficacy of rhizobacterial isolates against *R. solani* and *P. infestans*

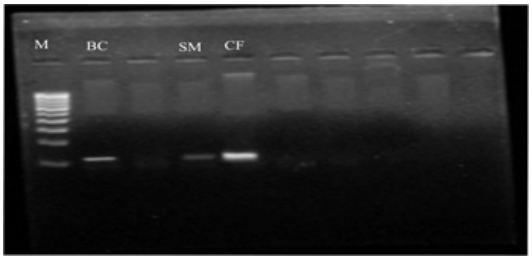

Fig. 7. PCR amplification for 16s rRNA gene of isolates (Lane 1: 1kb Ladder, Lane 2: *B. cepacia*, Lane 4: *S. marcescens*, Lane 5: *C. freundii)*

Table 2. Homology based study for selected isolates

Query value (%) Identity (%)	*B. cepacia*	*C. freundii*	*S. marcescens*
Burkholderia cepacia	100	81	77
	100	79	78
Citrobacter freundii	77	100	84
	79	100	91
Serratia marcescens	85	96	100
	78	91	100

CONCLUSION

Three rhizobacterial strains were isolated from soil sample of mustard plant rhizosphere. These isolates were subjected morphological and biochemical characterization followed by screened for plant growth promoting efficiency using several biochemical tests like phosphate solubilization, siderophore production, ammonia production, nitrogen fixation along with confirmatory tests like ninhydrin production, gibberellic acid production and indole acetic acid production tests. Our isolates were also screened for their ability to perform biocontrol activity using some antagonistic tests like HCN, chitinase, cellulase and lipase production tests. Genomic DNA of all three cultures were isolated and amplified through PCR with suitable primers. PCR products were then sequenced for 16s RNA gene and results were successfully submitted to DDBJ/NCBI under allotted accession numbers and bioinformatics based phylogenetic analysis was done to estimated 16s RNA sequence similarities of our isolates with those in the database and their evolutionary relationship. Based on molecular characterization selected three rhizobacterial strains were identified as *Burkholderia cepacia, Serratia marcescens and Citrobacter freundii* and these isolates were found capable for plant growth promoting activity and possessed significant antagonistic efficacy. Antibiotic susceptibility profile of *B. cepacia, C. freundii* and *S. marcescens* against various antibiotics namely gentamicin, ampicillin, erythomycin, kanamycin, tetracyclin, vancomycin and chloramphenicol was recorded. The ultimate achievement of these inoculates will be to be use as bio fertilizers that are cost effective and do not hinder soil fertility unlike chemical fertilizers. Further research and understanding of mechanism of PGPR mediated phyto-stimulation would pave the way to find out more competent rhizobacterial strains which may work under diverse agro-ecological conditions.

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