Agric. Sci. Digest., 37(3) 2017: 226-231
Print ISSN:0253-150X / Online ISSN:0976-0547

AGRICULTURAL RESEARCH COMMUNICATION CENTRE
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Plant growth promoting traits shown by bacteria *Brevibacterium frigrotolerans* SMA23 Isolated from *Aloe vera* rhizosphere

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Received: 17-03-2017 Accepted: 16-08-2017 DOI:10.18805/asd.v37i03.9223

ABSTRACT

Brevibacterium frigrotolerans SMA23 is a gram positive rod shaped bacteria isolated from *Aloe vera* rhizosphere. 16S rRNA sequencing confirmed the identity of the bacterium as *Brevibacterium frigrotolerans*. It was capable of growing at temperatures ranging from 10°C to 35°C, but maximum growth was observed at 30°C. It is endowed with multiple plant growth promotion attributes such as phosphate solubilization, IAA production and siderophore production, which are expressed differentially at sub-optimal temperatures. At 10°C it was found to exhibit all the plant growth promotion attributes. This bacterial isolate was able to positively influence and promote the growth and nutrient uptake parameters of wheat (HD 2967) under glasshouse conditions.

Key words: *Aloe vera,* IAA, PGPR, Phosphate, Siderophore.

INTRODUCTION

Microorganisms are important in agriculture to reduce the need for chemical fertilizers as much as possible and in order to promote the circulation of plant nutrients (Kumar *et al*., 2015). Soil microorganisms play very important role for the biogeochemical cycles of both inorganic and organic nutrients in the soil and also in the maintenance of soil quality and health (George *et al*., 2005; Diby and Sarma, 2006; Doni *et al*., 2014). Plant Growth Promoting Rhizobacteria (PGPR) is native soil bacteria that colonize the rhizosphere or plant roots resulting in stimulation of plant growth either directly and/or indirectly. PGPR can either directly or indirectly facilitate the growth of the plants (Glick, 1995). Indirect stimulation of plant growth includes mechanisms by which the bacteria prevent phytopathogens from inhibiting plant growth and development (Singh *et al.,* 2014; Kaur *et al*., 2015) while direct stimulation may include providing plants with fixed nitrogen, phytohormones, iron that has been sequestered by bacterial siderophores, and soluble phosphate (Lucy *et al*., 2004).

Aloe vera is a unique medicinal plant having large applications in medical and cosmetic industries. *Aloe vera* gel includes more than 75 biologically active substances such as vitamins, anthraquinones, minerals, enzymes, sugars, lignin, saponins, sterols, amino acids and salicylic acid. The gel present in the leaf pulp of *Aloe vera* is used for curative purposes and yellow latex present in bundle sheath cell is used for cathartic purposes. Thus, demand for this miraculous plant is increasing in both domestic and international markets (Rodríguez and Fraga, 1995). Hence, the quest to find a mechanism to increase its production is fundamental. Although much research has been conducted on the effect of PGPR on the crops, very little work has been done with medicinal plants. This study focuses on studying the properties possessed by bacteria isolated from *Aloe vera* rhizosphere.

Most of the previous studies have reported *Pseudomonads* (Meyer *et al*., 2004) as cold-adapted bacteria from the alpine and sub-alpine environments. The objectives of this study are to isolate and screen PGPR strains from rhizospheric soil, to check their growth ability under different temperatures and to assess their potential in growth promotion under *in vitro* conditions.

MATERIALS AND METHODS

Isolation: *Brevibacterium frigrotolerans* SMA23 used in this study was isolated in laboratory from *Aloe vera* rhizospheric soil using serial dilution agar plating method. In order to screen the isolate for psychotolerance, the growth pattern of isolate was observed at different temperatures (10°C-40°C). The overnight-grown broth cultures adjusted to optical density of 0.5 at 600 nm were inoculated into trypticase soy broth (TSB) and incubated at different temperatures (10°C-40°C) on a rotary shaker at 120 rpm for 48 h. Optical density of the cultures incubated at different temperatures was recorded. Morphological, physiological and biochemical characteristics of the isolate were carried out as per standard methodologies (Collins and Lyne, 1980).

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The sequences were analyzed against the NCBI database (Kumar *et al*., 2012).

Phosphate Solubilization and IAA production: Phosphate Solubilization was checked using tricalcium phosphate (TCP) as insoluble phosphate. Isolates obtained from *Aloe vera* rhizospheric soil samples were streaked on pikovskaya agar plates. Plates were incubated for 2-3 days at 28±2°C. Production of yellow halos around the colonies following pH drop through the release of organic acids indicated positive result for phosphate solubilization (Karpagam and Nagalakshmi, 2014). The concentration of the soluble phosphate in the supernatant was estimated every third day by the stannous chloride ($SnCl_2.2H_2O$) method (Gaur, 1990). Estimation of indole acetic acid (IAA) was done by inoculation of 200 µl of bacterial suspension (3×10^7cells ml^{-1}) in 10 ml Luria Bertani (LB) broth amended with L-tryptophan (100 µg ml^{-1}) and incubating it for a period of 48 h. The IAA content in the culture suspension was estimated by the standard procedure (Gordon and Weber, 1951). Quantitative estimation of tricalcium phosphate (TCP) solubilization and IAA production were carried out at different incubation temperatures *viz.* 10°C to 40°C. All the studies were repeated on independent temperatures to confirm the results.

Siderophore production and HCN production: Siderophores production was ascertained qualitatively on Chrome Azurol S (CAS) agar media. Bacterial cultures to be tested were spotted on media and incubated at 28±2°C for 4 days. Change in CAS reagent from blue to orange or golden yellow shows positive evidence for siderophore production (Kannahi *et al*., 2013). HCN production was using the method given by Lorck, 1948. The change in the color of the filter paper previously dipped in 2% sodium carbonate prepared in 0.05% picric acid, from yellow to dark brown indicates positive result for HCN production

Biochemical and molecular characterization: Isolate SMA23 selected on the basis of plant growth promoting characteristics was monitored for Gram staining, endospore staining, oxidase and catalase activity, indole production, methyl red, Voges-Proskauer test and citrate utilization test (IMViC), gelatin and starch hydrolysis, glycerol, sucrose, glucose, fructose, mannitol, lactose, xylose, galactose, mannose, rhamnose, sorbitol, maltose, and raffinose hydrolysis according to (Holt *et al*., 1994). For molecular characterization, bacterial genomic DNA was isolated (Chen and Kuo, 1993) and the 16S rDNA gene was amplified by PCR Using Forward Primers :27F(5'-AGAGTTTGA TCCTGGCTCAG-3'), 530F(5'-GTGCCAGCM GCCGCGG-3')and1114F (5'GCAACGAGCGCAACCC3') Reverse Primers: 1492R (5'-CCGTCAATTCC TTTRAGTT T-3'),519R(5'-GWATTACCGCGGCKGCTG-3') and 1100 R(5'-GGGTTGCGCTCGTTG-3')1488R(5'GTTACCTTG TTACGACTTCACC-3')under standard conditions (initial denaturation 94°C for 5 min, 30 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 40 sec, extension at 72°C for 90 sec, and final extension at 72°C for 7 min). The PCR product (1,500 bp) was purified and sequenced. The sequence obtained was compared with the existing database of 16S rDNA gene and submitted to GenBank.

Plant growth promotion ability of isolate SMA23: Plant growth promotion ability of Isolate SMA23 was determined under greenhouse conditions using wheat seedlings in non-sterile soil at sub-optimal temperature. Seeds of wheat (var. CSV-15) were surface-sterilized with 0.1% $HgCl_2$ and 70% ethanol and coated with talc based formulation (10^8cells/g) of *Brevibacterium frigrotolerans* SMA23 using 1% carboxymethyl cellulose as adhesive. The seeds were sown in 250ml plastic cups (surface-sterilized) filled with 200g nonsterile soil and placed in temperature controlled growth chamber of 20±2°C for 7 days. At the end of the experimental period the plants were uprooted, washed under running water and root/shoot lengths were measured. The plant biomass was dried at a constant temperature of 65°C and dry weights were recorded.

RESULTS AND DISCUSSION

Isolation and characterization of the bacterial isolate: Microscopic examination revealed that the isolate SMA23 was Gram positive tiny rods in chain, arranged in an irregular fashion, non-endospore former and produced small circular creamish white colonies with glistening surface on nutrient agar. It was able to grow over wide temperature range of 10°C-35°C, but maximum growth was observed at 30°C. It was positive for catalase activity, oxidase test, methyl red test, Vogus-proskauer test, citrate utilization, urease test, gelatin hydrolysis and nitrate reduction test. Negative reaction was observed for indole production test. SMA23 was able to utilize various carbon sources such as glucose, fructose, sucrose, maltose and mannitol as carbon source, but glucose was found to be most preferred carbon source among all. The 16S rRNA gene sequence of the Strain SMA23 was grouped most closely to a cluster containing *Brevibacterium frigoritolerans* CMGS4 and marine bacterium having 98% and 92% of sequence similarity, so the isolate has been confirmed as *Brevibacterium frigoritolerans.* Isolate having 1445 bp has been submitted to NCBI under accession number KC663437. Phylogenetic tree showing its similarity and its distance with closest relative has been shown in Figure 1.

Plant growth promoting attributes: The different plant growth promotion traits of the isolate were determined at different incubation temperatures of 10°C, 20°C, 30°C and 35°C. Zone of phosphorus solubilization around the bacterial colony varied from 10°C-35°C, although maximum amount of phosphorus solubilization was observed at temperature

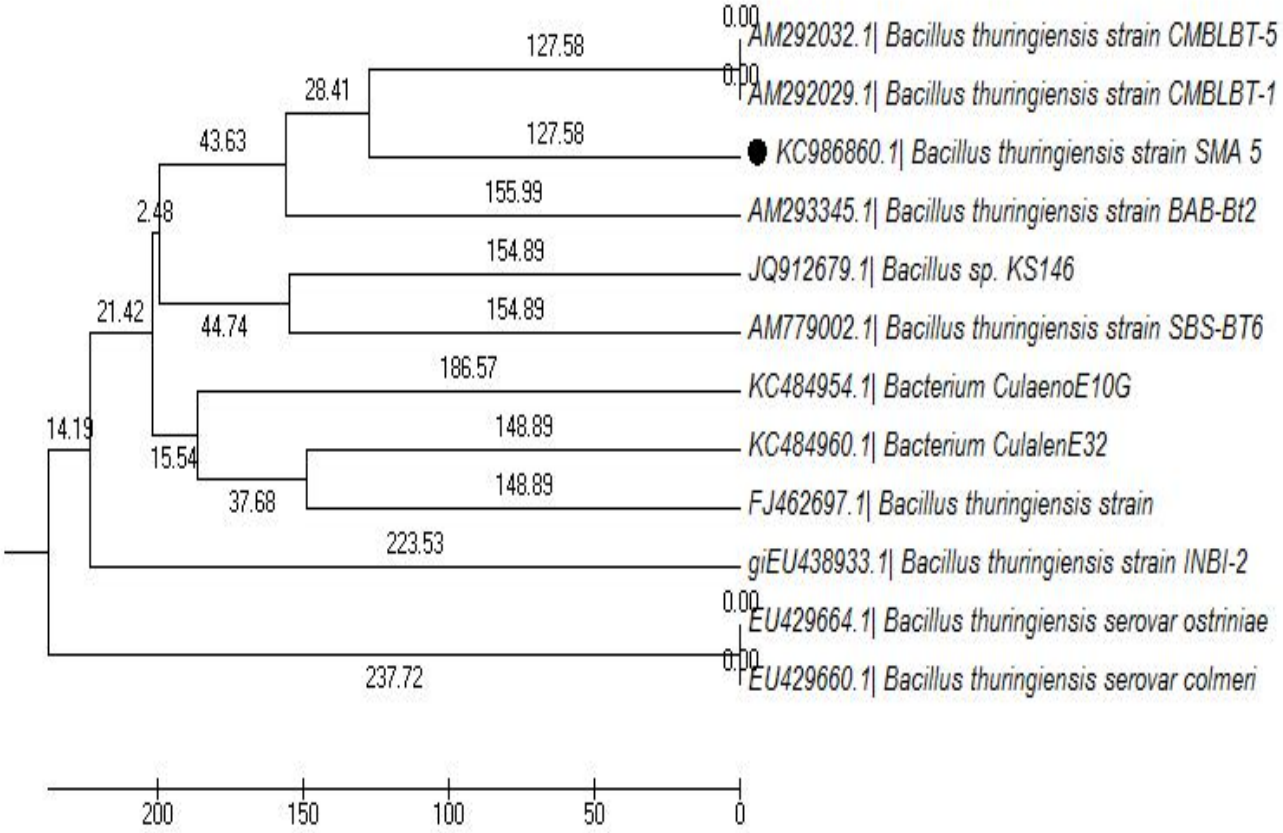

Figure 1: Phylogenetic analysis of isolate SMA5 using 16S- rRNA partial sequence. Genetic relatedness between isolate SMA5 and other *Bacillus* species was inferred from distance matrix using pair-wise distance. Phylogenetic tree was prepared using UPGMA programme.

of 30°C (Table 1) after incubation time of 36h (Figure 2a). Strain SMA23 was able to produce IAA in range of 29.8 µg ml^{-1} to 45.7 µg ml^{-1}. (Figure 2b). SMA23 produced pink color after addition of salowski reagent, which indicates positive result for IAA production. Maximum growth and amount of IAA production was observed at 30°C (Table 1).

Isolate SMA23 was able to produce orange halo around CAS media showing positive result for siderophore production (Figure 2c). Isolate SMA23 was found negative for HCN production. The bacterial strain was able to grow at all the temperature tested, and significant growth was observed at temperature of 28 ± 2°C. The survival of isolate under different temperatures was of significant importance especially in country like India where such a wide variation in temperature was observed across seasons. Although more research work is needed to evaluate their efficiency under temperature stress conditions but it was well documented that the relative effectiveness of plant inoculation was higher under extreme conditions of soil temperature in different experiments.

Plant growth promotion: Germination parameters studied includes the germination rate, germination speed, vigor index, total length and total weight of seedlings. Isolate SMA23 showed positive effect toward seed germination and significantly increased the radicle and plumule length as compared to control. A corresponding increase in the root

Table 1: Plant Growth Promoting Attributes of *Brevibacterium frigrotolernas* strain SMA23

Incubation temperature (°C)	P- solubilization (mg ml^{-1})	IAA production (µg ml^{-1})	Siderophore production (% SU)	HCN production
10	31±0.20	29.8±0.26	15±0.21	-
20	60±0.45	50.9±0.48	28±0.36	-
30	110±1.15	71.8±0.68	39±0.95	-
35	92±0.79	45.7±0.58	21±0.28	-
40	-	-	-	-

*All measures are given as means

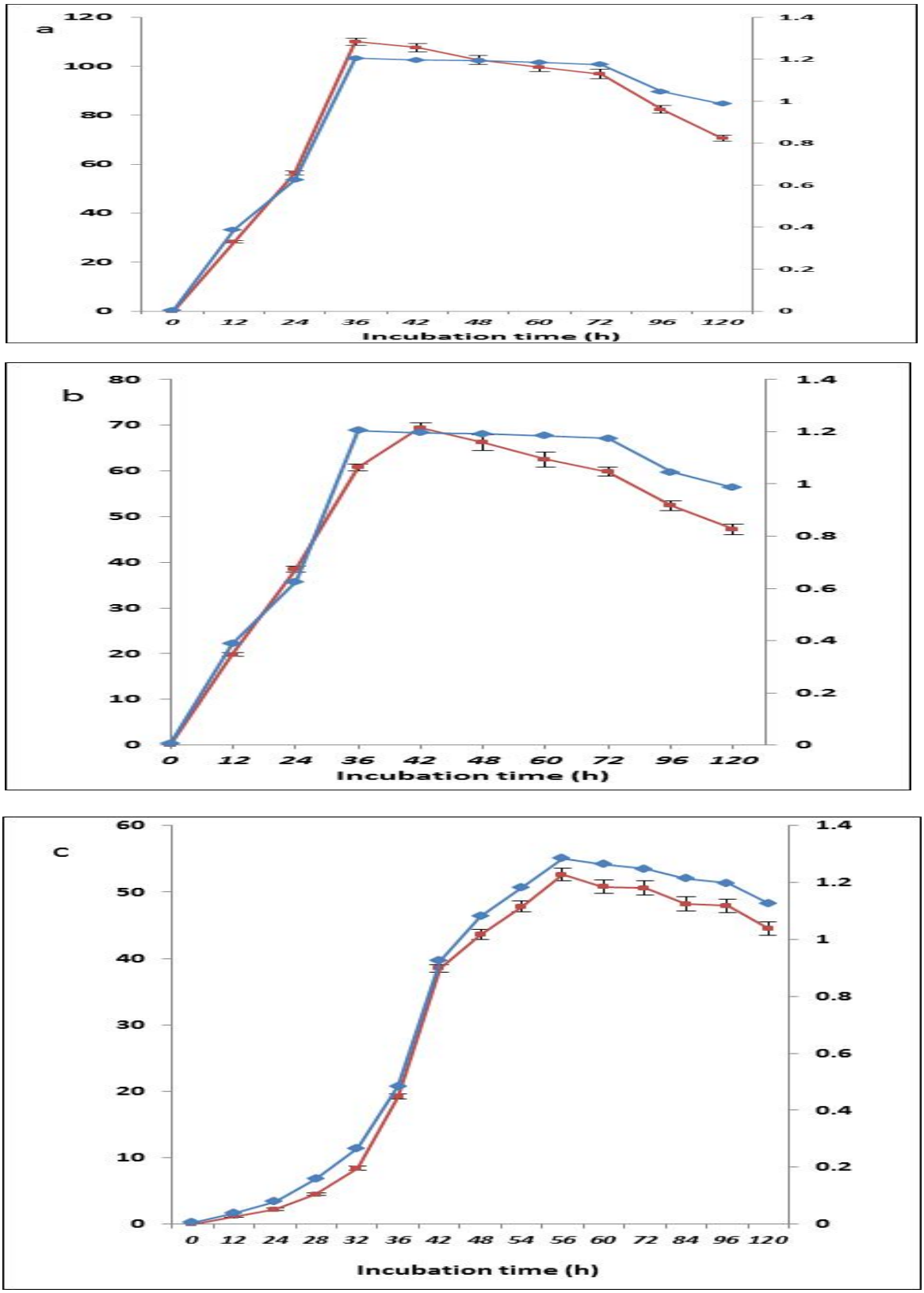

Figure. 2: a) Amount of P solubilized vs O.D. at 600 nm
b) Amount of IAA produced vs O.D. at 600 nm
c) Amount of Siderophore produced vs O.D. at 600 nm

Table 2: Effect of inoculation of the *Brevibacterium frigrotolernas* strain SMA23 on wheat (*Triticum aestivum* HD2967) after three weeks of sowing

Treatments	Root length(cm)	Shoot length(cm)	Wet biomass (g seed^{-1})	Dry biomass (g seed^{-1})	Seedling length (cm)
Control	5.8±1.12	7.2±1.26	0.68±1.02	0.34±0.94	13.0±1.08
SMA23	9.8±1.10	12.7±1.18	1.18±0.94	0.76±0.89	22.1±1.02

*Results were means of four replications.

and shoot biomass was also observed in bacterized seedlings. Average root length recorded in *Triticum aestivum* seedlings incubated with isolate SMA23, was 9.8 cm which showed 88.2% increase as compared to control. Various other parameters showing growth improvement by *Brevibacterium frigrotolernas* strain SMA23 as compared to control were shown in Table 2.

Medicinal plants harbor a distinctive microbiome due to their unique and structurally divergent bioactive secondary metabolites that are most likely to be responsible for the higher specificity of the associated microorganisms (Qi *et al*., 2012). Bacterial plant growth promotion is a complex phenomenon, and is often achieved by the activities of multiple plant growth promoting traits exhibited by the associated bacteria (Lifshitz *et al*., 1987). Present study is carried out to isolate rhizobacteria having specific plant growth promoting attributes from *Aloe vera* rhizosphere. PGPR are indispensable part of soil bacterial community that when grown in association with the host plants can stimulate the growth of the plants (Kloepper *et al*., 1980). PGPR have the ability to solubilize the insoluble phosphorus and maintain the soil health and quality. In this study *Brevibacterium frigoritolerans* belonging to genus *Bacillus* has been isolated from *Aloe vera* rhizosphere. It was for the first time reported that *Brevibacterium frigoritolerans* was able to solubilize phosphate (102.7±1.17 mg L^{-1}). During our studies it was observed that bacterial isolate release greater quantities of IAA in the presence of a physiological precursor, L-tryptophan, in culture media. Production of IAA varies greatly among different species and is also influenced by culture conditions, growth stage, and availability of substrates (Sridevi *et al*., 2008). Like plants, L-tryptophan is also considered as the IAA precursor in bacteria, because its addition to IAA producing bacterial cultures promotes and increases IAA synthesis (Park *et al.*, 2005; Tsavkelova *et al*., 2007). Root exudates are natural source of L-tryptophan for rhizosphere microflora, which may enhance auxin biosynthesis in the rhizosphere (Kravchenko *et al.*, 1991; Martens and Frankenberger, 1994).

Siderophores are iron chelating compounds produced by microorganisms that increase the availability and uptake of iron. These have been reported to facilitate biocontrol by sequestering iron from pathogens, thus limiting their growth. *Brevibacterium frigoritolerans* SMA23 produced 38.6% siderophore units which show its potential to be used as a biocontrol agent. Moreover, during our studies it was found that isolate SMA23 was able to grow over wide range of temperature, as it is able to grow at a temperature of 10°C, so it is better to say it a cold tolerant isolate.

In this study, isolate SMA23 stimulated the growth of *Triticum aestivum* HD2967 seedlings under pot culture conditions. The increased nutrient uptake parameters could be attributed to the enhancement of the root growth and development. Although other parameters could have positively influenced the growth of *Triticum aestivum*, nutrient enhancement by the isolates is proposed as a major means of attaining growth promotion. Future studies are required to prove the nature of these isolates and to harness their potential as bio-inoculants in agriculture.

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