Agric Res
https://doi.org/10.1007/s40003-022-00613-y

FULL-LENGTH RESEARCH ARTICLE

Plant Growth Promotion of *Vigna unguiculata* in Arid Sandy Soil Using Bacterial Species from Coastal Sand Dune

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Received: 27 November 2020 / Accepted: 22 January 2022

Abstract The coastal sand dune (CSD) ecosystem exhibits various stresses where organisms are exposed to dry, nutrient-deficient, intense UV rays, tidal effects, fluctuating pH, temperature and salinity conditions. Hence, it is considered unsuitable for agricultural practices. Despite this, microorganisms inhabiting CSD not only survive these but also support plant growth. From 13 different sand samples of CSD of Goa-India, 250 isolates were obtained on 7 distinct media. These isolates were screened for various plant growth-promoting (PGP) attributes in vitro. Five bacterial isolates exhibiting maximum PGP factors were selected for further screening for ACC deaminase production, antifungal activity and hydrolytic enzyme production. These isolates were subjected to the production of Indole acetic acid and exopolysaccharide in submerged cultivation. These 5 isolates were identified as *Pantoea* sp. K8AcR2AY004, *Chitinophaga eiseniae* K4NRBAY001, *Pantoea dispersa* K4NRR2AY011, *Bacillus marisflavi* K7SpZMAO002 and *Bacillus wiedmannii* K3AsBAP008 using biochemical and molecular characterization. These strains were studied for their abilities to promote cowpea growth in vitro and using sand-filled pots. Cowpea seeds treated with *C. eiseniae* K4NRBAY001, *P. dispersa* K4NRR2AY011 and *B. marisflavi* K7SpZMAO002 in sand exhibited higher germination rate, vigor index and growth parameters than uninoculated seeds. The present study demonstrates that bacterial strains from CSD have the potential to stimulate cowpea growth and could be exploited as bio-fertilizers in arid sandy soils.

Keywords *Bacillus* · Bio-fertilizers · *Chitinophaga* · Hydrolytic enzymes · *Pantoea*

Introduction

Considering the rapid increase in the worldwide population, there is a possibility that it could reach up to 9.1 billion globally by the year 2050 [1]. According to the United Nations (UN) report published in 2016, India will be the most populated country comprising 1.7 billion people. Although the population is increasing, food production is not significantly increasing to meet the demands in the coming years. Thus, there is an increase in crop demand globally. Over the decade, the amount of land available for agricultural use is declining rapidly; hence, there is an immediate need for exploring non-conventional land resources for crop cultivation. Arid and semi-arid areas such as deserts and coastal sand dunes have not been explored for agricultural purposes. Besides, the demand for agricultural land and fertilizers has surged to such an extent that it is not sustainable anymore. The scarcity of agricultural land is one of the critical factors in farming. According to the UN, 28.5% of the total landmass is being used for agricultural practices as of the year 2013. Arid and semi-arid sand regions such as CSD and deserts are not considered agricultural lands. Drastic climate change could convert existing agricultural land into semi-arid or arid regions, thereby significantly reducing the percentage of land on earth available for crop cultivation. Due to climate change, the rain pattern is changing and there is an increase in drought. Microbes play a vital role in several ecological

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Published online: 21 February 2022

processes in an agricultural ecosystem [20]. These include decomposition of organic matter, several nutrient cycles, etc. The contribution of microorganisms that could adapt to climate change by imposing resistance to several abiotic stresses and also promote plant growth is the need of the hour. Additionally, the soil type determines crop productivity. Several abiotic factors, such as soil salinity, pH and nutrients, are affecting crop production. A significant portion of fertile land may also turn barren due to overfarming and extensive use of chemical fertilizers. Six percent of the total landmass (800 Million hectares) is severely affected by salinity and hence unsuitable for agricultural practices [21]. Also, since chemical fertilizers pose a threat to the environment, it is necessary to formulate and develop eco-friendly and stable organic biofertilizers. Over the years, people have become increasingly aware of the harmful side effects of chemical fertilizers which have diverted attention toward the use of biofertilizers which are cost-effective, eco-friendly and environmentally sustainable. This could result in greater demand for bio-fertilizers which can sustain harsh environmental conditions and promote plant growth.

CSD ecosystems are less explored habitats harboring unique microorganisms. CSD is a low nutrient-containing habitat showing the prevalence of drought conditions, intense UV rays, tidal effects, and fluctuating temperature, pH, and salinity [16]. Plants growing on CSD are adapted to these harsh and fluctuating conditions. For example, *Vigna unguiculata* (cowpea) is a halophyte and is mostly grown in semi-arid regions [24]. Cowpeas are grown for their grains and as leafy vegetables. The cowpea leaves are rich in nutritional property which makes them ideal for reducing insecurities of food and nutrition. Green leaves of cowpea plants are used for the preparation of meals. Leaves of cowpeas have antioxidants such as alpha tocopherols, flavonoids, lycopene, and anticancer agents [32]. Preserved and fresh cowpea leaves are rich in beta-carotene and Iron [25]. These leaves provide $\geq$ 75% and 25% of RDAs for Vitamin A and Iron, respectively, of children aged 4–8. Microorganisms present in CSD are vital as they are well-known for supporting plant growth in drought conditions prevailing in the sand and can serve as effective plant growth-promoting microorganisms (PGPM) and hence bio-fertilizers [12]. Several organisms belonging to genera *Pseudomonas*, *Bacillus*, *Pantoea* and *Arthrobacter* are commercially used as bio-fertilizers. Glick [12] mentioned several plant growth-promoting (PGP) attributes produced by microorganisms that directly or indirectly benefit plant growth under unfavorable conditions. The direct mechanisms of PGPM include the production of phytohormones, HCN (Hydrogen Cyanide), ACC (1-Aminocyclopropanecarboxylic acid) deaminase, inorganic phosphate solubilization and protection against phytopathogens. And, the indirect mechanisms involved are the production of EPS (Exopolysaccharides), ammonia, siderophores and extracellular hydrolytic enzymes.

This study explores the usage of arid sandy soil for agriculture. The present study focuses on the isolation of bacterial strains from CSD, determining their effect on cowpea seed germination and plant growth-promoting parameters under saline and nutrient-deficient conditions by using sand for pot experiments.

Material and Methods

Isolation of Bacterial Isolates

The bacterial isolates used during the study were obtained from sand samples collected from the rhizosphere and non-rhizosphere region on CSD of Keri beach, Goa, India, during the pre-monsoon at low tide (15°42′39.84″ N, 73°41′41.29″ E). Ten percent sand suspension was serially diluted till 10^{-6} and spread plated on 7 different media (Nutrient agar, Zobell marine agar, Bennet's agar, Reasoner's 2 agar, Polypeptone yeast extract glucose agar and M1 agar). Nutrient agar is used to allow the growth of a wide range of microorganisms. Zobell marine agar was used to isolate heterotrophic marine bacteria. Bennet's agar is widely used in isolation of sporulating Streptomyces [17]. Reasoner's 2 agar was used to retrieve microorganisms that are unable to grow on nutrient-rich media [5]. Polypeptone yeast extract glucose agar (pH 10.5) was used to isolate alkaliphilic bacteria. M1 agar is designed in our laboratory to isolate heterotrophic bacteria with the composition of malt extract (1 g/l), yeast extract (1 g/l), tryptone (10 g/l), manganese chloride (1 g/l), magnesium chloride (1 g/l), sodium chloride (10 g/l), glucose (10 g/l) and pH 7.5. Morphologically distinct colonies were selected and purified on their respective isolating media.

Screening of Isolates for Plant Growth-Promoting Traits

Preliminary Screening

The isolates were screened for various plant growth-promoting attributes including solubilization of inorganic phosphate, production of IAA (Indole-3-Acetic Acid), siderophores, EPS and ammonia. Phosphate solubilization by the isolates was screened on Pikovskayas agar containing tricalcium phosphate. A zone of clearance around the colony indicates phosphate solubilization [26]. IAA production was checked by inoculating isolates in tryptophan broth. After incubation, Salkowski reagent was added to the cell-free filtrate and kept for 30 min. Pink coloration

indicates the production of IAA [26]. Isolates were screened for the production of siderophore using the Chrome Azurol S agar plate method [15]. The yellow zone around the colony indicates siderophore production. EPS production by the isolates was checked on Congo red agar containing sucrose (5%) [26]. Intense red coloration of the colonies indicates EPS production. Isolates were grown in peptone water broth for determining ammonia production. After 5 days of incubation, the Nessler's reagent was added and brown coloration indicates ammonia production [22]. The isolates exhibiting the maximum number of PGPFs (Plant Growth-Promoting Factors) were selected for further studies.

Screening of Selected Isolates for ACC Deaminase, Antifungal Activity and Hydrolytic Enzymes

Production of ACC deaminase by the selected isolates was tested using the protocol described by Hmaeid et al. [15]. Isolates were inoculated on Dworkin and Foster (DF) minimal medium containing 30 mM ACC as a sole nitrogen source. The growth of isolates on DF minimal media indicated a positive test. Negative control was maintained by inoculating the isolates into the DF minimal media not containing ACC. To carry out the antifungal activity, the bacterial isolates were spot inoculated on potato dextrose agar in the center while *Fusarium oxysporum* was spot inoculated on either side. The plates were incubated at 28 °C for 7 days, and the zone of inhibition was measured. The bacterial isolates were also screened for the production of protease, cellulase, chitinase, and amylase based on Alnahdi [2], Wood et al. [33], Yuli et al. [35] and Shaw et al. [31], respectively. To screen for protease activity, isolates were inoculated on skimmed milk agar and after incubation, checked for the zone of clearance indicating a positive test. To screen for cellulase activity, isolates were inoculated on nutrient agar containing CMC-Na (1%). After incubation, the plates were flooded with Congo red solution followed by washing with NaCl solution. To screen for chitinase activity, isolates were inoculated on nutrient agar containing colloidal chitin (1%) and checked for the zone of clearance. To screen for amylase activity, isolates were inoculated on starch agar. After the incubation, plates were flooded with Gram's iodine and the appearance of the zone of clearance indicates a positive test.

Production of Indole-3-Acetic Acid and Exopolysaccharide in Shake Flask

The selected isolates were checked for the production of IAA and EPS in broth. Bacterial isolates were inoculated in 50 mL nutrient broth and incubated at 30 °C at 150 rpm until the optical density at 600 nm reached 0.700. This initial inoculum was used for further experiments. One percent (v/v) of initial inoculum was used for the production of IAA and EPS. Strains were inoculated in 0.1% tryptophan broth and incubated for 24 h, 28 ± 2 °C and 150 rpm. The amount of IAA produced was determined by following the protocol described by Mendes et al. [19].

For EPS production, isolates were inoculated in 100 mL nutrient broth containing 5% sucrose and incubated at 28 °C for 72 h at 150 rpm. After incubation, the broth was centrifuged and the supernatant (S1) was collected. To the pellet, 10 mL of saline ethylene-diamine-tetra acetic acid was added, centrifuged at 10,000 rpm for 10 min at 4 °C and supernatant (S2) was collected. Both the supernatants, S1 and S2, were pooled together, to which twice the volume of chilled isopropanol was added [10]. After vigorous shaking, this mixture was kept at 4 °C overnight followed by centrifugation at 10,000 rpm for 15 min to obtain EPS which was dried and weighed.

Identification of the Selected Isolates

The selected isolates were identified using morphological, biochemical and molecular methods. Colony characteristics and cell morphology of isolates were recorded. Biochemical tests were performed. Sequences of 16S rRNA of the bacterial genome were used for molecular identification as per the protocol explained by Prabhu et al. [27]. The sequences obtained were compared with the type strain sequences from the National Center for Biotechnology Information BLASTn (2.2.18 +). The phylogenetic tree was constructed using MEGA 7 software [18] by the neighbor-joining method (1000 bootstrap). The sequences were submitted to GenBank, and accession numbers were obtained.

Effect of Inoculation of Selected Bacterial Strains on Cowpea

Inoculum Development

One percent of the initial inoculum was inoculated in 50 mL sterile nutrient broth and incubated at 28 °C for 48 h at 150 rpm. Twenty mL of culture broth was centrifuged at 8000 rpm for 10 min. The pellet was washed and re-suspended in sterile distilled water to give 10^8 CFU mL^{-1}.

Effect of Inoculation on Cowpea Seed Germination and Seedling Development In Vitro

Twenty cowpea seeds were surface-sterilized using mercury chloride solution based on the procedure described by

Nain et al. [22], followed by overnight soaking in a mixture containing 1 mL of culture and 4 mL of sterile distilled water. After air drying, the seeds were kept in sterile Petri plates (90 mm) containing sterile damp Whatman filter paper (No.1) for 7 days. The seeds were exposed to the light–dark cycle of 8–16 h daily. After 7 days, the number of germinated seeds, the RL (Root Length), SL (Shoot Length), number of developed cotyledons and wet weight of each seedling were recorded [16]. Germination rate and the VI (Vigor Index) were calculated using the following formulae:

$$\text{Germination rate (\%)} = (\text{Number of seeds germinated}/\text{Total number of seeds}) \times 100$$

$$\text{Vigor index} = \text{Germination rate (\%)} \times [\text{Mean RL} + \text{Mean SL}]$$

Containerized Studies on the Effect of Bacterial Strains on Cowpea Plant in Sand

Pot experiments were carried out in sterile sand to determine the effect of bacterial strains on cowpea seedling development.

Collection and Processing of Sand

Sand samples were collected during low tide from Vainguinim beach (15°27′18.3″ N, 73°48′52.8″ E), Goa-India. The sand was sieved with a sieve (0.2 mm) and sterilized by autoclaving in a glass beaker for 2 h every 24 h for three cycles. It was then packed in sterile black polythene bags having 10 cm diameter and holes (to remove excess water), which were sterilized by exposure to UV rays for 20 min.

Bio-inoculation of Cowpea

Bacterization of cowpea seeds was carried out by soaking them in the bacterial inoculum. Sand drenching was done by mixing 20 mL of bacterial inoculum in the sand. Ten bacterized cowpea seeds were sown per pot. Vermicompost (25 g/1.5 kg sand) and sterile distilled water (5 mL/20 seeds) were maintained as positive and negative controls, respectively. The sand was moistened with sterile distilled water every alternate day up to 25 days. After germination of the seeds, the pots were kept in sunlight for 12 h day-night cycle. After 25 days, the number of germinated seeds, plant height (RL and SL), number of leaves and leaf area [29] were measured. The roots and shoots were separated, dried overnight and weighed.

Statistical Analysis

Data were analyzed by SYSTAT 13 (13.2.01) using a nonparametric T test (two-sample Kolmogorov–Smirnov test) for determining pair-wise significant differences among the treatments. Additionally, an analysis of variance (ANOVA) F test was performed to determine significant differences.

Results

Isolation of Bacteria and Screening for Plant Growth-Promoting Factors

Two-hundred-fifty bacterial isolates were obtained from 13 different sand samples collected from CSD of Keri-Goa, India, on 7 different nutrient media. Among these, 66% of isolates were obtained from the rhizosphere and the remaining from the non-rhizosphere region of CSD. All bacterial isolates were tested for six PGPF. Among these, 5, 34, 31, 16 and 14% of the isolates exhibited production of IAA, siderophores, EPS, ammonia and solubilization of inorganic phosphate, respectively. Out of 250 isolates, five potential isolates, namely K4NRR2AY011, K8AcR2AY004, K3AsBAP008, K4NRBAY001 and K7SpZMAO002, were selected based on their ability to produce the maximum number of the above-listed PGPFs (Table 1). Three isolates, namely K8AcR2AY004, K3AsBAP008 and K7SpZMAO002, were obtained from the rhizosphere of *Acrocephalus capitatus*, *Anacardium occidentale* and *Spinifex littoreus*, respectively, while K4NRR2AY011 and K4NRBAY001 were isolated from the non-rhizosphere region of CSD.

All five selected isolates displayed ACC deaminase production (Table 1). Isolate K4NRBAY001 revealed the production of protease, cellulase and chitinase (Table 1), while isolate K3AsBAP008 showed protease, amylase and cellulase activity. Isolate K4NRR2AY011 was able to produce chitinase. However, no hydrolytic enzyme production was witnessed by isolate K8AcR2AY004 and K7SpZMAO002. Out of five, two isolates (K7SpZMAO002 and K4NRBAY001) exhibited antifungal activity against the phytopathogen *Fusarium oxysporum* with 30 and 24 mm zone of inhibition, respectively (Fig. 1).

Production of Indole-3-Acetic Acid and Exopolysaccharide in Shake Flask

Isolate K4NRR2AY011 produced significantly higher level of IAA, followed by K4NRBAY001, K3AsBAP008, K7SpZMA0002 and K8AsR2AY004 ($p < 0.05$) (Fig. 2).

Table 1 Plant growth-promoting factors of selected bacterial isolates

Bacterial isolates	IAA	PS Size (mm)		SP Size (mm)		EPS	Ammonia	Protease	Amylase	Cellulase	Chitinase	ACC Deaminase
		CS	ZS	CS	ZS							
K8AcR2AY004	+	10	20	15	45	+	+	−	−	−	−	+
K4NRBAY001	+	5	7	10	17	+		+	−	+	+	+
K4NRR2AY011	+	7	23	15	25	+++	+++	−	−	−	+	+
K7SpZMAO002	+	9	−	15	17	+	−	−	−	−	−	+
K3AsBAP008	+	10	14	12	21	++	−	+	+	+	−	+

IAA Indole Acetic Acid, *PS* Phosphate solubilization, *mm* millimeter, *CS* colony size, *ZS* zone size, *SP* Siderophore production, *EPS* Exopolysaccharide, (−) negative, (+) slight positive, (++) moderate positive, (+++) strong positive and *ACC* 1-Amino Cyclopropane Carboxylic acid

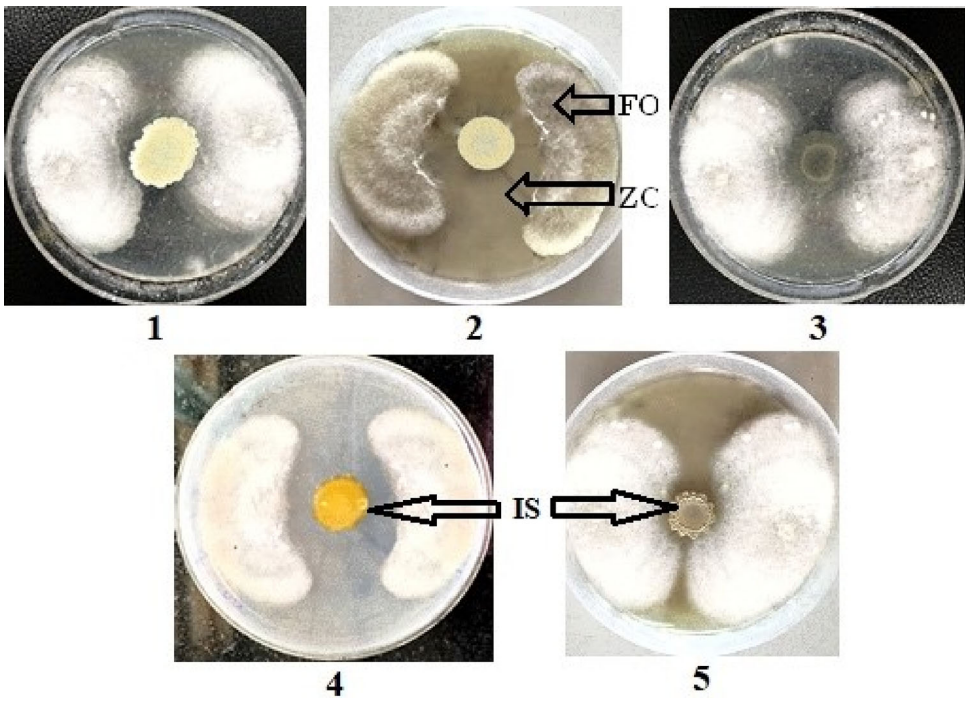

Fig. 1 Antifungal activity exhibited as a zone of clearance (ZC), by the selected bacterial isolates (IS) viz. (1) K8AcR2AY004, (2) K4NRBAY001, (3) K4NRR2AY011, (4) K7SpZMAO002 and (5) K3AsBAP008, obtained from a coastal sand dune, against fungal pathogen *Fusarium oxysporum* (FO)

EPS produced by isolate K4NRR2AY011 was significantly higher, followed by isolate K3AcBAP008, K8AsR2AY004, K4NRBAY001 and K7SpZMAO002 ($p < 0.05$) (Fig. 2).

Identification of CSD Bacterial Strains

All bacterial cells were found to be rod-shaped. Apart from isolates K7SpZMAO002 and K3AsBAP008 which are Gram-positive, the rest of the isolates were Gram-negative. Biochemical tests are provided in Supplementary Table 1. Based on biochemical tests and 16S rRNA gene sequencing (Fig. 3), the isolates were identified as *Pantoea* sp. K8AcR2AY004, *Chitinophaga eiseniae* K4NRBAY001, *Pantoea dispersa* K4NRR2AY011, *Bacillus marisflavi* K7SpZMAO002 and *Bacillus wiedmannii* K3AsBAP008. The sequence of these strains has been deposited in the NCBI GenBank under the accession numbers MH620780, MK106277, MK106282, MK106294 and MK106270, respectively.

Effect of Bacterial Strains on Seed Germination and Seedling Development in Cowpea

Cowpea seeds treated with the test strains and control (uninoculated) exhibited a 100% germination rate in vitro. Out of 20 cowpea seedlings, 17 seedlings showed cotyledon and leaf formation with the treatment of test strains, whereas only 6 seedlings showed cotyledon and leaf formation in control.

It was observed that the treatment of cowpea with selected bacterial strains exhibited a significantly higher

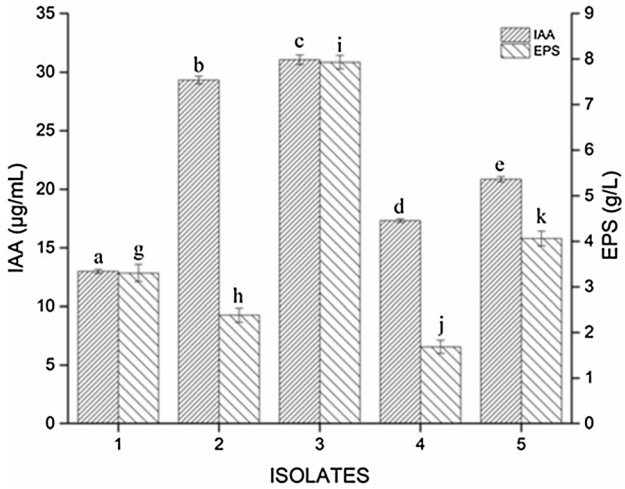

Fig. 2 Indole acetic acid and exopolysaccharides production by selected bacterial isolates viz. (1) K8AcR2AY004, (2) K4NRBAY001, (3) K4NRR2AY011, (4) K7SpZMAO002, and (5) K3AsBAP008, in shake flask. Bars are mean, error bars showing standard error. Alphabets denote significant differences at $p < 0.05$ (alphabets are independent for each parameter)

wet weight than the negative control ($p < 0.05$) (Table 2). Treating seeds with *P. dispersa* K4NRR2AY011, *B. marisflavi* K7SpZMAO002 and *B. wiedmannii* K3AsBAP008 displayed significantly higher wet weight than that with *C. eiseniae* K4NRBAY001 and *Pantoea* sp. K8AcR2AY004 ($p < 0.05$). Among the seedlings treated with *C. eiseniae* K4NRBAY001 and *Pantoea* sp. K8AcR2AY004, the wet weight was significantly higher than in treatment with the former ($p < 0.05$). There was no significant difference in the RL of seedlings treated with selected strains and negative control ($p > 0.05$) (Table 2). The SL of cowpea seedling was significantly higher in treatment with *C. eiseniae* K4NRBAY001, *P. dispersa* K4NRR2AY011, *B. marisflavi* K7SpZMAO002 and *B. wiedmannii* K3AsBAP008 when compared to the negative control ($p < 0.05$) (Table 2). The SL of cowpea treated with *Pantoea* sp. K8AcR2AY004 and negative control were found to be similar ($p > 0.05$). Treating cowpea seeds with *P. dispersa* K4NRR2AY011 and *B. marisflavi* K7SpZMAO002 gave significantly higher SL than *Pantoea* sp. K8AcR2AY004 treatment ($p < 0.05$). However, the seedlings which were treated with *Pantoea* sp. K8AcR2AY004 gave significantly larger RL than those with *P. dispersa* K4NRR2AY011 ($p < 0.05$). The VI of control cowpea seedling was 1392 in vitro, whereas treating cowpea with *B. marisflavi* K7SpZMAO002 exhibited the highest VI (1814) followed by *C. eiseniae* K4NRBAY001 (VI = 1799), *Pantoea* sp. K8AcR2AY004 (VI = 1611), *P. dispersa* K4NRR2AY011 (VI = 1582) and *B. wiedmannii* K3AsBAP008 (VI = 1530).

Containerized Studies on Inoculation of Cowpea Plant in Sand

A hundred percent seed germination was seen in cowpea treated with *B. marisflavi* K7SpZMAO002, *C. eiseniae* K4NRBAY001 and *P. dispersa* K4NRR2AY011. Uninoculated seeds and seeds treated with compost showed an 80 and 90% seed germination rate, respectively. Cowpea seed germination rate was below the control value with *Pantoea* sp. K8AcR2AY004 (70%). The germination rate of cowpea seed treated with *B. wiedmannii* K3AsBAP008 was the same as that of positive control.

Treatment with bacterial strains gave significantly higher root dry weight in cowpea than the negative control ($p < 0.05$) (Table 2). Treatment of *C. eiseniae* K4NRBAY001 and *P. dispersa* K4NRR2AY011 exhibited a significantly higher root dry weight than the positive control ($p < 0.05$). The RL of the cowpea plant treated with *C. eiseniae* K4NRBAY001 was found to be significantly higher compared to the positive control and other strains ($p < 0.05$) (Table 2). However, there was no significant difference observed in RL with the treatment of *C. eiseniae* K4NRBAY001 and negative control ($p > 0.05$). No significant difference was seen in root dry weight and RL with treatments of negative and positive control ($p > 0.05$). The SL of the cowpea plant treated with *P. dispersa* K4NRR2AY011 was significantly higher than the negative control, *Pantoea* sp. K8AsR2AY004 and *B. wiedmannii* K3AcBAP008 ($p < 0.05$) (Table 2). No significant difference was observed in SL treated with *C. eiseniae* K4NRBAY001, *P. dispersa* K4NRR2AY011 and positive control ($p > 0.05$). Moreover, there was no significant difference in the total wet weight, leaf number and shoot dry weight of the cowpea plant among the treatments ($p > 0.05$) (Table 2). The average number of leaves observed in the cowpea plant was $\sim$ 6 (Table 2). The maximum and the minimum number of leaves witnessed were 8 and 2, respectively. Treatments with *B. marisflavi* K7SpZMAO002, *C. eiseniae* K4NRBAY001, *P. dispersa* K4NRR2AY011 and the negative control gave significantly larger leaf area when compared to *B. wiedmannii* K3AcBAP008 (Table 2). Highest VI was exhibited by the cowpea plant which received *C. eiseniae* K4NRBAY001 (5995) followed by *P. dispersa* K4NRR2AY011 (5540), *B. marisflavi* K7SpZMAO002 (4890), positive control (4620), *B. wiedmannii* K3AsBAP008 (4220), negative control (3870) and finally *Pantoea* sp. K8AcR2AY004 (3090).

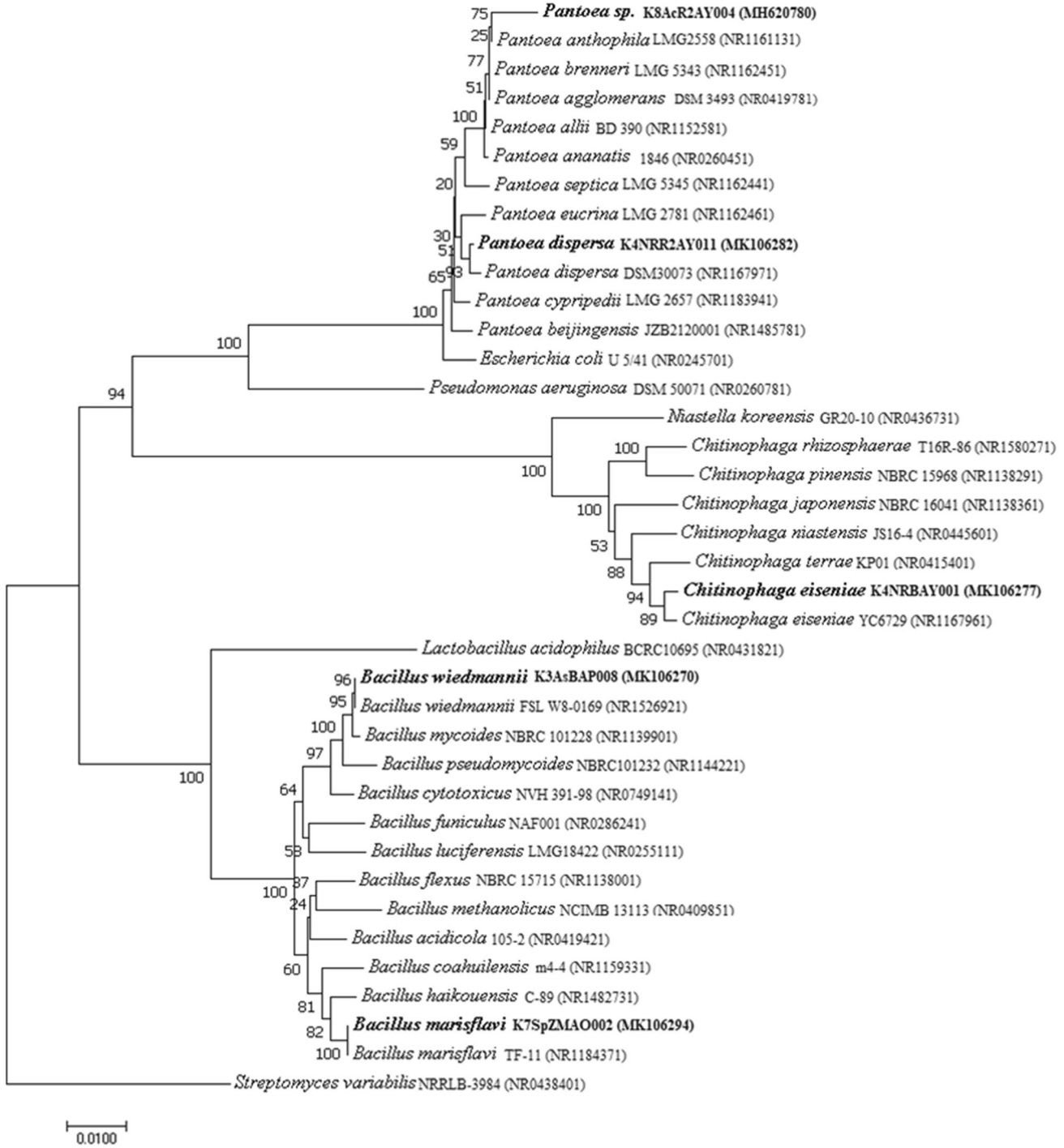

Fig. 3 The phylogenetic tree of 16S rRNA gene sequences of selected bacterial strains. The tree was constructed using 1000 bootstraps. The percentages of 1000 replicate trees are shown next to the branches. The bar shows substitutions per nucleotide position. Bold- Bacterial strains obtained during the present study and ()—accession numbers

Discussion

As a result of the increasing population, the demand for crops has amplified drastically. As time is progressing, the available agricultural land is declining at an alarming rate. Hence, there is a need to explore non-traditional areas that can be used for cultivation. Sand dunes and deserts are unique habitats that have not been explored for agricultural purposes. The three major requisites for carrying out agricultural practices are land, type of crops and microbial

Table 2 Effect of bio-inoculum on cowpea seeds during seed germination and seedling development and cowpea plant during containerized studies

Experiments	Strains	Negative control	Positive control	*Pantoea* sp. K8AcR2AY004	*Chitinophaga eiseniae* K4NRBAY001	*Pantoea dispersa* K4NRR2AY011	*Bacillus marisflavi* K7SpZMAO002	*Bacillus wiedmannii* K3AsBAP008
Seed germination and seedling development	Wet weight	0.490 ± 0.016^{a}	NA	0.610 ± 0.043^{c}	0.780 ± 0.043^{d}	1.180 ± 0.078^{b}	1.220 ± 0.063^{b}	1.070 ± 0.069^{b}
	Root length	10.650 ± 0.821^{e}	NA	11.525 ± 0.912^{ef}	12.310 ± 0.959^{e}	3.696 ± 0.959^{eg}	10.525 ± 0.980^{e}	8.800 ± 1.037^{e}
	Shoot length	3.270 ± 0.306^{h}	NA	4.590 ± 0.762^{hi}	5.685 ± 0.839^{ij}	3.122 ± 1.527^{j}	7.625 ± 0.773^{jk}	6.500 ± 0.707^{ijk}
Containerized study	Root length	18.625 ± 1.562^{l}	16.333 ± 0.833^{l}	15.143 ± 1.973^{l}	24.100 ± 1.657^{l}	18.300 ± 1.175^{l}	16.900 ± 0.837^{l}	16.333 ± 0.645^{l}
	Shoot length	29.750 ± 1.864^{mo}	35.000 ± 1.093^{mo}	29.000 ± 2.918^{mo}	35.850 ± 1.170^{mo}	37.100 ± 1.006^{no}	32.000 ± 1.318^{mo}	30.556 ± 1.015^{mo}
	Wet weight	2.993 ± 0.411^{p}	4.661 ± 0.546^{p}	3.351 ± 0.761^{p}	4.243 ± 0.617^{p}	6.200 ± 0.981^{p}	4.604 ± 0.945^{p}	2.804 ± 0.627^{p}
	Shoot dry weight	0.269 ± 0.037^{q}	0.317 ± 0.037^{q}	0.218 ± 0.049^{q}	0.382 ± 0.055^{q}	0.285 ± 0.045^{q}	0.345 ± 0.071^{q}	0.179 ± 0.040^{q}
	Root dry weight	0.036 ± 0.005^{r}	0.061 ± 0.007^{rt}	0.090 ± 0.021^{st}	0.123 ± 0.018^{s}	0.198 ± 0.031^{s}	0.110 ± 0.023^{st}	0.079 ± 0.018^{st}
	Leaf area	22.617 ± 2.703^{u}	24.080 ± 2.962^{uw}	19.888 ± 3.026^{uw}	25.113 ± 2.932^{uw}	24.563 ± 1.645^{u}	27.545 ± 2.427^{u}	14.602 ± 2.018^{vw}
	Number of leaves	6.250 ± 0.561^{x}	6.778 ± 0.494^{x}	6.571 ± 0.843^{x}	6.900 ± 0.434^{x}	6.800 ± 0.490^{x}	6.900 ± 0.348^{x}	6.222 ± 0.465^{x}

Superscript alphabets denote significant differences at $p < 0.05$ (alphabets are independent for each parameter), *NA* not applicable

inoculants. The present work focuses on exploring bacteria from CSD as bio-inoculants for cultivating cowpea plants in arid and sandy lands. Microorganisms from CSD of Goa-India have been reported to degrade hydrocarbons, bio-remediate metals, produce polyhydroxyalkanoates, release EPS and promote eggplant growth in mine reject soil [23]. CSD is a nutrient-deficient habitat that is exposed to drought-wet cycles, fluctuating pH, temperature, salt spray from the sea and unstable ground. Cowpea was selected for the present study since it is a staple food in India, has high nutritional value and can grow in drought and salt stresses [24]. The 250 isolates acquired in this study were screened for various PGPFs. Five isolates exhibiting the maximum number of PGPFs (Table 1) were selected and identified as *Pantoea* sp. K8AcR2AY004, *Chitinophaga eiseniae* K4NRBAY001, *Pantoea dispersa* K4NRR2AY011, *Bacillus marisflavi* K7SpZMAO002, and *Bacillus wiedmannii* K3AsBAP008 (Supplementary Table 1, Fig. 3). To the best of our knowledge, these species have not been earlier demonstrated from CSD habitat. In this study, the inoculation of cowpea seeds with 5 bacterial strains significantly improved the seedling characteristics except with *Pantoea* sp. K8AcR2AY004 (Table 2).

Pantoea dispersa K4NRR2AY011 gave positive results for all preliminary PGPF tested in the present investigation and also showed chitinase activity (Table 1). Since *P. dispersa* K4NRR2AY011 also produced IAA and EPS (Table 2); it could act as a good plant growth promoter in sandy regions, by providing phytohormone and creating sand aggregates through EPS and thereafter, leading to an increase in water retention. *P. dispersa* K4NRR2AY011 was able to significantly increase the SL and root dry weight of cowpea plants in sand-containing pots compared to the negative and positive controls (Table 2). In sand, cowpea plants treated with *P. dispersa* K4NRR2AY011 showed a surge of 43.15% and 19.91% in VI compared to the negative and positive controls, respectively. Among the selected strains, the best PGP capacity was observed in *P. dispersa* K4NRR2AY011. *P. dispersa* is known for its capability of detoxifying albicidin toxin produced by *Xanthomonas albilineans*, a causative agent of sugarcane leaf scald [36]. Chitinase production, indicative of antifungal activity, was reported in *P. dispersa* obtained from sea dumps in Bhavnagar, India, produced 108 units mL^{-1} of chitinase [14]. *P. dispersa* 1A isolated from the Himalayas producing IAA, HCN, siderophore and solubilizing phosphate revealed an increase in RL, SL and biomass in wheat seedlings [30]. *P. dispersa* 1A produced IAA between 3.7–4.4 μg mL^{-1} which is less compared to *P. dispersa* K4NRR2AY011 (31.056 μg mL^{-1} IAA).

Chitinophaga eiseniae K4NRBAY001 was capable of solubilizing inorganic phosphate at neutral pH, producing IAA, siderophore, EPS, ACC deaminase, protease and cellulose (Table 1). *C. eiseniae* K4NRBAY001 showed chitinase activity and possessed antifungal activity against phytopathogen *Fusarium oxysporum* (Fig. 1). This is the first demonstration of the antifungal activity of *C. eiseniae.* In cowpea, several fungal diseases are reported including Anthracnose (*Colletotrichum* spp.), Asochyta blight (*Asochyta phaseolorum*), Brown rust (*Uromyces* spp.), Cercospora and Pseudocercospora leaf spot (*Cercospora*), Charcoal rot (*Macrophomina phaseolina*), Fusarium wilt (*Fusarium oxysporum*), Powdery mildew (*Erisyphe polygani*), Rhizoctonia seedling blight (*Rhizoctonia solani*) and Southern blight (*Sclerotium rolfsii*). The majority of the mentioned diseases occur in China, Africa and Latin America. *Fusarium oxysporum* is a major pathogen reported in Asia which causes Fusarium wilt in cowpea. This disease gets worsened by warm, limited soil moisture and poor soil fertility [3]. These conditions are similar to that prevailing at sand dunes and these increase the incidences of Fusarium wilt disease. Therefore, the isolates obtained in the current study were screened for antifungal activity against *Fusarium oxysporum*. The only prevention against this disease is mixing soil with a fungicidal before planting. From the current study, *Chitinophaga eiseniae* K4NRBAY001 and *Bacillus marisflavi* K7SpZMAO002 have the antifungal ability against this pathogen. Thus, these strains could be effective as bio-fungicides and also bio-fertilizers. *C. eiseniae* K4NRBAY001 was able to significantly increase the RL of cowpea in sand compared to the positive control and rest of the strains (Table 2). It also showed a significant increase in root dry weight as compared to negative and positive controls (Table 2). During seed germination, the VI of cowpea seedling treated with this strain hiked by 29.2% in comparison to the negative control. Further, during the pot experiment, the VI of cowpea plants that received this strain showed an increase of 54.9% and 29.76% compared to the negative and positive control, respectively. This VI is the highest among the tested strains. Interestingly, there are no reports of this species apart from the one wherein it has been isolated from vermin-compost in Korea [34].

Bacillus marisflavi K7SpZMAO002 produced IAA, siderophore, ACC deaminase, EPS and showed antifungal activity (Table 1, Fig. 1). This is the first evidence of antifungal activity shown by *B. marisflavi*. However, it failed to solubilize inorganic phosphate at neutral pH. An alkaliphilic strain of *B. marisflavi* was able to solubilize inorganic phosphate at alkaline pH and not at neutral pH. In addition to this, it was capable of producing EPS [26]. *B. marisflavi* K7SpZMAO002 did not produce any of the hydrolytic enzymes tested (Table 1). During seed germination, cowpea seeds treated with *B. marisflavi* K7SpZMAO002 exhibited 30.3% higher VI than those

with the negative control. During the pot experiment in the sand, there was an improvement in the VI by 26.35% and 5.84% from negative and positive controls, respectively. The root dry weight of cowpea treated with *B. marisflavi* K7SpZMAO002 was found to be significantly higher than the negative and positive controls (Table 2). *Oryza sativa* treated with *B. marisflavi* revealed a significant increase in plant characters compared to uninoculated control and could serve as bio-fertilizer in alkaline soil [26]. Based on the above, *B. marisflavi* K7SpZMAO002 could be a suitable candidate as a bio-fertilizer in harsh environmental conditions.

Bacillus wiedmannii K3AcBAP008 produced IAA, siderophore, EPS, ACC deaminase, protease, amylase, cellulase and could solubilize phosphate (Table 1). The VI of cowpea seedling and the plant treated with *B. wiedmannii* K3AcBAP008 improved by 9% compared to negative control. Further, it was observed that treating cowpea with *B. wiedmannii* K3AcBAP008 gave a significantly lower leaf area compared to other treatments (Table 2). During the pot experiment, this strain did not give promising outcomes as compared to *P. dispersa* K4NRR2AY011, *C. eiseniae* K4NRBAY001 and *B. marisflavi* K7SpZMAO002. Hence, this strain may not be endorsed as bio-inoculants for cowpea crops.

Pantoea sp. K8AcR2AY004 was able to produce varieties of PGPF such as IAA, siderophore, EPS, ammonia, ACC deaminase and solubilize inorganic phosphate (Table 1). It exhibited a lower germination rate than the negative control during the pot experiment, implying a negative effect on the growth of cowpea. During molecular identification, *Pantoea* sp. K8AcR2AY004 showed the closest similarity with *P. ananatis* and *P. anthophila*. Both *Pantoea* species are phytopathogens, causing soft rots and various infections in a wide range of crops [37]. However, *P. ananatis* has also been reported to promote the growth of the potato, papaya and pepper [6]. In the current study, *Pantoea* sp. K8AcR2AY004 did not promote cowpea growth in the sand and hence is not recommended as a bio-fertilizer. Furthermore, the present study was able to detect the negative impact of the *Pantoea* sp. strain on cowpea growth.

Studies have revealed the bacterization of cowpea with *Exiguobacterium* sp. N11-0906, *Micrococcus* sp. NII-0909 and *Pontibacter niistensis* NII-0905 has a profound effect on its growth parameters [7–9]. Reports on cowpea seeds bacterized with *Enterobacter* strains have shown a significant increase in biomass and lengths [11]. *Bacillus* sp. RM-2, isolated from the rhizosphere of *Vigna radiata* grown in soils of the semi-arid region of Banasthali (Rajasthan, India), was studied for its effect on cowpea and found to be significantly influencing seed germination and other growth parameters [22]. In the current study, an increase in leaf area up to 22% and shoot dry weight up to 42% were seen due to inoculation with test isolates compared to a negative control. Effects of inoculating symbiotic microorganisms such as *Rhizobium* in addition to phosphate solubilizing bacteria and arbuscular mycorrhizal fungi on cowpea were studied in sandy-loam soil [4, 28]. Rego et al. [28] recorded that there was no significant effect of inoculation on shoot dry biomass of cowpea. Similar observations were noticed in the current study, and the shoot dry weight among the treatments did not show a significant difference.

Sand dunes are unstable and have distinct ecosystems harboring typical diversity. The stabilization of the sand dunes is because of the vegetation and microorganisms otherwise it moves and changes its characteristics [13]. In rural places, people are mostly dependent on green vegetables produced in the small farms. The rural people living in the vicinity of the sand dunes do use the part of the sand dunes for growing vegetables. However, the quality and amount are very poor. Further, there is a large land of sand dunes that is available for cultivation with the novel methodology for expanding agriculture such as drip irrigation, novel microbes-crop combination, etc. The cowpeas and the bacterial isolates tested are obtained from the same ecosystem and will not cause damage rather will help in stabilizing the dunes thus supporting the native flora and fauna. Arid and semi-arid ecosystems viz., deserts and CSDs are harsh environments with poor nutrients that provide limited scope for crop cultivation. A few plant species are growing in such habitats. Interestingly, cowpea can grow in such ecosystems. However, the scope of cultivating this crop in the sand has not been explored as yet. The present study establishes the fact that the growth of cowpea in sandy soil can be enhanced with the application of bio-inoculants. Three bacterial strains, *P. dispersa* K4NRR2AY011, *C. eiseniae* K4NRBAY001 and *B. marisflavi* K7SpZMAO002, from CSD revealed their ability to promote the growth of cowpea plants in sandy soils. Thus, these strains could be effective bio-fertilizers in sandy soil. This is the first case, wherein *C. eiseniae* has been investigated as a plant growth promoter in sandy soils. Cowpea is a leguminous plant capable of undergoing symbiosis with nitrogen-fixing bacteria, thus, conferring an additional benefit to other crops grown in sand dunes and thereby improving the nutritional quality of the soil. The present study suggests using bio-inoculants obtained from CSD environments for the growth of cowpea in sandy soil.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s40003-022-00613-y.

Acknowledgements This work was supported by University Grant Commission (UGC), National Fellowship for Students to pursue a

Ph.D. degree [Award Letter Number: F./2014-15/NFO-2014-15-OBC-GOA-486/ (SA-III/website)]. The authors would like to thank Dr. Ashwani Kumar and Dr. Ajeet Kumar Mohanty from the National Institute of Malaria Research Field Unit, Goa, for Polymerase Chain Reaction and 16S rRNA gene sequencing. The authors would also like to thank Dr. Priya D'Costa for her help in improving the quality of the manuscript.

Author Contributions All authors contributed to the study's conception and design. Preparation of materials, laboratory experiments, data collection, and analysis were performed by Sulochana A. Shet. The draft of the manuscript was written by Sulochana A. Shet and Sandeep Garg commented on previous versions of the manuscript. All the authors read and approved the final manuscript.

Availability of Data and Material GenBank submission: All the sequences have been submitted to GenBank and have appeared in the public database.

Declarations

Conflict of interest The authors declare that they have no potential conflict of interest.

Ethical Approval This article does not contain any studies with human participants or animals performed by any of the authors.

Consent to Participate Not applicable.

Consent for Publication The authors agreed to the publication of the manuscript in this journal.

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