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ISOLATION AND CHARACTERIZATION OF ENDOPHYTIC BACTERIA WITH PLANT GROWTH PROMOTING TRAITS FROM MAIZE (*Zea mays*)

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ABSTRACT

Endophytic microbes that colonize the inner tissues of plants without causing any disease or damage play an important role in the growth and development of host plants that grow under both normal and stressful conditions. Plant growth-stimulating effect of endophytes is manifested by coordinated action on phytohormone production, biological nitrogen fixation, phosphate solubilization, modulation of 1-aminocyclopropane-1-carboxylic acid deaminase (ACC) expression and siderophore production. In this study, 106 endophytic bacterial strains were isolated from leaves, stems, roots and kernels of maize plants collected from different regions of Andhra Pradesh. The ability of these strains were evaluated for plant growth promoting activities. Out of 36 effective isolates, 15 strains were found to grow on N-free semisolid media supplemented with bromothymol blue (BTB) indicator, showed solubilization index varying from 4-6 on Pikovskayas agar, potassium solubilizing index from 1.2-2.5 on Alexandrov agar. Majority of the isolates were found to be positive for IAA production, siderophore production, HCN production and growth on ACC supplemented DF (Dworkin and Foster) minimal agar indicating ACC deaminase activity. The multi trait plant growth promoting isolates can be used microbial inoculants after being tested for growth promotion in maize under field conditions.

Keywords: Endophytic bacteria, Plant growth promoting traits, *Zea mays*

Plants harbour several microbes, which reside within the cells in intercellular spaces or in vascular system, without affecting the plants are known as endophytes (Sandhya *et al*., 2017). Researchers reported that few bacteria, which live attached to or inside the plants, have the ability to promote host plant growth. While considering the maintenance of sustainable agriculture and environmental protection, these endophytic microorganisms have attracted attention because of the reduced use of chemical fertilizers when applied in conjunction with plant growth promoting bioinoculants (Szilagyi-Zecchin *et al.* 2014)

The quality and yield of crop may change due to the association of endophytic microbes that reside in plants asymptomatically. It can be said that the endophytic bacteria are considered as more favorable than the bacteria inhabiting the rhizosphere. Because endophytes live always in contact with plant tissue and exert a direct beneficial effects to the plant while rhizobacteria perform in rhizosphere whose beneficial effects might not be utilized by plants efficiently. As the endopyte plant association is the result of evolutionary process, these bacteria are considered to be more competitive than facultative or non-endophytic microorganisms (Rosenblueth and Martínez-Romero, 2006). Many endophytic bacteria, *Klebsiella* (Chelius and Triplett, 2000a), *Pantoea, Bacillus,Herbaspirillum* (Chelius and Triplett, 2000b) were isolated from maize cultivars previously. Maize stem was found to host group of bacterial species *Pseudomonas aeruginosa*, *P. fluorescens, B. subtilis, Bacillus pumilus* as the relatively more predominant (Rai *et al.,* 2007). Both abiotic and biotic factors may influence the highly diversified endophytic microbial communities residing in plants. Their colonization and plant growth promoting characters are dependent on soil type and the host plant (Grządziel and Gałązka, 2018).

Literature have shown that endophytic bacteria promotes plant growth by fixing atmospheric nitrogen, solubilizing inorganic phosphate, solubilizing potassium, synthesizing phytohormones and producing biomolecules like siderophores, exopolysaccharides and HCN. In addition to growth promotion, endophytic bacteria protect host plant from both biotic and abiotic stresses (Zhu and She, 2018). Therefore, isolation and evaluation of endophytic bacteria for their plant-growth-promoting abilities is a significant area of research for the plant health improvement. Hence present study was aimed to isolate endophytic bacteria from the maize

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plant niche and characterized based on plant growth promoting traits.

MATERIALS AND METHODS

Sample collection

In this study maize plant samples were collected from different regions of Andhra Pradesh (AP) during Kharif and Rabi of 2019. Samples were collected from major maize growing districts of Andhra Pradesh.

Isolation of endophytic bacteria

Soil adhered to the surface of plant roots was collected by vigorous tapping and the remaining adhered soil particles were removed by using sterile water. Each plant sample was divided into 3 sub samples leaves, stem and root (kernels as 4th sub sample if any) and surface sterilized separately. Plant tissues were cut using sterile knife and scissors and immersed in phosphate buffered saline (PBS) at pH 7.0 for 15 minutes to balance the osmotic pressure and prevent the passive diffusion of the disinfectant to the roots (Suman *et al.,* 2001). Each plant sample (about 0.5 g) was cut into pieces of about 1 cm, and surface sterilization of roots was done with 70% ethanol for 40 seconds, and then 2.5% sodium hypochlorite for 20 minutes. Leaves, stems and kernels are disinfected with 70% ethanol for 20 seconds and 2.5% sodium hypochlorite for 10 minutes. The slices were washed 3-4 times in sterile distilled water then grounded with the help of sterile pestle and motor with 1 ml of 0.9% sodium chloride. Dilutions 10^{-3} &10^{-4} of tissue extracts (100 µl) were spread plated onto nutrient agar plates. As a sterile control an aliquot (100 µl) of the last wash water was inoculated and incubated at 28°C for 72 hours. Obtained bacterial isolates were used for further screening (Barzanti *et al.,* 2007).

The bacterial isolates obtained from the surface sterilized plants are known as putative endophytic bacterial strains that displayed distinguished colony morphologies. Morphotypes of bacterial endophytes were selected on the basis parameters like shape, size, color, elevation, margin, texture, grams reaction and cell shape. Pure cultures of all the isolates were obtained by quadrant streaking on nutrient agar medium plates. Purified bacterial isolates were preserved in nutrient agar slants at 4 °C as working culture for further characterization.

Qualitative evaluation of endophytic bacteria for plant growth promoting (PGP) traits

The bacterial endophytes were screened for direct PGP attributes, including nitrogen fixation on N free Doberieners medium, solubilization of phosphorus on Pikovskaya agar and solubilization of potassium on Alexandrov agar medium. The isolates were evaluated for the production of biomolecules like siderophores, indole-3-acetic-acid (IAA), ACC deaminase and HCN production using the standard methods. All assays were carried out in triplicate. To generate bacterial suspensions for assaying PGP traits, all isolates were incubated in nutrient broth medium for 18 h at 30°C with shaking at 150 rev min^{-1}. Next, the bacterial cells were pelleted by centrifugation (8000 *g*, 10 min) and then resuspended in a 0.85% saline solution to a final OD 600 of 1.0 (approximately 10^8 CFU ml^{-1}) for subsequent assays.

Nitogen fixation

All isolates were inoculated in nitrogen free agar medium containing trace amount of BTB (Bromo Thymol Blue) as indicator (N-free broth: 0.1 g of K_2HPO_4, 0.4 g of KH_2PO_4, 0.2 g of $MgSO_4$, 0.1 g of NaCl, 0.02 g of $CaCl_2$, 0.01 g of ($FeCl_3$), 0.002 g of ($NaMoO_4$), and 10 g of glucose in 1000 ml of distilled water) incubated for 24 h at 30°C (Döbereiner *et al*.,1972). The color change phenomenon of BTB indicator from green to blue with increased pH above neutral represents nitrogen fixing ability of isolates.

Phosphorus solubilization

The ability to solubilize inorganic phosphate (Tri calcium phosphate @ 5gl^{-1}) by the endophytic bacteria was evaluated on Pikovskaya's agar plates by the procedure suggested by Pikovskaya (1948). Formation of a clear zone around the colonies were considered as positive result for phosphate solubilization and the efficiency of P solubilization was calculated using the formula

PSI (Phosphate Solubilization Index) = Z / C

Z- Diameter of clearance zone including bacterial growth

C- Colony diameter

Potassium solubilization

Bacterial isolates were evaluated for potassium solubilization on Aleksandrov agar medium plates. Potassium Alumino Silicate @ 2gl^{-1} was added in Aleksandrov medium as insoluble K substrate (Hu *et al.,* 2006). Each plate was divided into 4 sectors and each sector was inoculated with a spot of 5µl bacterial suspension containing nearly 10^8 bacterial cells. The plates were incubated at 30 °C for 5 days and formation of clearing zone around the colony is an indicator of K-solubilization and measured potassium solubilization index.

IAA production

Indole acetic acid (IAA) production was determined by method given by Gordon and Weber (1951). In 20 ml peptone water supplemented with 0.5 mg ml^{-1} L-tryptophan 1 ml of each bacterial cell suspension was inoculated and incubated for 24 h at 30 °C. Then, to 1 ml of the cell-free extracts 2 ml of Salkowski reagent and two drops of orthophosphoric acid were added at room temperature and incubated for 20 min. Change of color to pink after incubation indicates positive for IAA production.

Siderophore production

For qualitative assay of siderophore production 5µl of bacterial suspension of each isolate have nearly 10^8 cell density was inoculated on Chrome Azurol-Sulphonate agar medium (CAS blue agar and incubated for 48–72 h at 28 °C (Schwyn and Neilands, 1987). Appearance of orange halo zone around colony after incubation indicates siderophore production.

ACC Deaminase production

The bacteria are found to have ACC deaminase activity when they utilize 1-Aminocyclopropane -1-carboxylate as sole nitrogen source. Spot inoculate bacterial culture onto modified Dworkin and Foster (DF) agar plates supplemented with ACC (0.3 gl^{-1}). Spot inoculate DF plates with ammonium sulphate (0.3 gl^{-1}) and without additional N source as positive and negative controls respectively. Incubated the plates for 3 days at 30 °C and positive growth is considered as ability to produce ACC deaminase (Jacobson *et al.,* 1994).

HCN production

The overnight grown bacterial cultures were streaked on King's B agar plates supplemented with 4.4 gl^{-1} of glycine. Soaked petri plate sized Whatman no. 1 filter paper disc in in 0.5% picric acid prepare using 2% sodium carbonate and placed inside the lid of streaked petri plate. Sealed plates with parafilm tightly and incubated at 28°C for 4 days. Change of filter paper color from yellow to deep orange-dark brown indicated production of HCN (Bakker and Schipper, 1987).

RESULTS AND DISCUSSION

Sample collection and isolation of endophytic bacteria

At the time of sample collection parameters like growth stage of crop, previously grown crop, type of soil and the latitude and longitude were recorded (Table 1). Point map was constructed for locating area of sample collected using QGIS software (Fig. 1). The isolate code represents the place of isolation (1st letter indicating mandal), part of tissue (2nd letter indicating R-Root, S-Stem, L-Leaf, C-Kernel) and endophytic colonization. A total of 106 culturable endophytic bacteria were isolated from surface sterilized maize plant tissues. They were screened to 36 effective endophytic bacteria based on morphotypes and primary qualitative screening of plant growth promoting traits (Fig. 2). Bacterial strains possessing more than one PGP traits are capable to improve plant growth both under normal and stressed conditions.

In studies conducted by Marag and Suman(2018) from maize hybrid PEEHM-5 and composite PC-4 endophytic bacteria were isolated using root, stem and leaf tissues of plants at vegetative, flowering and maturity stages of growth and it was reported that Morphologically 188 different endophytic isolates (82 from PEEHM-5, 106 from PC-4) were screened for plant growth promoting attributes. A total of 437 culturable bacterial endophytes were isolated from maize sap and screened for the functional diversity in plant growth promoting traits including nitrogen fixation, phosphate and zinc solubilization, siderophore production, indole acetic acid production and antifungal activity against several plant pathogens (Ali *et al*., 2018). Fifty-five endophytic PSB that were isolated from sap, leaves, and roots of maize were tested for their ability to solubilize tricalcium phosphate and produce organic acid. Partial sequencing of the 16S rRNA-encoding gene showed that the isolates were from the genus *Bacillus* and different species of *Enterobacteriaceae* (De Abreu *et al*., 2017).

Qualitative evaluation of endophytic bacteria for plant growth promoting traits

Nitrogen Fixation

The color change (green to blue) of inoculated N-Free agar plates are considered to be positive for Nitrogen fixation. Of 36 prominent bacterial isolates 78% are found to have capability of N fixation (Table 2). The atmospheric N_2 is converted to ammonia by nitrogen fixing bacteria which results in increased pH *invitro.* Thus, color change phenomenon of BTB indicator from green to blue with increased pH above neutral represents nitrogen fixing ability of isolates. In many studies conducted on plant growth promoting endophytic bacteria many endophytic prokaryotes were reported to have N fixing ability due to less competition for nutrients and less O_2 concentration inside the plant (Kaun *et al.,* 2016; Yadav and Yadav, 2017; Döbereiner *et al.,* 1995). Although high percent of isolates were identified as N fixers in present study, further confirmation of character for presence of nif genes is required.

Table 1. Location wise details of the maize plant sample collection

S. No	District	Mandal	Crop growth stage	Previous crop	Soil type	Latitude and Longitude
1.	Visakhapatnam	Anakapalle (A)	Grain filling	Maize	Red soil	17.6896◦N 83.0024◦E
2.	Nandyal	Allagadda (Al)	Flowering	Sesame	Red soil	15.158173◦N 78.53815◦E
3.	Guntur	Tenali (T)	Grain filling	Rice	Black soil	16.2379◦N 80.6444◦E
		Repalle (R)	Grain filling	maize	Red loam	16.0174◦N 80.8295
		Venigandla (V)	Vegetative	Cotton	Black cotton	16.2129◦N 80.2847◦E
		Nidumukkala (N)	Grain filling	Maize	Black soil	16.1148◦N 80.2085◦E
4.	Vizianagaram	Bobbili (B)	Flowering	Brinjal	Sandy	18.3014◦N 23.1649◦E
		Saluru (S)	Grain filling	Maize	Sandy	18.3014◦N 23.1649◦E
		Gantyada (G)	Grain filling	Rice	Sandy loam	18.0014◦N 23.2686◦E
5.	Srikakulam	Chilakapalem (C)	Grain filling	Maize	sandy	18.2735 ◦N 83.8071◦E
		Ponduru (P)	Grain filling	Rice	Sandy loam	18.3030 ◦N 83.7063◦E
		Subadrapuram (Su)	Flowering	Rice	Red soil	18.2271 ◦N 83.7116 ◦E
		Pydibhimavaram (Py)	Grain filling	Maize	Clay	18.1460 ◦N 83.6281 ◦E
6.	East Godavari	Peddapuram (Pd)	Grain filling	Maize	Red soils	17.0712 ◦N 82.1479 ◦E
		Jaggampeta (J)	Flowering	Maize	Loamy soil	17.1417 ◦N 82.0798 ◦E
		Rangampeta (Rg)	Grain filling	Rice	Clay loam	17.0850 ◦N 81.9788 ◦E
7.	Anantapur	Garladinne (G)	Grain filling	Paddy	Black soil	14.8256◦N 77.5935◦E
		Lolur (L)	Flowering	Cotton	Red soil	14.8007 ◦N 77.5982 ◦E
		Vadiampeta (Va)	Grain filling	Bhendi	Red soil	14.7599 ◦N 77.6165 ◦E

Solubilization of phosphorus

In the present study, 7 isolates have shown low solubization indices (SI <2) and 7 isolates have shown moderate SI (2-3), whereas 19 endophytic isolates were found have high phosphorus SI (>3). Maximum solubilization was shown by the strain KL3E1 (6.0) ,KL3E2 (6.0) and LS3E3 (5.8) (Table 2). According to Silva Filho and Vidor (2000), solubilization indices below 2 are considered low, between 2 and 3 medium and above 3 high. The phosphorus solubilizing bacteria can be used as bioinoculants for the crops growing in phosphorous limited areas as Oteino *et al.* (2015) demonstrated that the inoculation of endophytes into the rhizosphere increased growth in plants suffering from limited phosphate supply. The endophytic bacteria belonging to genera *Bacillus*, *Klebsiella*, *Microbacterium, Pantoea*, *Paenibacillus*, and *Pseudomonas*, have been reported to solubilize phosphorus (Kour *et al.* 2020 a, b).

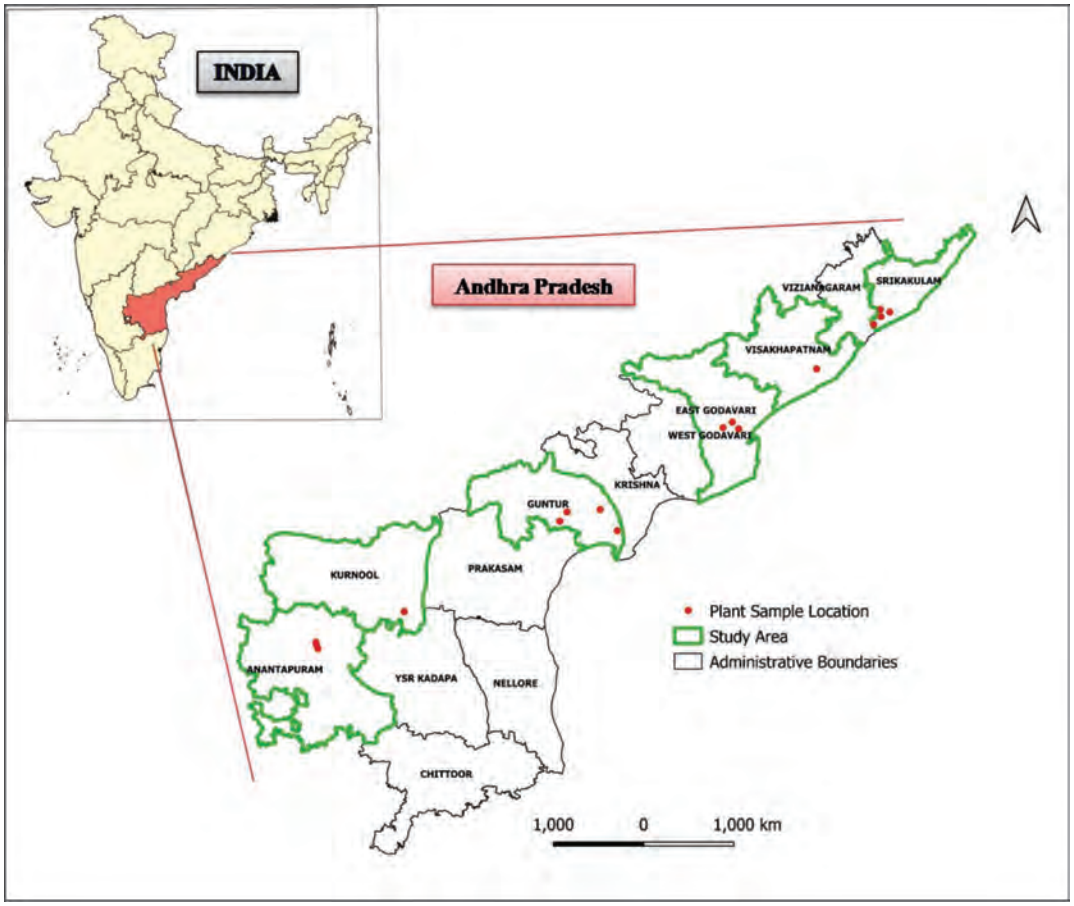

Fig. 1. Location map of maize plant samples collection in Andhra Pradesh

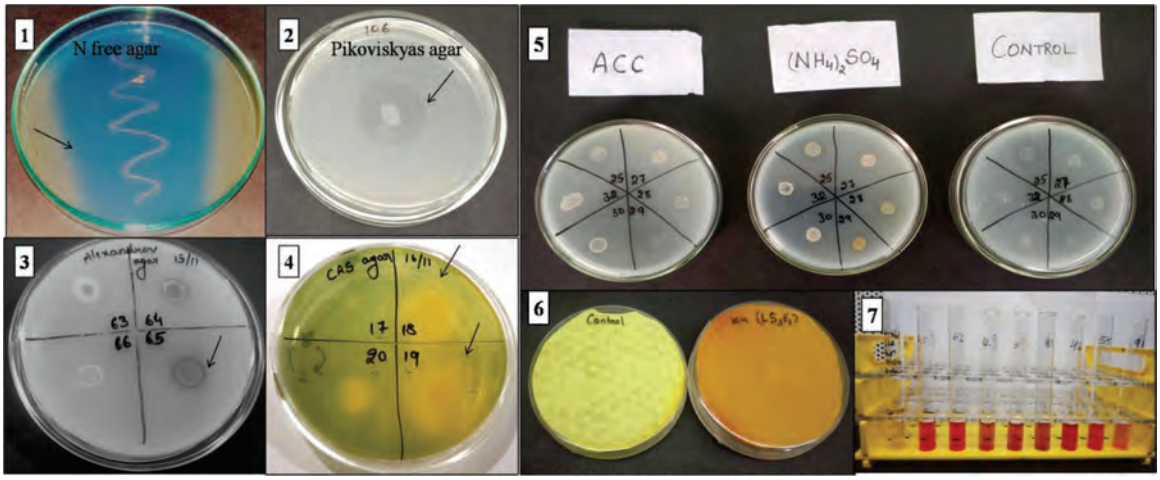

Fig. 2. Plant growth promoting characters of endophytic bacteria in maize

1. N fixing bacterial growth resulted in change of color (Green to Blue) in NFree agar (with BTB indicator) Plates.
2. Clearance zone on Pikovskyas agar plate indicating P solubilization.
3. Clearance zone on Alexandrov agar plate indicating K solubilization.
4. Orange halos on CAS agar plates representing Siderophore production.
5. Bacterial strains grown on Dworkin Foster (DF) minimal with different N source (ACC, $(NH_4)_2SO_4$ and Control)
6. Development of deep orange to dark brown color indicating production of HCN.
7. Pink color development on addition of Salkowski reagent representing IAA Production.

Solubilization of potassium

The potassium solubilization indices of maize endophytic bacteria were recorded in the range of 1.0 to 1.6. and the highest SI was shown by the strains JC3E2 (1.59) and LS3E3 (1.60). Only 50 % of the maize endophytic bacterial strains were found to have capability of potassium solubilization (Table 2). Number of K solubilizers are found to be less as compared to P solubilizers and N fixers in present investigation. Similar results were reported by Marag and Suman, (2018) that maize endophytic bacteria were with less K solubilizing ability as compared to P and Zn solubilization. From different host plants K-solubilizing endophytic bacteria have been isolated and characterized in various studies and the efficient K solubilizing bacterial genera reported are *Bacillus*, *Klebsiella, Enterobacter, Microbacterium, Alcaligenes*, *Achromobacter* and *Acinetobacter* (Kour *et al.,* 2020c; Verma *et al.* ,2019; Yaish *et al.,* 2015; Verma *et al.,* 2014).

Production of Indole Acetic Acid

Based on the color intensity developed after adding the salkowski reagent and incubation the IAA production is categorized into low/no production (no color), Moderate (pale pink), High (dark pink). Of the effective 36 isolates 12 strains were found to be low or no producers of IAA. Moderate level of IAA production was shown by 16 strains while 8 bacterial isolates (PdC3E2, PdS3E2, RgL3E4, RgC3E2, JC3E2, LS3E2, LS3E3 and LL3E1) were found to be having high production of IAA.More than 75% of isolates are reported as significant IAA producers in current work and bacteria colonizing leaf and stem were found to be elite producers of IAA.Similar results were obtained by Beneduzi*et al.* (2013) as they found 71% of endophytic bacteria isolated from sugarcane stem and root were positive for IAA production in the range of 0.1-264 µg ml^{-1} in 3 days. The endophytic bacterial genera *Bacillus, Microbacterium, Psychrobacillus*and *Lysinibacillus*have been isolated from maize roots and reported to have ability to produce IAA (Yu *et al.,* 2016).

Production of siderophore

The formation of orange halo zone around the colonies on CAS agar indicates siderophore production and 26 isolates were found to be positive in plate assay and selected for quantitative estimation of siderophore production (Table 2). The production of siderophores by endophytic bacteria helps the plants to acquire bioavailable iron by process of sequesteration and inhibits the growth of pathogens by competing for nutrients and thus limiting the iron availability to them. The endophytic bacterial genera reported as iron sequesters in previous studies are *Pantoea, Klebsiella, Enterobacter, Pseudomonas* isolated from corn sap (Ali *et al.* 2018), *Bacillus licheniformis K. pneumoniae* and *Pseudomonas* sp. isolated from *P. nigrum* (Jasim *et al.,* 2013), *Pseudomonas* isolated from banana (Catherine *et al.,* 2012)

ACC Deaminase Production

The ability of strains to utilize ACC as a source of N was assessed on the basis of bacterial growth on plates containing different substrate ACC and $(NH_4)_2SO_4$. On the basis of preliminary qualitative plate assay 2 isolates (RgC3E2, JC3E1) have shown high growth, 16 isolates have shown moderate growth, 14 isolates have shown low growth and 4 strains were found to have no growth (Table 2). According to few studies for endophytic bacteria ACC deaminase activity plays pivotal role in its ability to stimulate plant growth (Hardoim *et al.,* 2008). We found more than 50% of the isolates were positive for ACC deaminase activity while the strains RgC3E2 and JC3E1 were elite producers based on the qualitative evaluation. The previous studies reported many genera which showed positive result for ACC deaminase activity including *Bacillus megaterium* and *Pantoea agglomerans* (Silambarasan *et al.,* 2019a), *Pseudomonas stutzeri* (Han *et al.,* 2015), *Klebsiella oxytoca* and *Klebsiella variicola* (Zheng *et al*., 2014) salt tolerant endophytic bacteria *Bacillus aryabhattai* (Siddikee *et al.,* 2010) and *B. megaterium* (Chinnaswamy *et al.,* 2018).

HCN production

HCN is a volatile product that exhibits antifungal action and its production is considered as indirect mechanisms of growth promotion. In present study endophytic bacterial strain LS3E1 is the only isolate which has shown positive result for HCN production. Few bacteria inhibit the disease development indirectly by synthesizing HCN, there by strengthening the host's disease resistance mechanism (Whipps *et al.,* 2001).

Application of bioinoculants with multi trait plant growth promoting traits to the maize crop may improve the plant growth and production even under nutrient limiting conditions as it is the nutrient exhaustive crop. This study provides background information of effective plant growth promoting endophytic bacterial isolates. More than 50% of isolates were positive for N fixation, P solubilization, IAA production and siderophore production. The effective isolates should be further studied quantitatively and identified at molecular level. The culturable isolates can be further analyzed for their abiotic and biotic stress mitigating ability like osmotolerance, salinity tolerance and disease resistance.

Table 2. Qualitative evaluation of plant growth promoting traits of endophytic bacterial isolates from maize plants.

S.No	Isolate code	Plant Tissue	N fix	PSI	KSI	IAA	Siderophore	ACC Deaminase activity	HCN
1	AR3E2	Root	+	2.92	-	+	-	++	-
2	AlR2E5	Root	+	1.58	-	+	-	++	-
3	GC3E2	Kernel	+	2.33	-	+	+	++	-
4	BS2E1	Stem	-	1.26	-	-	+	++	-
5	VS1E1	Stem	-	3.69	1.29	-	+	++	-
6	VR1E1	Root	+	1.35	1.25	-	+	+	-
7	NL3E2	Leaf	+	3.93	1.18	+	+	+	-
8	NL3E3	Leaf	+	5.45	-	+	++	+	-
9	NL3E4	Leaf	+	5.16	-	+	+	-	-
10	NL3E5	Leaf	+	3.92	-	-	-	++	-
11	NC3E1	Kernel	-	2.27	-	+	-	+	-
12	NC3E2	Kernel	+	5.63	-	+	++	+	-
13	NR3E1	Root	+	1.73	-	+	+	++	-
14	NR3E3	Root	-	2.11	-	-	-	++	-
15	PdC3E2	Kernel	-	1.87	-	++	-	++	-
16	PdC3E3	Kernel	+	2.67	1.22	+	+	-	-
17	PdC3E4	Kernel	-	1.43	1.42	-	-	++	-
18	PdS3E1	Stem	+	4.41	-	+	++	-	-
19	PdS3E2	Stem	+	3.79	1.21	++	+	+	-
20	RgL3E4	Leaf	++	4.92	1.35	++	++	++	-
21	RgC3E2	Kernel	+	4.43	-	++	+	+++	-
22	JC3E1	Kernel	+	1.32	1.22	-	+	+++	-
23	JC3E2	Kernel	+	4.77	1.59	++	++	++	-
24	PL3E2	Leaf	+	2.27	1.15	-	+	+	-
25	CC3E3	Kernel	+	5.47	1.18	-	+	+	-
26	VaR3E1	Root	++	3.33	-	+	++	++	-
27	VaL3E1	Leaf	-	3.62	1.22	+	+	+	-
28	VaS3E1	Stem	+	1.53	1.14	-	-	++	-
29	KL3E1	Leaf	+	6.0	1.10	-	++	+	-
30	KL3E2	Leaf	-	6.0	1.25	+	-	-	-
31	KS3E1	Stem	++	3.92	1.25	+	++	+	-
32	KS3E2	Stem	+	1.47	1.29	-	-	+	-
33	LS3E1	Stem	++	4.08	-	+	++	++	++
34	LS3E2	Stem	+	2.43	-	++	+	+	-
35	LS3E3	Stem	++	5.8	1.60	++	++	++	-
36	LL3E1	Leaf	++	4.21	-	++	+	+	-

-No production, + Less production, ++ Medium production, +++ More production.
N Fix- Nitrogen Fixation, PSI- Phosphorous Solubilization Index, KSI- Potassium Solubilization Index

Authors' contribution

Conceptualization and designing of the research work (TN, VGA, VRJ, RRG, ST); Execution of field/ lab experiments and data collection (USM); Analysis of data and interpretation (USM, TN); Preparation of manuscript (USM, VGA).

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