

Original Research Article

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Diverse Novel Bacterial Endosymbionts and their Plant Growth Promoting Traits in association with Banana Plant

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ABSTRACT

Endophytes are endosymbiotic groups of microbes colonized in plants. These endophytes are of major interest due to multifaceted plant growth promotion (PGP) activities like production of various metabolites, their defense response towards plant pathogens and also help to combat the stress in plants. South Gujarat region is declared as an export fruit zone for mango, banana and sapota. Hence, present study aims to isolate the endophytes from the pseudostem and suckers of banana grown in South Gujarat region. Total 21 different bacteria were isolated, purified and identified by 16S rRNA. Majority of the genera belongs to *Bacillus* followed by *Klebsiella*. All these isolates were evaluated for their various PGP activities like P solubilization, Indole Acetic Acid (IAA) production, organic acid production, biofilm formation and their antagonistic activity against some plant pathogenic fungi. Isolates were examined for their ability to produce various organic acids. They showed promising results in various PGP activities. Organic acid production profile was also monitored using HPLC for various organic acid secrets by isolated organisms. They were also tested for their antagonistic activity against *Macrophomina phaseolina*, *Alternaria spp.* and *Sclerotinum spp.* of plant pathogen and showed promising results in inhibition of these pathogens. Among all the tested isolates isolates BN2 and BN5 belongs to *Klebsiella* and BN9 & BN15 belongs to *Bacillus sp.* showed more promising results and can be used as application of biofertilizer for banana plant.

Keywords

Antagonist activity,
Endophytes,
Organic acid,
PGPR, Phosphate
solubilisation

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Introduction

Plants are associated with a diverse community of microorganisms. Rhizospheric microbes play a key role in plant growth and also in suppression of plant pathogens by various mechanisms. All these mechanisms are influenced by root exudates and they proliferates microbial communities. Microbes are also responsible for root architecture of plants and hence provide high nutrition to plants. Among this plant growth promoting rhizobacteria (PGPR), some also reside inside the plant as endophytes and form symbiotic association with plants. Endophytes reside in plants and provide some nutrients to plants and they get shelter in host plants. Their interactions are unique in each ecosystem and are less explored. These endophytes are a great source of various natural metabolites which yet not fully explored for their beneficial effect on plants (Jasim *et al.*, 2014). As they reside inside the plants, they are more important to ameliorate the plant metabolism and plant health. Moreover, there is microbial succession of genera based on tissue type, age of plant, season of sampling.

Banana (*Musa*. sp) is an economically important fruit crop that is cultivated in many tropical and subtropical countries. India is a leading producer of Banana accounting for 27.43% of total banana production in the world. The important banana growing states are Tamil Nadu, Maharashtra, Gujarat, Andhra Pradesh, Karnataka, Bihar and Madhya Pradesh which together accounted for about 87.01 per cent of total banana production in the country. Among different states in the country, Gujarat has accounted for 13.44 per cent of total banana production and it ranks third in production. South Gujarat region of Gujarat State is known as export zone for mango, banana and sapota. Banana production and productivity in India are challenged by various biotic and abiotic stresses. One

approach involves the use of beneficial plant-associated microorganisms such as endophytes which can offer an environmentally safe method to increase productivity and alleviate different plant stresses in addition to reducing chemical inputs. The use of such plant growth promoting endophytes as bioinoculants could reduce the use of chemical fertilizers, environmental pollution and at the same time increase the yield and productivity of medicinal and other agricultural plants (de Souza *et al.*, 2015). Hence, present study aims to explore endophytes from banana pseudostem and its PGP traits for plant growth.

Materials and Methods

Sample collection

Different species of banana plant suckers and pseudostems were collected from Navsari Agriculture University farm at Navsari, Gujarat. Around ten species of banana suckers and its pseudostem were taken in the glass bottles and were transferred to the laboratory in cool boxes for the microbial isolation.

Isolation of endophytes from banana suckers

The banana suckers and pseudostem were surface sterilized to remove other surface bacterial load. First, the outer layer of tissues was removed with a sterile knife followed by a surface sterilized with 0.1% mercuric chloride for 10-15 min. Subsequently, it was washed thrice with sterile distilled water. 4 cm sterile sucker/pseudostem was added in sterile distilled water and crushed for endophytic bacteria. Sample was serially diluted and streaked on sterile Nutrient agar plates followed by incubation at room temperature for 24 to 48 hrs. Isolated colonies with different morphology were purified and stored on nutrient agar slant at 4 °C until use.

Identification of isolates

Isolated bacteria were identified by morphological, biochemical and molecular characterization. Colony morphology on an agar plate and cell morphology was recorded by Gram's reaction. Biochemical tests were performed as per Bergey's Manual of Systematic Bacteriology.

For molecular identification, genomic DNA was extracted using the CTAB (CetylTrimethyl Ammonium Bromide) method as described by Doyle and Doyle (1987). DNA was amplified with universal primer 27f and 1592r (Vyas and Murthy 2015). PCR product was purified and sequenced using ABI 3130xl genetic analyzer (Applied Biosystems, CA).

Sequence was searched for homology using the BLAST tool on the National Centre for Biotechnology Information (NCBI). Sequence was submitted to GenBank for accession number.

PGPR traits of endophytes

Isolated endophytic bacteria were screened for their following multiple PGP traits.

Indole acetic acid production

Isolates were screened for IAA production using methods described by Bric and co-workers (1991). Briefly 1% of 18 hrs grown culture of isolated bacteria was inoculated in 50 ml of nutrient broth and nutrient broth supplemented with tryptophan (1 mg/ml) as described by Vadnerker and co-worker (2018). After 3 days incubation, quantification was done using Salkowsky's reagent and optical density was measured at 535 nm. The concentration of IAA was extrapolated from the standard curve of IAA prepared using 100 µg/ml of standard IAA.

Phosphate solubilization

Phosphate solubilization was quantified using the method described by Subba Rao (1988). Isolates were grown in Pikovskaya's Broth and incubated for 7 days. The concentration of the soluble phosphate was estimated by stannous chloride method at 430 nm. Soluble P was extrapolated from the standard curve (0 – 20 µg/ml).

Organic acid profiling

Organic acid produced by bacteria lowers the pH and thus solubilize the P and other elements and thus make it available for plants. Various organic acids produced by bacteria were measured by HPLC. For organic acid profiling, cells were grown in Pikovskaya's Broth and after incubation cell free supernatant was collected. Supernatant was further passed through 0.2 µm pore size Millipore filter to remove any cell present in supernatant. Organic acid was estimated using C18 reversed phase HPLC column (250 mm x 4.6 mm with 5 µm particle sizes) using 0.008% sulphuric acid as mobile phase at flow rate of 1.0 ml/min. Organic acid present in the sample was detected 190 nm using PDA detector (Scherer 2012). Fumaric acid, Citric acid, Propionic acid, Acetic acid, Lactic acid, Malic acid, Pyruvic acid, Oxalic acid, Glutamic acid was run standard.

Ammonia production

Ammonia production was quantified by growing cells in peptone water for 3 days at room temperature. Supernatant was collected by centrifugation at 8000 rpm for 10 min. 0.5 ml of Nessler's reagent was added in 3 ml supernatant (Cappucino and Sherman 1992). If ammonia is produced by isolates, they develop brown to yellow color which is quantified at 450nm. The concentration of ammonia was extrapolated from the standard curve of ammonium.

Hydrogen cyanide (HCN) production

To examine HCN production, isolated bacteria were individually streaked on Nutrient Agar supplement with Glycine. A Whatman filter paper number 1 soaked in 2% w/v sodium carbonate in 0.5% picric acid solution was placed inside the lid of a petri plate. Petri plates were sealed with parafilm and incubated at room temperature for 2 days. A change in the filter paper color from yellow to brown was considered positive for HCN production (Lorck 1948).

Biofilm formation

Biofilm formation was observed by growing cells in LB broth in the test tube for 24 hrs. After 24 hrs, media broth was removed and biofilm formation on the test tube wall was visualized by staining using methylene blue. Isolates that were able to form biofilm showed purple color ring or purple color walls. Potential isolates were examined for quantitative EPS production by phenol-sulfuric assay (Dubois *et al.*, 1951).

EC tolerance

All the potential isolates were tested for their ability to tolerate EC. For EC tolerance, LB medium was adjusted to various EC such as 2, 4, 6 EC. Each flask was inoculated with 0.1 ml of 24 h old bacterial culture broth and incubated at room temperature. Growth was monitored in terms of increased OD at 660 nm at 24 hrs interval upto 72 hrs.

Antagonistic activity

The endophytic isolates were tested for their antagonistic response against plant pathogens *Macrophomina phaseolina*, *Alternaria* and *Sclerotium* by dual culture method (Fokkema 1978). All the plates were incubated at room temperature for 7 days and observed for

determination of antagonistic activity. Potential isolates were further tested for quantitative evaluation for their antagonistic potential. The quantitative evaluation of antagonism by test strain was carried out by the method described by Trivedi and co-worker (2008) with some modification. The test strains were allowed to grow for 24 hours in the LB broth. Simultaneously the phytopathogens (*Macrophomina phaseolina*, *Alternaria sp.* and *Sclerotium sp.*) were allowed to grow for 48 hrs in PDA broth. 50 µl of overnight grown bacterial cultures were inoculated into the PDA broth containing respective pathogenic fungi. The broth having only fungi was kept as control for determination of inhibition percentages. After incubation of 48 hrs, the culture was passed through pre-weighed filter paper and filter paper was dried. Biomass was measured as difference with control flask. The dry weight of fungi + bacteria (W2) and fungi alone (W1) were recorded and % reduction in weight was calculated using the formula: $(W1-W2/W1) \times 100$.

Results and Discussion

Isolation of endophytes from banana suckers

From the various samples of banana sucker and pseudostem, total 21 (designated BN1 to BN21) different bacteria were isolated. All these bacteria were selected based on different colony morphology and were isolated and purified. These isolates were stored on nutrient agar slant until use.

Identification of isolates

All the bacteria were identified for their morphological, biochemical and molecular characterization. Colony characteristics of the 21 bacterial endophytes from suckers and pseudostem of banana plant were noted. They

were round or irregular, smooth, medium, moist or dry, translucent to opaque, flat, raised to convex. Biochemical characteristics like methyl red(MR), Vogesproskauer(VP), oxidase, catalase, indole production, urea hydrolysis, gelatin hydrolysis, citrate production, nitrate reduction, and fermentation of sugar like ribose, lactose, galactose, sucrose, maltose etc were recorded (data not shown here). All these isolates were identified using 16S rRNA and sequence was submitted to GenBank for accession number. Among divers endophytes isolated, the highest number of isolates belongs to genera *Bacillus*, followed by *Klebsiella* sp. (Fig. 1).

PGPR traits of endophytes

Indole acetic acid production

IAA is important for plant growth promotion activity. All the isolates were evaluated for their ability for production of IAA. All isolates were able to produce IAA as they form pink color on reaction with salkowski reagent. However among all these isolates, BN1 produced highest IAA (259 ppm) whereas, BN12 produced least (17 ppm) (Fig. 2a).

Phosphate solubilization

All the isolates were tested for their ability to solubilize phosphate and revealed that all were able to solubilize Tri calcium phosphate. Among all the isolates BN6 (275 ppm) showed highest P solubilization followed by BN13 (244 ppm) (Fig. 2b). Least was reported in BN20 (15ppm).

Organic acid production

One of the important traits of PGPRs is organic acid production. Organic acid production lowers the soil pH and thus increases availability of P to plant. Hence, all the isolates were tested for their ability to

produce nine different organic acid viz., acetic, citric, formic, glutamic, lactic, malonic, oxalic, propionic and pyruvic acid. Isolates grown in pikovskaya broth supplemented with tri-calcium were observed using HPLC. Data revealed that all the isolates produced one or more organic acids in the medium. Among all the acids evaluated, all the isolates except four were able to produce formic acid. Another major acid production by majority isolates was citric acid followed by oxalic acid and propionic acid. Thus production of difference acid by isolates fell in the category from higher to lower as formic acid > citric acid > oxalic acid and propionic acid > glutamic acid > lactic acid > pyruvic acid > malonic acid > acetic acid. Thus only three isolates were able to produce acetic acid (Table 1).

Ammonia production

All the isolates showed positive for ammonia production. Higher ammonia production was reported in BN17 (1.60 ppm) followed by BN8 (1.21 ppm) (Fig. 3a). Least was reported in BN7 (0.36 ppm). After three days of incubation on estimating ammonia, maximum concentration of ammonia was produced 1.065 ± 0.135 ppm by BN 8 and BN 17 and isolates, minimum amount ammonia production 0.43 ± 0.04 ppm produced by BN 4.

Hydrogen cyanide (HCN) production

HCN production was examined for all the isolated bacteria. All these isolates changed the yellow color filter paper to brown color and showed positive for HCN production.

Biofilm formation

All the isolates were tested for their ability to form biofilm. When they have stained with crystal violet, BN9 showed the positive for biofilm. BN9 which showed positive for biofilm was further evaluated for the amount

of exopolysaccharide produced by isolate. Phenol sulfuric assay revealed that it produced 27 µg/ml of EPS. Thus, BN9 was able to secrete polysaccharide which probably plays a role in their biofilm formation.

EC tolerance

EC, pH and salt tolerance was also examined for all the isolated PGPRs. All the isolates were able to grow in broth having 2, 4 and 6 EC. Increased in growth was monitored after 24 hrs at 24 hrs interval up to 72 hrs at 660 nm. Highest growth was achieved after 72 hrs of incubation in 2, 4 and 6 EC (Fig. 3b).

Antagonistic activity

All the isolates were examined for their antagonistic activity against plant pathogens *Macrophomina phaseolina*, *Alternaria* and *Sclerotium* by dual culture method. *Macrophomina phaseolina* pathogen was inhibited by BN2, BN3, BN15 and BN16 PGPRs, whereas, *Sclerotium* was inhibited by BN4 and BN5.

Alternaria was inhibited by BN9, BN10, BN15 and BN16. These isolates which showed inhibition of pathogenic fungi were further tested for their quantitative ability to inhibit plant pathogens.

Rests of the isolates were not able to show antagonistic activity. In quantitative antagonistic assay, highest inhibition of *Macrophomina* was observed by BN2 (91 %) followed by BN3 (Fig. 4). *Sclerotium* was inhibited 90 % by BN4 and BN5, whereas, *Alternaria* was highest inhibited by BN9 (98 %) followed by BN15 (Fig. 4). Thus, some endophytes were able to suppress growth of tested plant pathogens. Banana is important plant and hence, interaction of microbes with banana plants plays a crucial role in either beneficial or deleterious effects on plants. In

the present study, diverse endophytic bacteria associated with banana pseudostem and suckers were isolated for their various PGP traits. Total 21 isolates based on their diverse colony morphology were selected and purified for further studies.

As PGPR, isolated endophytes can increase the growth of plants directly, indirectly or synergistically (Bhattacharyya and Jha 2012).

Some direct mechanisms of PGPRs are P solubilization, IAA production, N fixation, etc. (Mullen MD 2005; Glick BR 1995), whereas, some indirect PGP activities are production of antimicrobial compounds, HCN production, chitinase production, siderophore production etc.

Total 21 PGPR isolates belonged to 10 different genera. Among them, the major belonged to *Bacillus* (7 isolates), followed by *Klebsiella* (4 isolates). Their morphological examination revealed that out of 21 isolates, 8 were Gram positive whereas, rests 13 were Gram negative.

Thus, Gram negative bacteria were dominating in association with banana plants. Some unique associations were also observed like the presence of *Kosakonia oryzae* and *Pantoea* which are majority reported in association with paddy. *Klebsiella* (4 isolates) and *Rhizobium* (2 isolates) were also present indicating their ability to fix nitrogen. Su and co-worker (2017) have also reported 11 different genera from banana endophytes and evaluated their antagonistic effects against nematodes. Zakaria and Aziz (2018) have worked on fungal endophytes of banana leaves.

They have reported isolates belonged to 10 different genera and comprised 17 different species. Karthik and co-worker (2017) have also reported 8 genera and 10 species.

Table.1 Organic acid ($\mu\text{g/ml}$) production by various isolated endophytic bacteria from banana plant

Isolates	Acetic acid	Citric acid	Formic acid	Glutamic acid	Lactic acid	Malonic acid	Oxalic acid	Propionic acid	Pyruvic acid
BN1	-	12.6	204	57.11	-	-	-	32.4	8.76
BN2	-	-	0.72	0.261	23.96	-	11.9	0.37	-
BN 3	1.66	64.4	98.6	-	31.84	0.052	-	1.66	-
BN 4	-	1.83	111	-	-	-	-	-	51.1
BN 5	-	12.6	134	-	15.88	-	-	-	-
BN 6	5.64	-	92.4	-	-	-	5.64	-	-
BN 7	-	32.3	111	-	-	-	-	-	-
BN 8	-	9.22	-	-	14.06	-	1.49	1183	-
BN 9	-	-	-	-	-	-	1.30	2.33	-
BN 10	-	-	111	-	2.602	-	1.69	176	-
BN 11	-	-	101	-	-	6.29	-	-	38.0
BN 12	-	-	40.9	0.059	-	0.5799	0.98	0.10	66.6
BN 13	-	1.05	-	0.077	0.3949	-	-	-	110
BN 14	-	0.02	100.	-	-	-	-	-	-
BN 15	-	56.2	83.2	-	-	-	2.46	-	-
BN 16	-	33.2	91.1	-	-	-	2.11	-	-
BN 17	-	165	91.9	-	-	-	-	-	-
BN 18	11.2	-	91.1	-	-	-	-	5.29	-
BN 19	-	2.29	125	39.56	-	2.3692	-	2.39	-
BN 20	-	-	92.3	0.699	-	-	-	-	-
BN 21	-	-	-	0.270	-	-	6.73	-	-

Fig.1 Phylogenetic tree of the endophytes isolated from banana plants

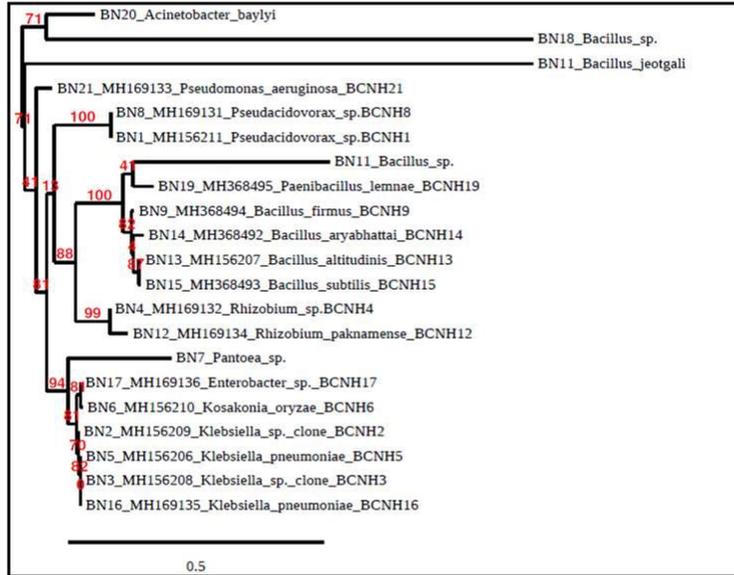


Fig.2 Production of IAA (a) and P solubilization (b) by banana endophytes

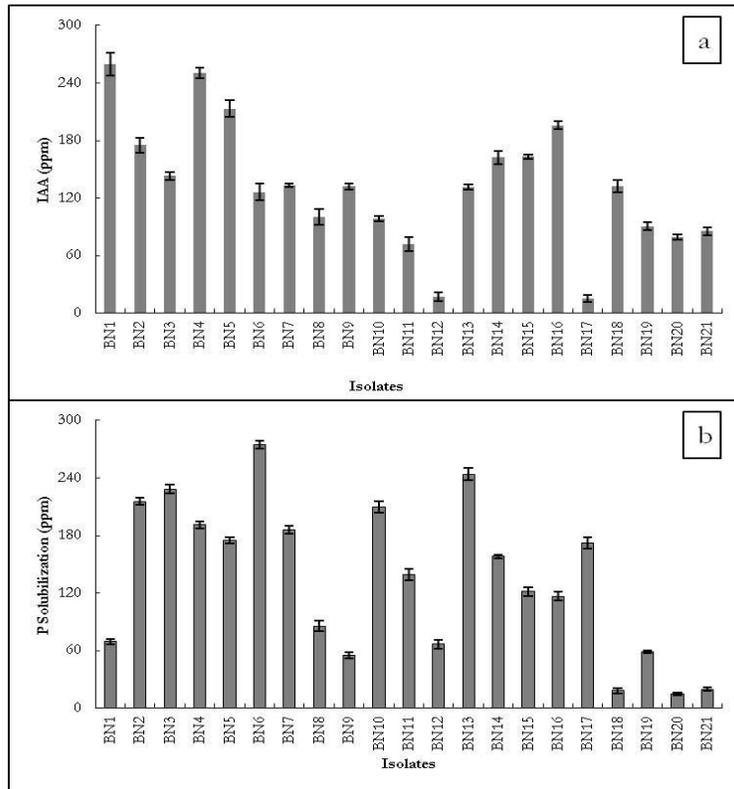


Fig.3 Ammonia production (a) and EC tolerance (b) by banana endophytes

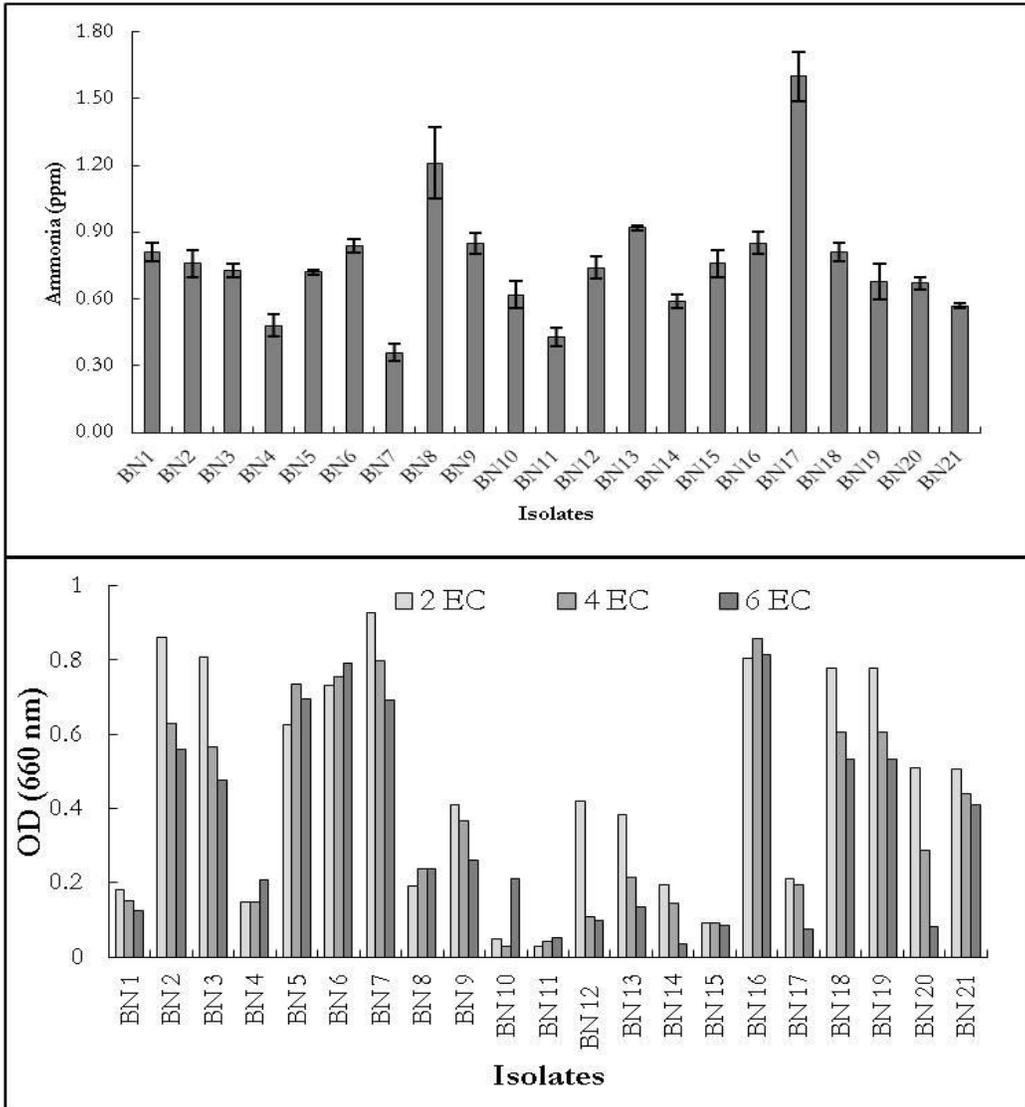
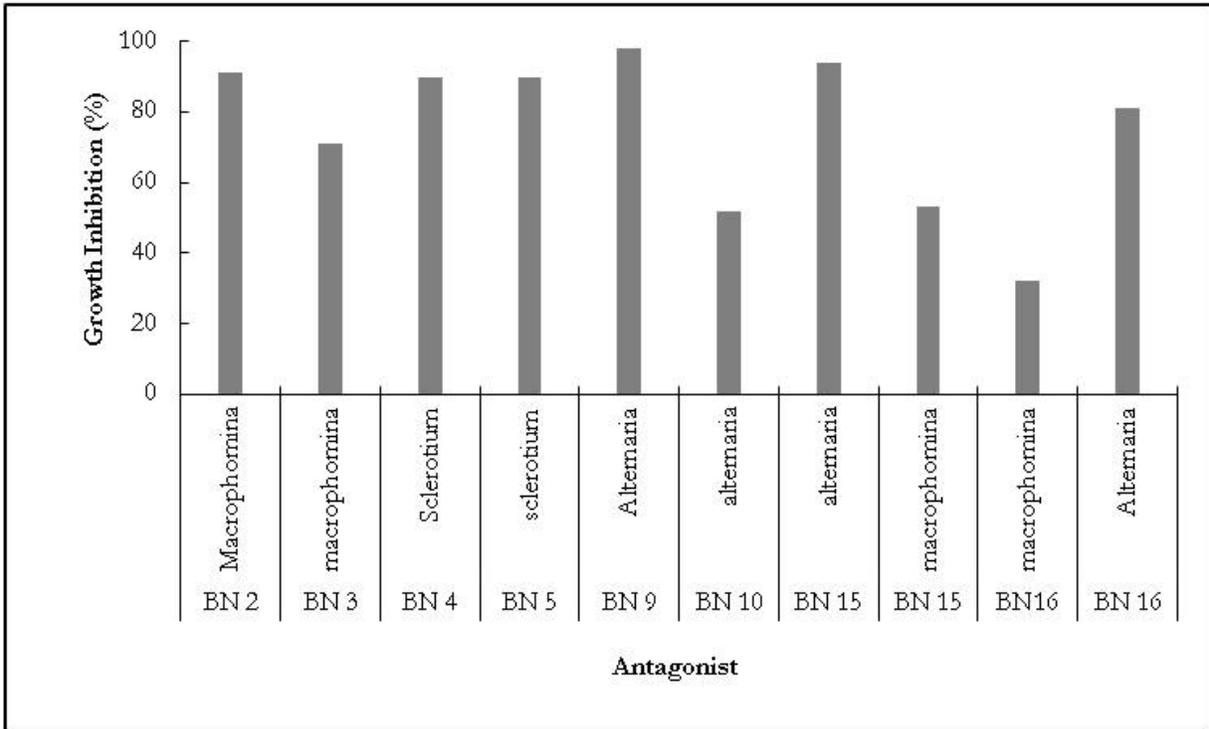


Fig.4 Antagonistic activity of isolates against plant pathogens



All these data are in accordance with present findings where they have reported *Bacillus*, *Klebsiella*, *Pseudomonas* and *Rhizobium* are common genera (Zakaria and Aziz 2018; Su *et al.*, 2017). Vyas and co-worker (2017) have also reported that application of organic manure has more growth promotion in banana plant compared to control or inorganic manure application.

Another PGP trait of bacteria is production of phytohormone IAA. IAA is an important plant hormone in play a role in differentiation of cell, cell division, apical growth, development of root, initiation of lateral root formation etc. Apart from this, IAA is also required in some physiological processes like photosynthesis, pigment synthesis, formation of various metabolites and providing resistance in stress conditions (Ryu and Patten 2008). As IAA is responsible for increased root length and initiation of lateral root formation, consequently uptake of nutrients is also

increased and thus overall plant growth (Vessey, 2003). In the present report all the isolates showed positive for IAA production. BN1 was reported as a potential IAA (281µg/ml) producer.

In soil, the majority of the phosphate is either immobilized or unavailable for plants. However, PGPR produces organic acid and solubilizes P and makes it in available form. However, total organic acid production by the various microbes is very important to determine the solubilization of phosphate rather than considering individual acid (Oburger *et al.*, 2009; Chen *et al.*, 2006; Song *et al.*, 2008). There is always ambiguity regarding various organic acids produced by various isolates due to complex growth requirements and physiological status (Pérez *et al.*, 2007). Our results are in accordance with de Abreu and coworkers (2017). They have reported that various bacterial isolates belongs to *Bacillus* and *Enterobacter* were

able to solubilize P. However, majority of the reports showed that gluconic acid, 2-ketogluconic acid and oxalic acids are the major acid produced by PGPR during P solubilization (Behera *et al.*, 2017; de Abreu *et al.*, 2017). Thus present data are in contrast to the mentioned reports.

In present study isolates were evaluated for their ability to produce ammonia and HCN. These both activities were positive for all the isolated bacteria. The current study showed that isolated twenty one bacteria *Klebsiella* spp. had remarkable antagonism against test pathogens *Alternaria* sp., *Macrophomina* sp. and *Sclerotium* sp. Among them, *Klebsiella* spp. was found to be more antagonistic against most of the test pathogens followed by the *Bacillus*, which predominantly inhibit growth of *Alternaria* sp. Similarly, *Rhizobium* exhibited antagonistic activity against *Sclerotium* sp. Antagonistic activities of endophytic bacteria facilitate the plants to survive in the biotic stress. Similarly, *Bacillus*, *Klebsiella* sp and *Pseudomonas* sp showed anti fungal effect against test pathogen *Sclerotium rolfsii*, *Macrophomina phaseolina*, *Alternaria* sp, *Fusarium solani* and *Fusarium oxysporium* (Karthikeyan *et al.*, 2006; Hameeda *et al.*, 2006; Senthilkumar *et al.*, 2009; Das *et al.*, 2015). There may be inhibitory volatile and diffusible metabolites produced by the isolated microbes which sometimes inhibit the toxin production of the fungi (Shifa *et al.*, 2015). The isolates may produce of bacteriocins, lytic enzymes as well as siderophore. Among the reported various fungal and bacterial isolates so far, BN2, BN9, BN4, BN5 and BN 15 could be the most potential isolates to inhibit growth of test fungi. By the use of such biocontrol agents with PGPR activities could substantially reduce the cost of chemicals. Shifa and co-worker (2015) tested efficacy of biocontrol strain *Bacillus subtilis* strain G-1 and recorded an inhibition of 28% in mycelial growth of *S.*

rolfsii. Endophyte of root nodules of *Vigna mungo* L. identified as *Klebsiella pneumonia* possessed antagonistic effect and inhibited 73.3% and 50.8% growth of *M. phaseolina* and *A. alternate* respectively. Their study is consistent with the results of the current work. There are several reports supports our claim of antagonism of *Bacillus* Sp. against *Sclerotium* and *Macrophomina phaseolina* by *Bacillus subtilis* (Liu *et al.*, 2008).

Plant growth promoting bacterial endosymbiont are gaining interest due to their multiple beneficial effects on plant growth and as biocontrol agents. Present study reported novel diversity of banana endophytes and their plant growth promoting activities. All possess different PGP activity however, together synergistically they provide the majority of the macro and micro nutrients requirement to the plant. Along with these, they are also able to suppress some plant pathogens like *Macrophomina*, *sclerotium* and *alternaria*. Among all the isolates *Bacillus* and *Klebsiella* possess majority of all PGP traits and antagonistic activity. Hence, together both these isolates can be used as biofertilizer and biocontrol agent for banana plant. Present study reports both, diversity of endophytes and their PGP traits which are not reported yet.

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