Journal of Root Crops, 2013, Vol. 39 No. 2, pp. 154-162
Indian Society for Root Crops
ISSN 0378-2409

Isolation and Characterization of N Fixing Bacteria from Elephant Foot Yam (*Amorphophallus paeoniifolius* (Dennst.)Nicolson) Growing Regions of South India

I.P. Anjanadevi[1], Neetha Soma John[1], K. Susan John[1], M.L. Jeeva[1] and R.S. Misra[2]

[1]Central Tuber Crops Research Institute, Sreekariyam, Thiruvananthapuram 695 017, Kerala, India
[2]Regional Centre of Central Tuber Crops Research Institute, Bhubaneswar 751 019, Odisha, India
Corresponding author: I.P. Anjanadevi, e-mail: adipmicrobiome@gmail.com
Received: 5 September 2013; Accepted: 31 December 2013

Abstract

Isolation and screening of 130 bacteria from the rhizosphere region of elephant foot yam (*Amorphophallus paeoniifolius*) growing area of South India for their ability to fix atmospheric N resulted in the identification of 62 isolates having varying levels of N fixing ability in Jensen's N free medium. The most potent N fixing isolates were tested for their efficiency to release plant growth promoting substances like indole acetic acid (IAA), ammonia (NH_3), hydrogen cyanide (HCN) and catalase enzyme. Morphological and molecular characterization of the two potent bacterial strains led to their identification as *Bacillus cereus* strain ANctcri1 (HQ286640) and *Pseudomonas aeruginosa* ANctcri11 (JX974833). A study on the effect of various physiological conditions on the growth of these potent bacteria revealed their facultative alkaliphilic halo tolerant nature.

Keywords: N fixing bacteria, Kjeldhal technique, PGPR, DNA isolation, 16srRNA characterization, physiological conditions

Introduction

Elephant foot yam(*Amorphophallus paeoniifolius* (Dennst.) Nicolson) is an important tropical tuber crop that offers unprecedented scope for adoption as a cash crop due to its high production potential and popularity as a vegetable in various cuisines. It is a medicinal plant and possesses antibacterial and antioxidant activities. These prospects make this crop an important tuber crop not only in India but also all over the world. In India, it is grown mostly in West Bengal, Andhra Pradesh, Karnataka, Kerala, Maharashtra, Tamil Nadu, Uttar Pradesh, Punjab, Bihar, Assam and Odisha.

Nitrogen is a major mineral nutrient required for the growth and productivity of elephant foot yam and its deficiency both in the soil as well as in the crop adversely affects the growth and yield of the crop. It is well known that, biological N fixation is one way of converting elemental inert atmospheric N into plant usable form as organic compounds through its enzymatic reduction by N fixing microbes (Gothwal et al., 2007). Since chemical fertilizers are costlier and its imbalanced application can affect soil health, application of beneficial N fixing microorganisms as a substitute to chemical N fertilizer in an integrated approach is a viable strategy. Research work on crop nutrition associated with N fixers has increased during the last two decades after the research work of Dobereiner in *Azospirillum* (Bashan, 1998; Baldani et al., 1997).

Important N fixing bacteria associated with plants are *Bacillus megaterium, Bacillus cereus, Bacillus pumilus, Bacillus circulans, Bacillus licheniformis, Bacillus subtilis, Bacillus brevis, Pseudomonas, Azospirillum, Azotobacter, Klebsiella, Enterobacter, Alcaligenes, Arthrobacter, Burkholderia* and *Serratia* (Xie et al., 1998; Kloepper et al., 1989; Glick, 1995). Asilah et al. (2012) proved that acquisition of N through N fixation by *Bacillus* sp. can support plant growth without the application of fertilizer N at the vegetative stage of rice.

Apart from the N fixing activity, plant growth promotion also was enhanced directly through the production of metabolites such as auxins (Asghar et al., 2002), cytokinins and indirectly through the elimination of pathogens by the production of cyanide (Owen and Zlor, 2001) and siderophores. Plant growth promoting rhizobacteria (PGPR), which was first defined by Kloepper and Schroth (1978) hold great promise as biocontrol agent and is an innovative approach to promote plant growth, crop yield and control of various plant diseases (Prashanth and Mathivanan, 2010). There are reports on the increased microbial activities in the plant rhizosphere because of the activities of different soil bacteria (Waksman and Starkey, 1931; Katznelson et al., 1948). Though many N fixers have been already identified, the native isolates were found to be more competent in colonizing the rhizosphere.

Taking into account the significance of N fixing bacteria as biofertilizer and biocontrol agents in crop production, the study was taken up with the objective of isolating and characterizing N fixers from the rhizosphere of elephant foot yam (*Amorphophallus paeoniifolius*) cultivated in the two major southern states of India, viz., Kerala and Tamil Nadu. The potent N fixing isolates identified were further screened *in vitro* for theirmajor plant growth promoting traits like production of indole acetic acid (IAA), ammonia (NH_3), hydrogen cyanide (HCN) and catalase as well as their morphological and molecular characterization. The effect of different physiological conditions on the growth of the potent bacteria was also studied.

Materials and Methods

Sampling

Rhizosphere soil samples (three samples each from each site) were collected in sterile clean containers from the major elephant foot yam growing areas of Kerala and Tamil Nadu for undertaking the studies described below.

Isolation and screening

The isolation of bacteria from the collected soil samples was performed by serial dilution and plate counting technique. The rhizosphere bacteria isolated were purified in nutrient agar (NA) medium and were maintained in nutrient agar slants at 4° C. These isolates were then streaked on Jensen's agar medium. After streaking, the plates were incubated at 28°C for 48 h followed by visual observation for bacterial growth. Isolates that grew successfully were subcultured and maintained in Jensen's agar slants for further studies.

Quantification of N fixing capacity

The isolates which showed positive and predominant growth during the first 3 to 4 days were selected for quantification using the Kjeldahl N digestion and distillation system (Kelplus system, Classic Dx[VA]).The selected isolates were incubated in 10 ml of Jensen's broth in a rotary shaker at 150 rpm for 5 days at 28° C. The amount of N fixed in the microbial tissues contained in the broth was determined by the method described by Kizilkaya (2009). From the quantification result, two potent N fixing isolates having the highest N fixing ability were selected for further characterization.

Morphological characterization of potent bacteria

The two isolates which showed highest N fixing activity among the 62 isolates were streaked in nutrient agar plate and incubated for 24 h at 28°C. After incubation, the colony morphology was studied. Gram staining was also performed to identify the morphology of the two potent strains.

Molecular characterization

Molecular characterization of the potent bacteria was carried out using a modification of the method suggested by Babu et al. (2004) as follows.

DNA isolation

The two potent N fixers isolated were incubated overnight in Luria broth and were used for extracting genomic DNA. After incubation, the broth was centrifuged at 12,000 rpm for 10 min. The pellet was resuspended in 450 μl tris EDTA buffer. To this, 45 μl of 10% SDS and 25 μl of 10 mg ml^{-1} proteinase K were

added and mixed well by inverting the tube and incubated at 37^{0}C for 1 h. After incubation, extracted twice with 500 μl phenol: chloroform mixture (1:1) and twice with 500 μl chloroform by centrifuging the sample each time at 12,000 rpm for 10 min. The nucleic acid was precipitated by adding 25 μl of 5M NaCl and 1ml of 95% ethanol. The mixture was centrifuged for 10 min at 12,000 rpm at room temperature. The supernatant was discarded and the pellet was suspended in TE buffer (30 μl). The quality and integrity of the DNA isolated was checked using 0.8% agarose gel electrophoresis with1kb size DNA ladder (Fermentas).

PCR amplification and sequencing

The 16S rRNA was amplified from the genomic DNA using universal primers (8F: 5'-AGAGTTTGATCCTGGCTCAG-3' and 1492R: 5-' CGGCTACCTTGTTACGACTT-3') (Dojka et al., 1998). The PCR was performed in 25 μl reaction volume consisting of the following components: DNA–2 μl, Taq buffer-2.5 μl, dNTP mix–1 μl, primer (forward)-1 μl, primer (reverse)– 1 μl, Taq polymerase– 0.5 μl. PCR regime used was as follows: initial denaturation at 92°C for 2 min 10 seconds followed by 35 cycles of denaturation at 94°C for 1 min 10 seconds. Annealing temperature of primers was 49°C for 30 seconds and extension at 72°C for 2 min. A final extension was performed for 10 min at 72°C. The amplified products were eluted using the QIA quick gel extraction kit and sequenced directly by outsourcing (Chromous Biotech, Bengaluru, India). Sequence analysis was performed using sequences available in the National Centre for Biotechnology Information (NCBI) database using BLAST algorithm. Phylogenetic analyses of the 16S rRNA gene sequences were conducted with MEGA 5.2 and trees were generated by neighbour-joining method. The robustness of the branches was inferred by bootstrap replication (2000 replicates).

Assay for plant growth promoting activities

Tests for plant growth promoting attributes like indole acetic acid (IAA), ammonia (NH_3), hydrogen cyanide (HCN) and catalase production were conducted for the two potent bacteria.

Indole acetic acid

Indole acetic acid (IAA) production was detected as described by Ahmad et al.(2005) with modifications. Bacterial cultures were grown for 4 days in their respective media with L-tryptophan (1 mg ml^{-1}) at 28°C. Fully grown cultures were centrifuged at 4000 rpm for 15 min. The supernatant (2 ml) was mixed with two drops of orthophosphoric acid and 4 ml of the Salkowski reagent (mixture of 50 ml, 35% of perchloric acid and 1 ml 0.5 M $FeCl_3$ solution) and development of pink colour in the mixture indicated IAA production and its concentration was determined using a spectrophotometer at 540 nm wavelength against a standard curve.

Ammonia

Bacterial isolates were tested for the production of ammonia as described by Joseph et al. (2007).

Hydrogen cyanide (HCN)

HCN production by the isolates was detected by the method of Bakker and Schipper (1987).

Catalase

Catalase test was performed according to the method reported by Kannahi and Kowsalya (2013).

Effect of physiological conditions on the growth of potent N fixing Bacteria

Effect of various growth conditions such as temperature, salt tolerance and pH on the growth of the most potent N fixers were checked in nutrient broth. For studying the effect of temperature, potent bacteria were incubated at temperatures viz., 15°C, 20°C, 25°C, 35°C, 45°C, 55°C, 65°C and 75°C for 24 h at 150 rpm. Nutrient broth supplemented with different concentrations of NaCl (ranging from 0-15%) was used for salt tolerance studies and the hydrogen ion concentration in the range of 3-14 was selected for pH studies. The flasks were incubated at room temperature for 24 h in a rotary shaker at 150 rpm. The growth and activity of the potent N fixing bacteria in the given growth conditions were observed by taking the optical density of the medium.

Results and Discussion

Isolation, screening and quantification of N fixers

The total bacterial population (average of three samplings) (cfu g^{-1}) obtained by serial dilution technique ranged from 9-14$\times$10^8 from soils of Kerala and 2-5$\times$10^8 from that of Tamil Nadu. A total of 130 bacterial

strains obtained from the two states were purified by repeated streak culture on nutrient agar (NA) medium. It is seen by preliminary observation of morphological analysis of the streaked isolates that the population of *Bacillus* spp. dominated in the rhizosphere of elephant foot yam collected from Kerala. Bahig et al. (2008) reported the presence of diverse population of *Bacillus* spp. and *Pseudomonas* spp. from the cultivated soil treated with canal water and waste water. All isolates were tested for their N fixing activity by an agar assay using Jensen's N free medium. A total of 62 bacteria showed the capability to fix atmospheric N in the media. However, only 40 isolates that exhibited prominent growth during the first 2-4 days were selected for further studies. By using Kjeldhal's method, the N fixing capacity of each of the 40 bacteria were quantified and the results of major 24 are represented in Fig. 1. It can be seen that, the first 1 to 14 isolates represents the efficient strains from soils of Kerala and 15 to 24 from Tamil Nadu. Among the different isolates screened, NF12 showed the maximum N fixing capacity of 21.72 μg g^{-1} followed by NF23 (19.75 μg g^{-1}). These isolates were identified as potent N fixers for further investigation on morphological, molecular and plant growth promotion characteristics.

In the present study, from the total isolated bacteria, 46 % of isolates were found capable of fixing atmospheric N. The isolates which grew well in the media for longer duration were selected for determining the N fixing efficiency. It was found that among the total N fixers, only 67% of the isolates were capable of living up to 3 - 4 days in the N free media. The bacteria NF12 and NF23 showed the highest N fixing ability. Kizilkaya (2009), assessed the N fixing potential of *Azotobacter* spp. in pure culture isolated from different soils by Kjeldhal method and some of the isolates showed similar N fixing capacity akin to our cultures. Dobereiner (1969) also found that free living organisms were able to fix 12-30 mg N g^{-1} of C source. Rhizobial strains were also studied for their rate of N fixation by inoculating them in YEM media using Kjeldahl method by Hajare and Ade (2012).

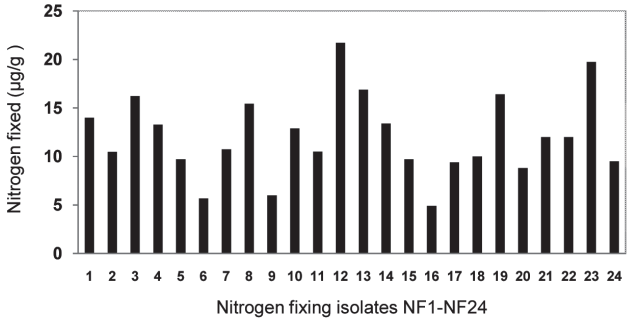

Fig.1. Nitrogen fixing capacity of bacterial isolates. 1 to 14 isolates represents the efficient strains from soils of Kerala and 15 to 24 from Tamil Nadu. Values are average of three replicates

Morphological and molecular characterization

When morphological characteristics were studied by gram stain method, NF12 was found as rod shaped, violet coloured gram positive cells and on nutrient agar media, it appeared as large irregular, raised and opaque colonies. NF23 was rod shaped, pink coloured gram negative bacteria and the colonies were small, round, slightly raised and blue green pigmented. The PCR amplified products were gel checked (Fig. 2) and purified by gel extraction before sequencing. On sequencing, partial sequences of about 1434 (from NF 12) and 770 (from NF23) bases were obtained from the 1.5kb amplicon. The gene sequences obtained were used as BLAST queries against the NCBI database. The results indicated that the partial sequences of 16S rDNA of NF12 and NF23 showed 99% similarity towards *Bacillus cereus* and *Pseudomonas aeruginosa* respectively. Phylogenetic tree were constructed with closely and distantly related species and genera of *Bacillus* and *Pseudomonas* sp. (Fig. 3). The obtained sequences were deposited in the GenBank with accession numbers HQ286640 and

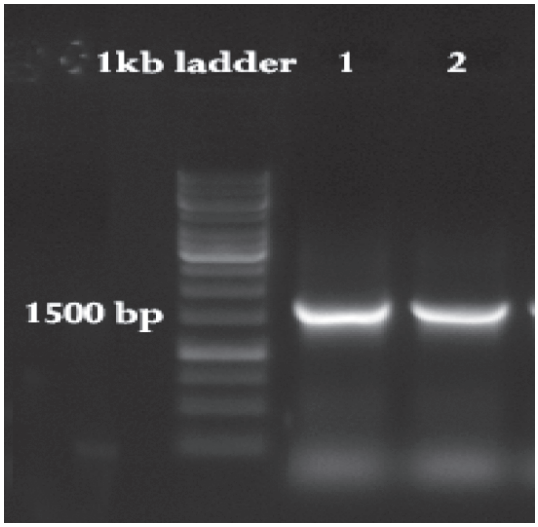

Fig. 2. Agarose gel electrophoresis of 16s rDNA of 1. *B. cereus* (ANctcri1); 2. *P. aeruginosa* (ANctcri11)

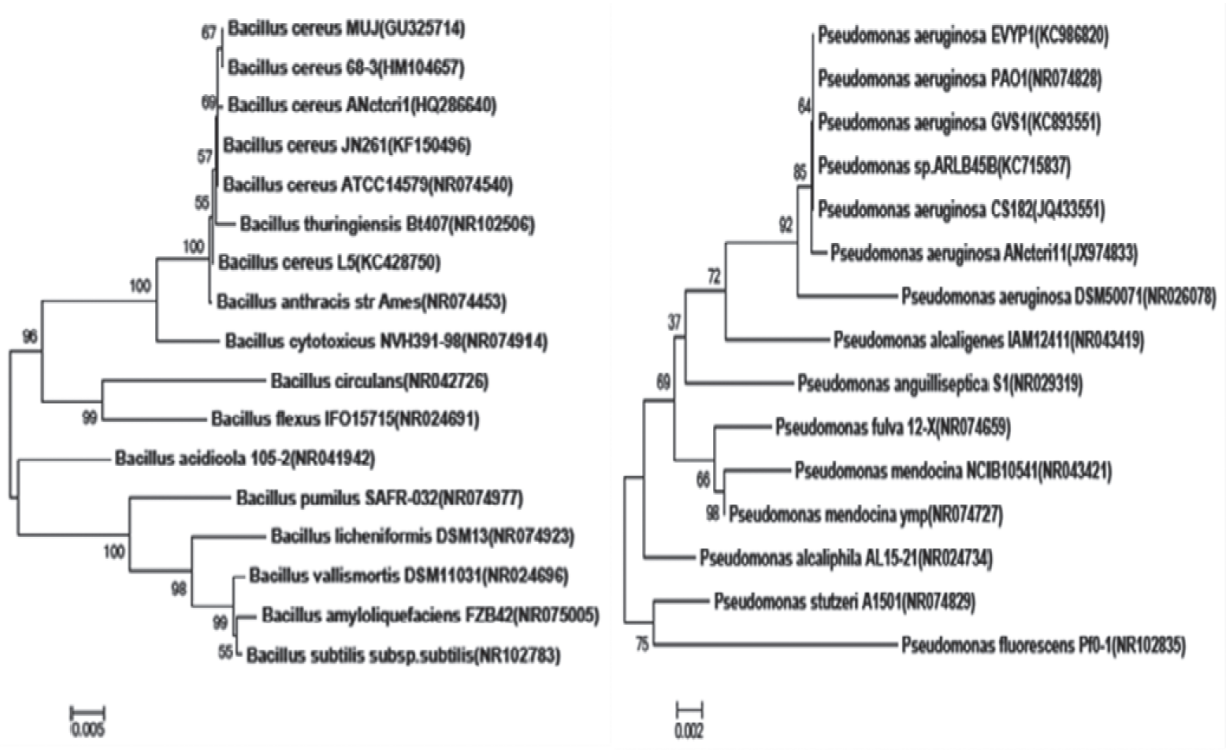

Fig.3. Phylogenetic relationships of *Bacillus cereus* strain ANctcri1 and *Pseudomonas aeruginosa* strain ANctcri11 based on partial 16S rDNA sequences (neighbour-joining method with 2000 bootstrap replication)

JX974833 under the name, *Bacillus cereus* strain ANctcri1 and *Pseudomonas aeruginosa* strain ANctcri11 respectively. The potent isolates were morphologically and molecularly identified as *Bacillus cereus and Pseudomonas aeruginosa*. Bosshard et al. (2003) stated that 16s rRNA gene sequence analysis was better for the identification of bacteria compared to the usual phenotypic methods. Hurek et al. (1993) identified and characterized 14 strains of N fixing bacteria from different Brazilian agricultural plant species including cassava, maize and sugarcane using 16srRNA sequencing technique. Xie et al. (1998) and Ding et al. (2005) reported *B.cereus* as N fixing bacteria based on nitrogenase activity and also by identifying the nifH gene.

Plant growth promoting activities

The potent isolates were tested for varying levels of PGPR traits and they showed varying degrees of these activities. *P. aeruginosa* (ANctcri11), produced maximum quantity of IAA (16.8 μg ml^{-1}) followed by *B. cereus* ANctcri1(12 μg ml^{-1}). Production of ammonia was also detected in both strains showing yellow-orange colour in *P. aeruginosa* (ANctcri11) and orange brown colour in *B. cereus* (ANctcri1), which in turn indicated higher evolution of ammonia in *B. cereus* (ANctcri1) compared to *P. aeruginosa* (ANctcri11). Both the strains produced the enzyme catalase in the presence of H_2O_2, but *P. aeruginosa* (ANctcri11) alone produced HCN. PGPR activity of potent isolate is given in Table 1.

Apart from N fixing activity, the potent isolate *B. cereus* (ANctcri1) had IAA, NH_3 and catalase producing ability. Many species of *Bacillus* and *Bacillus* derived genera have a role in plant growth promotion by solubilization of nutrients, production of IAA like substances, antifungal antibiotics and siderophores (Prashanth and Mathivanan,

Table 1. *In vitro* production of IAA, siderophore, HCN and ammonia by the potent N fixers

Bacteria	Nitrogen fixed (μg g^{1})	Production of IAA (μg ml^{-1})	Ammonia	HCN	Catalase
B. cereus (ANctcri1)	21.72	12	+++	-	+++
P. aeruginosa (ANctcri11)	19.75	16.8	++	+++	++

2010). It is observed that, ammonia and catalase producing capacity were high with *B. cereus* (ANctcri1) compared to *P. aeruginosa* (ANctcri11). In the characterization of plant growth promoting rhizobacteria associated with chickpea, Joseph et al. (2007) reported that ammonia production was high in *Bacillus* isolates (95%) followed by *Pseudomonas* (94.2%). According to Loewen and Switala (1987) and Rochat et al. (2005) most of the *Bacillus* strains were efficient catalase producers. There are also reports that *B. cereus* could increase nodulation (Halverson and Handelsman, 1991) and root length of soybean (Halverson, 1991). Guo et al. (2011) also identified *B. cereus* as a promising biocontrol agent for root knot disease of vegetables.

The second potent bacteria, *P. aeruginosa* strain (ANctcri11) could also fix high amount of N *in vitro*. Previous studies by several workers (Watanabe, 1987; Hatayama et al., 2005) have identified a number of N fixing *Pseudomonas* spp. namely *P. diazotrophicus*, *P. azotifigens* and *P. stutzeri* which were phylogenetically close to *P. aeruginosa*. Kannapiran and Sri Ramkumar (2011) also reported the PGPR activity of *P. aeruginosa*. *P. aeruginosa* strain (ANctcri11) exhibited all the four plant growth promotion activities such as indole acetic acid (IAA), ammonia (NH_3), hydrogen cyanide (HCN) and catalase production.

The present study revealed that IAA and HCN production was high in *P. aeruginosa* strain (ANctcri11) compared to *B. cereus* strain (ANctcri1). Karnwal (2009) also reported varying amounts of IAA production by fluorescent *Pseudomonads*. Higher level of IAA production by *Pseudomonas* was reported by other workers (Xie et al., 1996). It showed significant production of NH_3which might have also indirectly influenced the plant growth. HCN production is a predominant character associated with *Pseudomonas* strain. The potent bacteria *P. aeruginosa* strain (ANctcri11) showed high degree of HCN production. Cyanide produced by *Pseudomonas* seems to play a major role in the suppression of root rot in tobacco caused by *T. basicola* (Ahl et al., 1986). Bhakthavatchalu et al. (2013) reported the NH_3, IAA and HCN production capacity of *P. aeruginosa*. Ahemad and Khan (2010) reported that the phytotoxicity of herbicides to legumes could be reduced by applying growth promoting herbicide tolerant strain of *P. aeruginosa* (PS1).The seed treatment of chickpea with co-inoculants of *Rhizobium*, *Azotobacter* and *P. aeruginosa* gave significantly better performance with respect to nodulation, yield and nutrient uptake (Verma and Yadav, 2012).

Effect of various physiological conditions on growth

When the bacteria were grown at different temperatures for 24 hours, *B. cereus* (ANctcri1) showed noticeably good growth under temperature ranging from 20-55°C, and the maximum growth was obtained at 45°C, whereas *P. aeruginosa* (ANctcri11) showed good growth under the temperature range of 35 - 45°C and highest growth occurred at 35°C. *P. aeruginosa* (ANctcri11) did not show growth at 20°C.

Both the bacteria could not grow at temperature above 55°C. *B. cereus* (ANctcri1) appeared to grow reasonably well at pH ranging from 5-9 and it could also tolerate wide range of pH from 3-11. Growth of *P. aeruginosa* (ANctcri11) was highest at pH ranging from 6-10 and showed tolerance to pH 3-12. Both the bacteria showed maximum growth at pH 7.

When *B. cereus* (ANctcri1) and *P. aeruginosa* (ANctcri11) were incubated at different concentrations of NaCl, it was observed that, both the bacteria were able to survive without NaCl and had tolerance for salt concentration up to 8% for *P. aeruginosa* (ANctcri11) and 7% for *Bacillus cereus* (ANctcri1). However, maximum growth of these two potent bacteria were observed at 0.5% NaCl. This study also revealed that the potent N fixing bacteria identified were able to survive wide range of physiological conditions except the temperature tolerance of *P. aeruginosa* (ANctcri11). The potent bacteria *B.cereus* (ANctcri1) was showing the growth characteristic feature of moderate thermophilic facultatively alkaliphilic halo tolerent bacteria. On the other hand *P. aeruginosa* strain (ANctcri11) exhibited facultatively alkaliphilic halo tolerant properties. Similar kind of results have also been reported by other researchers (Okanlawon et al., 2010; Todar, 2008; Bhakthavatchalu et al., 2013). The effect of physiological conditions on the growth of *B. cereus* (ANctcri1) and *P. aeruginosa* (ANctcri11) are depicted in Fig. 4.

Conclusion

The findings of the present study reinforce the fact that soils of southern regions of India are enriched source of

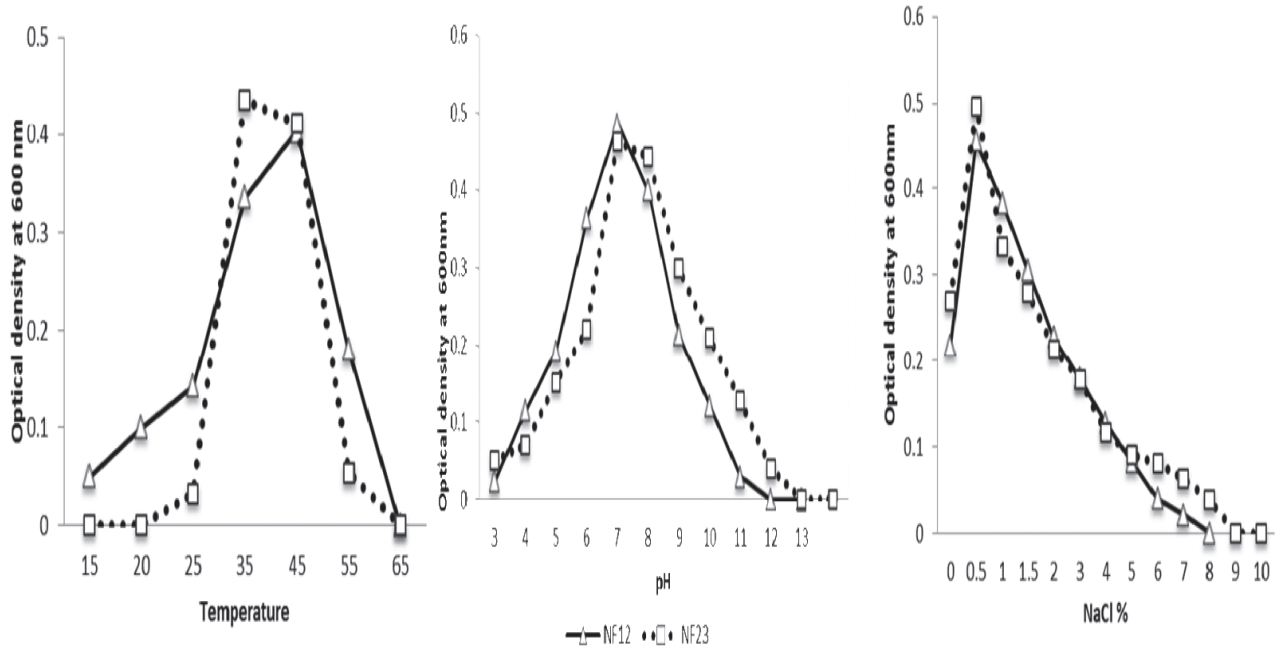

Fig.4. Effect of various physiological conditions on the growth of NF12 (*B. cereus* ANctcri1) and NF23 (*P. aeruginosa* ANctcri11)

plant growth promoting bacteria. Characterization of these bacteria led to the identification of two potent N fixers viz. *B. cereus* (ANctcri1) from Kerala and *P. aeruginosa* (ANctcri11) from Tamil Nadu. They exhibited various plant growth promoting activities and were also able to tolerate wide range of physiological conditions. These findings suggest that the potent N fixers identified in this study could serve as potential biofertilizer candidates for elephant foot yam cultivation. Application of these N fixers as bioinoculants can certainly increase the soil availability of N, can help in minimizing the use of chemical N fertilizers and can promote sustainable plant growth and yield.

Acknowledgement

The financial assistance from the National Bureau of Agriculturally Important Microorganisms, Indian Council of Agricultural Research and the research facility and support provided by Director, Central Tuber Crops Research Institute are gratefully acknowledged.

References

Ahemad, M. and Khan, M.S. 2010. Phosphate-solubilizing and plant-growth-promoting *Pseudomonas aeruginosa* ps1 improves green gram performance in quizalafop-p-ethyl and clodinafop amended soil. *Arch. Environ. Contam. Toxicol.,* **58**: 361-372.

Ahl, P., Voisard, C.and Défago, G. 1986. Iron-bound siderophores, cyanide and antibiotics involved in suppression of *Thielaviopsis basicola* by a *Pseudomonas fluorescens* strain. *J. Phytopath.*, **116**: 121–134.

Ahmad, F., Ahmad, I. and Khan, M.S. 2005. Indole acetic acid production by the indigenous isolates of *Azotobacter* and fluorescent *Pseudomonas* in the presence and absence of tryptophan. *Turk. J. Biol.*, **29**: 29-34.

Asghar, H.N., Zahir, Z.A., Arshad, M. and Khaliq, A. 2002. Relationship between *in vitro* production of auxins by rhizobacteria and their growth promoting activities in *Brassica junce*a L. *Biol. Fert. Soils*, **35**: 231-237.

Asilah, A.M., Radziah, O. and Radzali, M. 2012. Effect of nitrogen fertilizer on hydrolytic enzyme production, root colonisation, N metabolism, leaf physiology and growth of rice inoculated with *Bacillus* sp. (Sb42).*Bacteriol.*, **190**: 2663–2670.

Babu, T. G., Nithyanand, P., Kannapiran, E., Ravi, V.A. and Pandian, K.S. 2004. Molecular identification of bacteria associated with the coral reef ecosystem of Gulf of Mannar Marine Biosphere Reserve using16S rRNA sequences. *In: Proceedings National Seminar on New Frontiers in Marine Bioscience Research*, Alagappa University, Karaikudi, India. pp. 47-53.

Bahig, A.E., Aly, E.A., Khaled, A.A. and Amel, K.A. 2008. Isolation, characterization and application of bacterial population from agricultural soil at Sohag Province, Egypt. Malays. *J. Microbiol.*, **4**: 42- 50.

Bakker, A.W. and Schippers, B. 1987. Microbial cyanide production in the rhizosphere in relation to potato yield reduction and

Pseudomonas sp. mediated plant growth stimulation. *Soil Biol. Biochem.*, **19**: 451–457.

Baldani, J.I., Caruso, L.V., Baldani, V.L.D., Goi, S.R. and Döbereiner, J. 1997. Recent advances in BNF with non-legume plants. *Soil Biol. Biochem.*, **29**: 911–922.

Bashan, Y. 1998. Inoculants of plant growth-promoting bacteria for use in agriculture. *Biotechnol. Adv.*, **16**: 729-770.

Bhakthavatchalu, S., Shivakumar, S. and Sullia, B. S. 2013. Characterization of multiple plant growth promotion traits of *Pseudomonas aeruginosa* FP6, a potential stress tolerant biocontrol agent. *Ann. Biological Res.*, **4**(2): 214-223.

Bosshard, P.P., Abels, S., Zbinden, R., Bottger, E.C. and Altwegg, M. 2003. Ribosomal DNA sequencing for identification of aerobic gram-positive rods in the clinical laboratory (an 18-Month Evaluation). *J. Clin. Microbiol.*, **41**: 4134–4140.

Ding, Y., Wang, J., Liu, Y. and Chen, S. 2005. Isolation and identification of nitrogen-ûxing *bacilli* from plant rhizospheres in Beijing region. *J. Appl. Microbiol.*, **99**: 1271–1281.

Dobereiner, J. 1969. Nonsymbiotic nitrogen fixation in tropical soils. In: *Biology and Ecology of Nitrogen. Proceedings. of the Conference National Academy of Sciences*, Washington D.C.

Dojka, M.A., Hugenholtz, P., Haack, S.K. and Pace, N.R. 1998. Microbial diversity in a hydrocarbon- and chlorinated-solvent-contaminated aquifer undergoing intrinsic bioremediation. *Appl. Environ.Microbiol.*, **64**: 3869–3877.

Glick, B.R. 1995. The enhancement of plant growth by free living bacteria. *Can. J. Microbiol.*, **41**: 109–114.

Gothwal, R.K, Nigam, V.K., Mohan, M.K., Sasmal, D. and Ghosh, P. 2007. Screening of nitrogen fixers from rhizospheric bacterial isolates associated with important desert plants. *Appl. Ecol. Environ. Res.*, **6**: 101-109.

Guo, J.H., Xue, Q.Y. and Liu, H.X. 2011. XY21, a strain for biocontrol bacterial wilt of vegetables in greenhouse. State Intellectual Property Office of the People's Republic of China. Patent number: 200710130838.2

Hajare, B. and Ade, A. 2012. Confirming location of nitrogen fixing genes on plasmids in rhizobium isolated from *Pisum sativum*. *Biosci. Discovery*, **3**: 160-164.

Halverson, L.J. 1991. Population biology of *Bacillus cereus* uw85: A case study for biotechnology policy. *Ph. D. Thesis*. University of Wisconsin-Madison.

Halverson, L.J. and Handelsman, J. 1991. Enhancement of soybean nodulation by *Bacillus cereus* UW 85 in the field and a growth chamber. *Appl. Environ. Microbiol.*, **27**: 2767–2770.

Hatayama, K., Kawai, S., Shoun, H., Ueda, Y. and Nakamura, A. 2005. *Pseudomonas azotifigens* sp. nov., a novel nitrogen-fixing bacterium isolated from a compost pile. *Int. J. Syst. Evol. Microbiol.*, **55**: 1539-1544.

Hurek, T., Burggraf, S., Woese, C.R. and Reinhold-Hurek, B. 1993. 16S rRNA-targeted polymerase chain reaction and oligo-nucleotide hybridization to screen for *Azoarcus* spp., grass-associated diazotrophs. *Appl. Environ. Microbiol.*, **59**: 3816-3824.

Joseph, B., Patra, R.R. and Lawrence, R. 2007. Characterization of plant growth promoting rhizobacteria associated with chickpea (*Cicer arietinum* L.). *Int. J. Plant Prod.,* **2**: 141-152.

Kannahi, M. and Kowsalya, M. 2013. Efficiency of plant growth promoting rhizobacteria for the enhancement of *Vigna mungo* growth. *J. Chem. Pharm. Res.*, **5**: 46-52

Kannapiran, E. and Sri Ramkumar, V. 2011. Inoculation effect of nitrogen-fixing and phosphate-solubilizing bacteria to promote growth of black gram (*Phaseolus mungo* Roxb; Eng).*Ann. Biological Res.*, **2**(5): 615-621.

Karnwal, A. 2009. Production of indole acetic acid by fluorescent *Pseudomonas* in the presence of L-tryptophan and rice root exudates. *J. Plant Pathol.*, **91**: 61-63.

Katznelson, H., Lochhead, A.G. and Timonin, M.I. 1948. Soil microorganisms and the rhizosphere. *Bot. Rev.*, **14**: 543-587.

Kizilkaya, R. 2009. Nitrogen fixation capacity of *Azotobacter* spp. strains isolated from soils in different ecosystems and relationship between them and the microbiological properties of soils. *J. Environ. Biol.*, **30**: 73-82.

Kloepper, J.W., Lifshitz, R. and Zablotowicz, R.M. 1989. Free-living bacterial inocula for enhancing crop productivity.*Trends in Biotech.,* **7**: 39-44.

Kloepper, J.W. and Schroth, M.N. 1978. Plant growth-promoting rhizobacteria on radishes. *Proceedings of the Fourth International Conference on Plant Pathogenic Bacteria*, **2**: 879-882.

Loewen, P. C. and Switala, J. 1987. Multiple catalases in *Bacillus subtilis*. *J. Bacteriol.*, **169**: 3601–3607.

Okanlawon, B.M., Ogunbanwo, S.T. and Okunlola, A.O. 2010. Growth of *Bacillus cereus* isolated from some traditional condiments under different regimens. *Afr. J. Biotechnol.*, **8**(14): 2129-2135.

Owen, A. and Zlor, R. 2001. Effect of cyanogenic rhizobacteria on the growth of velvetleaf (*Abutilon theophrasti*) and corn (*Zea mays*) in autoclaved soil and the influence of supplemented glycine. *Soil Biochem.*, **33**: 801-809.

Prashanth, S. and Mathivanan, N. 2010. Growth promotion of groundnut by lAA producing rhizobacteria *Bacillus licheniformis* MM12501. *Arch. Phytopathol. Plant Protection*, **43**: 191- 208.

Rochat, T., Miyoshi, A., Gratadoux, J.J., Duwat, P., Sourice, S., Zevedo, V.A. and Langella, P. 2005. High-level resistance to oxidative stress in *Lactococcus lactis* conferred by *Bacillus subtilis* Catalase Kate. *Microbiology,* **151**: 3011–3018.

Todar, K. 2008. "*Pseudomonas aeruginosa.Online Text Book for Bacteriology*.http://textbookofbacteriology.net/pseudomonas.html.

Verma,J.P. and Yadav, J. 2012**.** Evaluation of plant growth promoting rhizobacteria and their effect on plant growth and grain yield of

chickpea *(Cicer arietinum* L.) under sustainable agriculture Production. IJREISS. **2**(9): 2250-0588**.**

Waksman, A. and Selman Starkey Lyman R. 1931.*The Soil and the Microbe*. John Wiley New York. 260 p.

Watanabe, I., Rolando, S.O., Ladha, J.K., Katayama-Fujimura, Y. and Kuraishi, H. 1987. A new nitrogen-fixing species of *Pseudomonad*: *Pseudomonas diazotrophicus* sp. nov. isolated from the root of wetland rice. *Can. J. Microbiol.*, **33**: 670-678.

Xie, G.H., Su, B.L. and Cui, Z.J. 1998. Isolation and identification of N-fixing strains of *Bacillus* in rice rhizosphere of the Yangtze River valley. *Acta Microbiol. Sin.*, **38**: 480–483.

Xie, H., Pasternak, J.J. and Glick, B.R. 1996. Isolation and characterization of mutants of the plant growth-promoting rhizobacterium *Pseudomonas putida* CR12-2 that over produce indoleacetic acid. *Curr. Microbiol.*, **32**: 67–71.