Salt tolerant polyembryonic mango rootstock (ML-2 and GPL-1): A putative role of endophytic bacteria by using BOX-PCR

R. Kannan*1, T. Damodaran2, A. Nagaraja1 and S. Umamaheswari4

School of Agricultural Sciences, Kalasalingam Academy and Research Education, Krishnan Kovil, Virudhunagar-626 126, Tamil Nadu, India.

Received: 27-02-2018  Accepted: 23-04-2018

ABSTRACT
This study was conducted to assess the movement of Na+ and K+ between leaves, stem and root segments of tolerant and susceptible polyembryonic mango accessions under saline-sodic environment. Among the 15 diverse polyembryonic from different location of South Andaman used for screening under saline-sodic condition with the soils of pH 9.45 under pot culture experiments in polyhouse, at the time of completion of experiment (240th DAS) the accessions GPL-1, ML-2 and ML-6 were selected which exhibited tolerance to sodicity with lower mortality percent of 6.67 to 13.33% and the rest of polyembryonic seedling were having huge mortality percent ranging from 96.67 to 100.00%. The Na+/K+ ratio in roots of tolerant accessions registered lower Na+/K+ ratio ranging from 0.47 (GPL-1) to 0.61 (ML-6) and susceptible accessions was higher Na+/K+ which ranged from 3.06 (GTP-2 to 6.53 (GPL-4). The same was reverse in the stem and leaves in tolerant and susceptible accessions. Results indicated that the isolates belonged to four major phylogenetic group: low G+C Gram positive bacteria, Firmicutes, Proteobacteria and Bacteroidetes.

Key words: BOX PCR, Endophytes, Plant growth Promotion, Polyembryonic mango, Sodicity.

INTRODUCTION
Andaman and Nicobar Islands are recognized as an important centre for variability in mango genotypes (Damodaran et al., 2012). In India, the Andaman and Nicobar group of Islands is a Genetically Diverse Hot Spot (GDHS) for wild mangoes. The Islands are rich in biodiversity with extremely fragile habitats harboring over 2500 angiosperm taxa of which about 245 are endemic (Ahlawat,2001). Mango originated in the Indo-Burma region is an important fruit crop of the tropical and subtropical regions of the world and was introduced in these islands by the settlers from rich diversity areas of Indo-Myanmar region (Damodaran et al., 2007). Soil salinity is considered as an important constraint in mango production under the coastal ecosystem (Kannan et al., 2014). It is also a major constraint in arid environment (Hoult et al., 1997). Salt affected soils contain excess sodium and chloride that affect the normal growth of the plant and lead to mortality (Samara, 1985). In India, limited attempts were made to identify tolerant type polyembryonic mangoes (Varu and Barad, 2010). Efforts were taken to collect the polyembryonic accessions from the post tsunami inundated regions of the Andaman Islands and screen them for salt tolerance (Damodaran et al., 2007). The micro-organism and plant interaction in the rhizosphere under salt stress can be beneficial, neutral, or deleterious for plant growth. Under salt stress, PGPR (Plant growth promoting Rhizo-bacteria) have shown positive effects in plants on parameters like germination rate, tolerance to drought, weight of shoots and roots, yield and plant growth (Raju et al., 1999). PGP functions by synthesizing specific compounds which enhance the nutrient mobilization under saline environment and increases plant growth (Kannan et al., 2014). This study aimed to understand the endophytic diversity and their role in imparting salt tolerance in susceptible and tolerant accessions of polyembryonic mango to salinity.

MATERIALS AND METHODS
Collection and evaluation of polyembryonic mango accessions: In the present experiment, 15 polyembryonic mango accessions from different locations of Andaman were observed under experiment. They were evaluated for their tolerance to abiotic stress (sodicity soil) under pot culture experiment with completely randomized design at the experimental farm of the ICAR-Central Soil Salinity Research Institute, Regional Research Station, Lucknow. List of accessions with location are given in Table 1. The stones collected from fruits of different mango accessions were sown in nursery beds after the extraction of pulp and washed thoroughly in tap water. After three months of sowing the nucellar seedlings of uniform vigour were uprooted (along with soil adhered to roots) from nursery bed and planted in polythene lined pots filled with 5 kg of sodic soil. The sodic soil used for ex-situ screening, which was Typic Natrustalfs with pH 9.51, electrical conductivity (ECe) 6.7 dSm−2;
sodium (Na+) 21.20 meq/L and potassium (K+) 0.126 meq/L at the beginning of the experiment. The soil parameters like pH, ECe, sodium and potassium contents were recorded regularly at the interval of 60 days to 240 days. Observations on mortality percentage (M) were also recorded at an interval of 60, 120, 180 and 240 days after planting in sodic soil.

The leaf (3rd leaf from terminal portion of seedling) samples were collected at the end of the experiment from the plants for analyzing the content of Na+ and K+ by extracting the oven-dried (65°C) samples in 100 mmol m⁻³ acetic acid kept in a water bath for 2 h at 90°C. Na+ and K+ content in the extract were determined using a flame photometer. At 240 days after sodicity treatment, three samples from each accession were removed from the pots for recording the root and shoot length (cm). The experiment was laid out in completely randomized design with three replications having ten plants in each.

Isolation of plant growth Promoting (PGP) endophytic bacteria: The uprooted healthy sodicity tolerant mango plants at the end of experiment (240th DAS) were briefly washed with sterile water and the root, stem and leaves were cut into 2-3 cm long pieces. These pieces were rinsed in sterile water and then surface disinfected by soaking in 70% ethanol for 30 s and then treated with sodium hypochlorite (3-5% available chlorine) for 3 minutes. Samples from each accession were removed from the pots for analyzing the content of Na+ and K+ by extracting the oven-dried (65°C) samples in 100 mmol m⁻³ acetic acid kept in a water bath for 2 h at 90°C. Na+ and K+ content in the extract were determined using a flame photometer. At 240 days after sodicity treatment, three samples from each accession were removed from the pots for recording the root and shoot length (cm). The experiment was laid out in completely randomized design with three replications having ten plants in each.

| Cultivar  | Abbreviation of cultivar name | Geographic origin | Embryo type | Flowering behavior |
|----------|-------------------------------|-------------------|-------------|-------------------|
| Kalatang | KL-2                          | South Andaman     | Polyembryonic| Differential      |
| Chidyatappu | GTP-2                      | South Andaman     | Polyembryonic| Single            |
| Guptapara | GPL-4                        | South Andaman     | Polyembryonic| Differential      |
| Chidyatappu | GTP-1                      | South Andaman     | Polyembryonic| Differential      |
| Guptapara | GPL-2                        | South Andaman     | Polyembryonic| Differential      |
| Guptapara | GPL-1                        | South Andaman     | Polyembryonic| Differential      |
| Manjeri  | ML-2                         | South Andaman     | Polyembryonic| Differential      |
| Manjeri  | ML-4                         | South Andaman     | Polyembryonic| Differential      |
| Chidyatappu | GTP-3                      | South Andaman     | Polyembryonic| Differential      |
| Garacharma | GCL-3                      | South Andaman     | Polyembryonic| Differential      |
| Guptapara | GPL-3                        | South Andaman     | Polyembryonic| Differential      |
| Baratang | B-1                          | South Andaman     | Polyembryonic| Differential      |
| Garacharma | GCL-2                      | South Andaman     | Polyembryonic| Differential      |
| Garacharma | GCL-1                      | South Andaman     | Polyembryonic| Differential      |
| Manjeri  | ML-3                         | South Andaman     | Polyembryonic| Differential      |

In that, Indole Acetic Acid (IAA) production was detected as described by Brick et al. (1991). The phosphorus solubilization potential of PSB strains was tested by liquid assay using National Botanical Research Institute’s phosphate (NBRIP) medium supplemented with known amount of TCP as a substrate (Fiske and Subbarow, 1925). Sideroproduction assay on nutrient agar with amended chrome-azurol-S (CAS) as indicator dye (Schwyn and Neilands, 1987).

**BOX-PCR:** Endophytic bacterial DNA elite for BOX-PCR analysis was done as described by Rademaker and De Bruijn (1997) using the BOX-AIR primer 50-CTA CGG CAA GGC GAC GCT GAC G-30. PCR amplification was performed with a GeneAmp1 PCR system 9700 (Applied Biosystems, USA) using an initial denaturation step at 95°C for 6 min and subsequently 35 cycles of denaturation at 94°C for 1 min, annealing at 53°C for 1 min and extension at 65°C for 8 min followed by final extension at 65°C for 16 min. A 10-ml aliquot of amplified PCR product was separated by gel electrophoresis on 1.5% agarose gels in 0.5% Tris–Acetate–EDTA buffer for 6 h, stained with ethidium bromide, and photographed under UV transillumination. The reproducibility of the results was verified in three independent experiments. Analysis of BOX-PCR molecular weight and RF values of each band was determined using “Molecular Analyst software” (Version 1.5). The character state “1” was given for a band, which could be clearly detected in the gel and “0” was assigned if it was not possible to determine. The similarity coefficient and cluster analysis was performed (Janda, 1991) by unweighted pair group method with arithmetic average of NTSYS–pc 2.02e (Applied Biosciences, Inc., New York, USA).

**Statistical analysis:** The data were analyzed using analysis of variance (ANOVA) using SAS 9.2 statistical software. Valid conclusions were drawn using Duncans Multiple Range Test at \(P=0.05\).
Table 2: Effect of levels of soil sodicity dominated by different ions on tissue sodium and potassium contents of polyembryonic mango accessions.

| Category              | Accession No. | Stem Na+ (meq / l) | Stem K+ (meq / l) | Na+ / K+ |
|-----------------------|---------------|--------------------|-------------------|---------|
| Tolerant mango accessions | GPL-1         | 1.80               | 12.66             | 0.14    |
|                        | GPL-3         | 1.78               | 12.92             | 0.14    |
|                        | ML-3          | 2.59               | 17.39             | 0.15    |
|                        | ML-4          | 2.79               | 19.74             | 0.14    |
|                        | ML-2          | 3.33               | 7.49              | 0.44    |
|                        | GPL-4         | 4.29               | 7.15              | 0.60    |
|                        | GTP-1         | 3.02               | 1.00              | 3.02    |
| Susceptible mango accessions | GPL-2        | 2.13               | 0.74              | 2.88    |
|                        | GTP-3         | 2.01               | 0.68              | 2.96    |
|                        | GCL-3         | 2.95               | 0.97              | 3.03    |
|                        | B-1           | 2.77               | 0.48              | 5.77    |
|                        | GCL-2         | 3.62               | 0.73              | 4.96    |
|                        | GCL-1         | 3.10               | 1.02              | 3.04    |
|                        | KL-2          | 3.26               | 0.69              | 4.72    |
|                        | GTP-2         | 4.24               | 1.32              | 3.21    |

Values are the means of three replicates. Means in the columns followed by the same letter are not significantly different according to Duncan’s multiple range test at \( P=0.05 \).

RESULTS AND DISCUSSION

A total of 15 polyembryonic mango accessions collected from different locations of the Andaman Islands were used for screening pot culture experiment at ICAR-Central Soil Salinity Research Institute, Regional Research Station, Lucknow. Screening based on sodium potassium content revealed that the accessions ML-2, ML-4, GPL-1 recorded lower sodium and potassium ratio in the shoots than the other accession used for screening (Table 2). The accessions ML-2 and ML-6 were reported to have low mortality rate in sodic and saline conditions (Damodaran et al., 2013).

A total of 45 endophytic bacteria were isolated from highly salt tolerant polyembryonic mango accessions (ML-2 and ML-6 and GPL-1), respectively (Table 2). More endophytic bacteria were isolated from root tissue followed by stem and leaves. The diversity of culturable endophytic bacteria was low G+C Gram positive bacteria, Firmicutes, Proteobacteria and Bacteroidetes. In PGP traits (Table 3) with supplement of 3.0 M sodium chloride concentration, four isolates showed IAA, siderophore production and phosphate solubilization and none of the isolate showed HCN production. The isolates CSR-M-16 (74.0 µM-L1), CSR-M-08 (65.7 µg mL-1), CSR-M-06 (49.5 µg mL-1) and CSR-M-09 (25.2 µg mL-1) had extensive formation for IAA production. Further, the isolates CSR-M-06, CSR-M-08 and CSR-M-16 showed higher phosphate solubilization.

Table 3: Assessment of plant growth promoting properties of endophytic bacteria from salt tolerant polyembryonic mango accessions (GPL-1 and ML-2).

| Bacterial Isolate | IAA Production (µg / ml³) | Siderophore Production | HCN Production |
|-------------------|---------------------------|------------------------|----------------|
| CSR-M-01          | 0.00                      | -                      | -              |
| CSR-M-02          | 0.00                      | -                      | -              |
| CSR-M-03          | 0.00                      | -                      | -              |
| CSR-M-04          | 0.00                      | -                      | -              |
| CSR-M-05          | 0.00                      | -                      | -              |
| CSR-M-06          | 49.5                      | +++                    | -              |
| CSR-M-07          | 0.00                      | -                      | -              |
| CSR-M-08          | 65.7                      | +++                    | -              |
| CSR-M-09          | 25.2                      | ++                     | -              |
| CSR-M-10          | 0.00                      | -                      | -              |
| CSR-M-11          | 0.00                      | -                      | -              |
| CSR-M-12          | 0.00                      | -                      | -              |
| CSR-M-13          | 0.00                      | -                      | -              |
| CSR-M-14          | 0.00                      | -                      | -              |
| CSR-M-15          | 0.00                      | -                      | -              |
| CSR-M-16          | 74.0                      | +++                    | -              |

IAA: Indole-3-Acidic Acid; HCN: Hydrogen Cyanide; PO₄: Phosphate; -: No production; +: 0.3-0.5 cm; ++: 0.6-0.9 cm; +++: >1 cm.
mechanisms such as production of phyto hormones, suppression of deleterious organisms, production of IAA, activation of phosphate solubilization and pro-motion of the mineral nutrient uptake are believed to be involved in plant growth promotion by PGPR (Glick 1995). Here we use the term PGPB (plant growth-promoting endophytic bacteria) as bacteria can competitively colonize plant root, promote plant growth such as IAA segregation, phosphate solubilisation, iron chelating agent during a biotic stress condition. Plant growth-promoting bacteria genera: Bacillus (Idriss et al., 2002), Enterobacter (Gupta et al., 1998) and Corynebacterium (El-Banana et al., 1988) have been reported to benefit plants by enhancing plant growth and improving plant health through various direct and indirect mechanisms. Auxin is the most effective plant growth hormone and among them IAA is a common one. Positive root growth promoting response of rice seedlings could be due to the production of auxin-like compounds by the entophytic bacteria isolates (Jannathul and Bhore Subhash, 2017). IAA may function as important signal molecule in the regulation of plant development (Usha RM et al., 2012). Phosphate solubilization by Bacillus sp. isolated from salt stressed environment had been observed by earlier researchers (Son et al., 2006). Siderophore chelates iron and other metals thereby contributes to disease suppression and acquisition of Fe\(^{2+}\) to plants for increasing the crop growth under stressed conditions (Hofte et al., 1992; Duffy 1999). BOX-PCR provided well-resolved amplicons in the range of 400–1800bp. The box PCR generated patterns examined by cluster analysis showed a high genotypic diversity (Fig.1). The dendrogram showed three major clusters, Cluster I (primary cluster) consist of four salt tolerant endophytes (CSR-M-08, CSR-M-09, CSR-M-06 and CSR-M-16) growing above 3.0 M NaCl and positive for IAA, P-solubilising and siderophore production under salt stress. Cluster II and cluster III consisted of 12 endophytic bacterial isolates that are negative for all PGP properties and growing salt concentration below 0.1 M NaCl. Overall, the strains showed wide variations in fingerprinting pattern due to their high degree of genetic variability. Naik et al., (2008) also observed high degree of diversity in BOX-PCR among the IAA, P-solubilising and siderophore bacteria. Therefore, we can presume that, in the salt tolerant polyembryonic mango rootstock (ML-2 and GPL-1), not only exist a high diversity of endophytic bacteria but also a large variety of plant growth promoting traits under salt stress conditions. It has been previously reported that some genera harbour plant growth promoting activity, for example, presence of higher salt stress the higher activity of IAA, siderophore production and phosphate solubilisation endophytic bacteria isolates from salt tolerant polyembryonic mango accessions (Kannan et al., 2014). The most promising isolate CSR-M-16 was evaluated and used as a phosphate solubilizing and growth promoting (auxin producer) bacteria in the bioformulation CSR-BIO which is currently being used in 22,000 ha area of the country through public private partnership commercialization (Damodaran et al., 2013).

REFERENCES
Ahlawat, S. P. S. (2001). Biodiversity of Andaman and Nicobar Islands. Journal of Andaman Science Association, 17: 1-2.
Brick, J.M., Bostock, R.M. and Silversone, S.E. (1991). Rapid In-situ assay for indole acetic acid production by bacteria immobilized on nitrocellulose membrane. Appl. Environ. Microbiol., 57: 535-538.
Cyrus, H. Fiske and Yellapragada Subbarow (1925). The colorimetric determination of phosphorus. J. Biol. Chem., 66: 375.
Damodaran, T., Kannan, R., Israr Ahmed, Srivastava, R.C., Rai, R.B. and Umannaheshwari, S. (2012). Assessing genetic relationships among mango (Mangifera indica L.) accessions of Andaman Islands using inter simple sequence repeat markers. New Zealand J. Crop Hort. Sc., 1175-8783: 1-12.
Damodaran, T., Medihi, R.P., Singh, D.R., Rai, R.B., Damodaran, V. and Kapil Dev. (2007). Identification of molecular markers linked with differential flowering behaviour of mangoes in Andaman and Nicobar islands. Carr. Sci., 92: 1053-1056.
Damodaran, T., Rai, R.B., Jha, S.K., Sharma, D.K., Mishra, V.K., Dhamra, K. and Singh, A.K., Sah, V. (2013). Impact of social factors in adoption of CSR BIO – A cost effective, ecoefficiently bio-growth enhancer for sustainable crop production. South Asian Journal of Experimental Biology, 3(4): 158-165.
Damodaran, T., Shailendra Rajan, Ram Kumar, Sharma, D. K., Misra, V. K., Jha S. K. and Rai, R. B. (2013). Post-tsunami collection of polyembryonic mango diversity from Andaman Islands and their ex-situ reaction to high sodium in sodic soil. *Journal of Applied Horticulture*, **15**(1): 21-25.

Duffy, B. K. (1999). Environmental factors modulating antibiotic and siderophore biosynthesis by *Pseudomonas fluorescens* biocontrol strains. *Appl. Environ. Microbiol.*, pp. 2429-438.

El-Banana, N. and Winkelmann, G. (1988). Pyrrolnitrin from *Burkholderia cepacia*: antibiotic activity against fungi and novel activities against streptomycetes. *J Appl Microbiol.*, **85**: 69-78.

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

Gupta, A., Saxena, A.K., Gopal, M. and Tilak K.V.B.R. (1998). Effect of plant growth promoting rhizobacteria on competitive ability of introduced *Bradyrhizobium* sp. (Vigna) for nodulation. *Microbiol Res.*, **153**: 113-117.

Hofte, M., Boelens, J. and Verstraete, W. (1992). Survival and root colonization of mutants of plant growth promoting *Pseudomonads* affected in siderophore biosynthesis or regulation of siderophore production. *J. Plant Nutr.*, **15**: 2253-2262.

Hoult, M. D., Donnelly, M. M. and Smith, M.W. (1997). Salt exclusion varies amongst polyembryonic mango cultivar seedlings. *Acta Hort.*, **455**: 455-458.

Hung, P. Q. and Annapurna, K. (2004). Isolation and characterization of endophytic bacteria in soybean (*Glycine* sp.). *Omon Rice.*, **12**: 92-101.

Idriss, E. E. S, Makarewicz, O and Farouk A (2002). Extracellular phytase activity of *Bacillus amyloliquefaciens* FZB 45 contributes to its plant growth-promoting effect. *Microbiology* **148**:2097–2109.

Janda, J. M. (1991). Recent advances in the study of the taxonomy, pathogenicity and infectious syndromes associated with the genus *Aeromonas*. *Clin Microbiol Res.*, **4**: 397-410.

Jannathul Firdous and Bhore Subhash, J. (2017). Screening of cultivable endophytic bacterial isolates for their plant growth promoting activity in rice. *Indian J. Agric. Res.*, **51**(5): 413-418.

Kannan, R., Damodaran, T., Pandey, B. K., Rai, R. B., Umamaheshwari, S., Sharma, D. K., Mishra, V. K., Jha, S. K. and Sah, V. (2014). Isolation and characterization of endophytic plant growth-promoting bacteria (PGPB) associated to the sodicity tolerant polyembryonic mango (*Mangifera indica*) root stock and growth vigour in rice under sodic environment. *African Journal of Microbiology Research*, **8** (7): 628-636.

Naik, P. R., Raman, G., Narayanan, K. B. and Sakthivel, N. (2008). Assessment of genetic and functional diversity of phosphate solubilizing fluorescent pseudomonads isolated from rhizospheric soil. *BMC Microbiol.*, **8**: 230.

Rademaker, J. L.W. and De Bruijn, F. J. (1997). Characterization and classification of microbes by REP-PCR genomic fingerprinting and computer-assisted pattern analysis. In: Akkermans ADL, van Elsas JD, de Bruijn FJ, editors. DNA markers: protocols, applications and overviews. New York: J. Wiley and Sons, pp. 1-26.

Raju, N. S., Niranjan, S. R., Janardhan, G. R., Prakash, H. S. and Mathur, S. B. (1999). Improvement of seed quality and field emergence of *Fusarium moniliformae* infected sorghum seeds biocontrol agents. *J. Sci. Food.*, **79**: 206-212.

Samara, J. S. (1985). Effect of irrigation water and soil sodicity on the performance and leaf nutrient composition of mango cultivars. *Acta Hort.*, **231**: 306-311.

Schwyn, B. and Neilands, J. B. (1987). Universal chemical assay for detection and determination of siderophore. *Anal. Biochem.*, **160**: 47-56.

Son, H. J., Park, G. T., Cha, M. S. and Heo, M. S. (2006). Solubilization of insoluble inorganic phosphates by a novel salt and pH tolerant *Pantoea agglomerans* R-42 isolated from soybean rhizosphere. *Biores. Technol.*, **97**: 204-210.

Usha, R. M. and Arundhati, Reddy, G. (2012). Screening of rhizobacteria containing plant growth promoting (PGPR) traits in rhizosphere soils and their role in enhancing growth of pigeon pea. *A. J. Biotechnol.*, **11**(32): 8085-8091.

Varu, D. K. and Barad, A. V. (2010). Standardization of mango rootstock for mitigating salt stress. *Indian J. Hort.*, **67**: 79-83.