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RESEARCH PAPER

Indigenous rhizobacteria possessing abiotic stress tolerant traits promote vigorous growth of coconut seedlings via increased nutrient uptake and positive plant–microbe feedback

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Abstract
A total of 20 bacteria including seven fluorescent *Pseudomonas* spp. and 13 *Bacillus* spp., out of 483 previously isolated bacteria from rhizosphere and roots of healthy and high yielding coconut palms from major coconut growing States in India, were tested for their growth promotion efficacy on West Coast Tall variety coconut seedlings. Firstly, they were characterized for (i) ability to grow under different environmental stress parameters, (ii) carbon use pattern determined with BIOLOG and (iii) their genetic diversity using RFLP analysis. Seedling response to PGPR inoculation was measured by recording the leaf numbers, girth, root and shoot fresh and dry biomass weights in relation to nutrient uptake efficiency and plant–microbe feedback. Tolerance studies for abiotic parameters showed that *Pseudomonas* spp. were efficient in growing in 5.2 to 8.2 pH, 15–30 °C temperatures and 0.5 to 10% NaCl concentration, whereas, *Bacillus* spp. grew well in pH 4.2–8.2, temperatures 20–40 °C and 0.5 to 10% NaCl concentration. The BIOLOG analysis highlighted *Bacillus* spp. to be more suited to coconut rhizosphere milieu compared to *Pseudomonas* spp. based on the carbon oxidizing capabilities. Among the four different enzymes used in RFLP analysis, HaeIII and HinfI were observed to be most discriminatory for *Bacillus* and *Pseudomonas* PGPR genera, respectively. Inoculation of coconut seedlings resulted in significant increase in seedling length (1–16% increase), girth of the seedlings (7–27%) and dry weight (7–38% increase) compared with control. Between the two genera, the gram-positive *Bacillus* spp. were seen to outperform the gram-negative *Pseudomonas* spp. in the growth promotion studies. The endophytic *B. megaterium* TEB2 (T2) resulted in highest dry weight biomass increase (38%) in coconut seedlings followed by another endophytic *B. cereus* HEB10 (T3) and a rhizospheric isolate *B. licheniformis* RSB14 (T17) indicating their potential for development of bioinoculant formulation. Our studies, thus, offer a bouquet of indigenous PGPR, possessing abiotic stress tolerant traits, to choose upon to develop either an effective singular bioinoculant or a minimum effective microbiome set (MEMS) for raising vigorous and robust coconut seedlings for supply to coconut farmers.

Keywords Biofertilizer · Coconut seedlings · Plant growth promoting rhizobacteria · Plant nutrients · Stress tolerance

Introduction

Microorganisms are essential for the functioning of natural ecosystems and constitute an imperative resource for the sustainable management of agriculture and environment. All the plants specifically select microbes out of the almost infinite pool of soil microbial diversity by providing sundry of exudations in the form of organic acids, amino acids, sugars, growth factors, vitamins, etc. (Bais et al. 2006). As a result, a specific microbiome of their own is developed by all crops and consists of both rhizobacteria and endophytes that support the plants by directly and indirectly improving their growth (Gupta et al. 2000; Backer et al. 2018). These highly qualified microbes can be isolated and can be

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efficiently used to maximize agricultural productivity, conserve natural resources and reduce the need for chemical inputs (Thomashow et al. 2018). The most common non-symbiotic genera with plant growth promotion activity included *Pseudomonas, Bacillus, Azospirillum*, *Azotobacter*, *Enterobacter* and *Serratia. Pseudomonas* spp. and *Bacillus* spp. attracted considerable attention because of their advantages over other plant growth promoting rhizobacteria (PGPR) strains by virtue of their mutiple plant beneficial factors (Gouda et al. 2018; Ferreira et al. 2019). PGPR are now being tested for plant growth promotion worldwide (Lucy et al. 2004; Bhattachrya and Jha 2012) in many field and horticulture plants including apple, banana, paddy, long yard bean, wheat, corn and medicinal plants, etc. (Karakurt and Aslantas 2010; Kavino et al. 2010; Jasrotia et al. 2021; Kumar et al. 2017; Yadav et al. 2017; Naeem et al. 2018; Lin et al. 2018; Pagnani et al. 2018). However, the use of PGPR for improvement of perennial palms such as coconut (*Cocos nucifera* L.) is meagre and offers an avenue to be exploited as bioinoculant technology (Gupta et al. 2000).

Coconut palm is a woody, perennial monocotyledon grown throughout the tropical world and is well known for its versatile uses. In India, about 2.14 million ha is under coconut cultivation and ranks third among the major coconut producing countries after Indonesia and Philippines (https://coconutcommunity.org/, 2016). Coconut cultivation plays a significant role in agrarian economy of India and about 10 million people are dependent on coconut farming and allied activities (Sunoj et al. 2017). Being a perennial crop with a long juvenile phase, it is of quintessence that farmers get good quality seedlings to plant right at the start. Any shortcoming in seedling quality leads to uncertain and poor yield during the active production life of coconut palm spanning more than 70 years, which impact the farmers' socio-economic status significantly. In addition, one of the main strategies suggested for increasing the productivity of coconut is through generation of quality coconut seedlings (Bavappa 2005), as research has clearly suggested that the vigour of coconut seedlings is highly correlated with adult palm characters such as early flowering, nut yield and copra production (Liyanage and Abayawardena 1957). Combining PGPR to produce good quality seedlings has been reported in forestry plants such as loblolly, pine and spruce (Enebak et al. 1998; Shishdo and Chanway 2000; Probanza et al. 2002). Similar to forestry crops, seedlings of coconut too can be primed with effective PGPR to raise robust and healthy seedlings.

Towards this direction, rhizosphere and root samples were collected from healthy and high yielding coconut palms growing in Kerala, Karnataka, Tamil Nadu, Andhra Pradesh, Maharashtra and Odisha for isolation of bacteria possessing plant growth promotion (PGP) properties. About 483 morphologically distinct bacteria could be obtained from rhizospheric and endophytic niches which were thoroughly characterized in vitro for direct and indirect PGP properties. (George et al. 2018). From among the 483 isolates, 20 PGPR including 13 *Bacillus* spp. and 7 fluorescent *Pseudomonas* spp. with multiple PGP traits were identified as potential candidates for further studies with coconut seedlings and for developing PGPR-based bioinoculant technology. A commercially viable PGPR technology will be helpful to the resource-poor farmers to enhance the growth and productivity of this crop in an eco-friendly manner. The entire set of 483 microbial isolates from coconut rhizosphere and roots also form plant microbiome vault (Gopal and Gupta 2019) specific for coconut, which can be exploited with inputs from metagenomic analysis of coconut rhizosphere, for larger possible use in coconut breeding programme (Gopal and Gupta 2016).

Materials and methods

Isolation of PGPR

The PGPR used in the study were originally isolated from the rhizosphere and roots of healthy and high yielding coconut palms growing in Kerala, Karnataka, Tamil Nadu, Andhra Pradesh, Maharashtra and Odisha and previously screened and characterized for their plant growth promoting properties (George et al. 2018). They were identified based on 16S rRNA gene sequence analysis and the identity, similarity percentage and NCBI GenBank database accession numbers were reported (George et al. 2018). The direct and indirect plant growth promotion activities possessed by the isolates are furnished in Table 1. The culture stocks were maintained at – 23 °C with 15% glycerol for any further studies.

Characterization of isolates for abiotic stress tolerance

Selected isolates were multiplied in nutrient broth and tested for their purity by streaking on agar plate and colony numbers enumerated. Cultures having the population count of 10^6 c.f.u. ml^{-1} were added to Trypticase Soy Broth (TSB) prepared using citrate buffer (pH 4.2 and 5.2), phosphate buffer (pH 6.2 and 7.2) and Tris–HCl buffer (pH 8.2 and 9.0) for maintaining respective pH. After 3 days incubation, 1 ml of the culture was centrifuged at 15,000 rpm at 4 °C for 15 min. Pellet was dissolved in 3 ml of the sterile distilled water and optical density was read at 600 nm. Tolerance to temperature was studied by streaking the isolates on TSA plates and incubating them at 4, 15, 30, 35, 40, 50, 55 and 60 °C. Salt tolerance was detected on TSA plates having different concentrations of NaCl viz. 0.5%, 1%, 2%, 4%, 6%,

Table 1 Details of plant growth promoting properties of different PGPR used in this study

Treatments	PGPR	Root (EB)/rhizosphere (RS)	PGP properties	Tolerance to		
				Temperature (°C)	NaCl (%)	pH
Trial 1						
T1	*B. megaterium* TSB16	RS	H, Am, Ac, I, G, T	15–50	0.5–10	6.2–8.2
T2	*B. megaterium* TEB2	EB	N, Am, I, Ac, G, T	20–40	0.5–6	6.2–8.2
T3	*B. cereus* HEB10	EB	Am, Ac, I, C, g, T	15–40	0.5–6	6.2–9.0
T4	*B. cereus* ESB15	RS	H, Am, S, Ac, C, G, T	15–60	0.5–12	5.2–7.2
T5	*B. cereus* KnSB6	RS	H, Am, Ac, C, G, T	15–40	0.5–8	4.2–9.0
T6	*Bacillus* sp. KiEB23	EB	P, N, Am, S, G, T	20–40	0.5–1	4.2–8.2
T7	*B. megaterium* KiEB31	EB	P, N, Am, Ac, G, T	20–40	0.5–10	6.2–7.2
T8	*P. plecoglossicida* RSF266	RS	P, Am, S, I, Ac, Ab, G, T	15–35	0.5–4	5.2–8.2
T9	*P. plecoglossicida* KiSF27	RS	P, N, Am, S, I, Ac	15–35	0.5–4	5.2–8.2
T10	Uninoculated Control					
Trial 2						
T11	*B. megaterium* KiSB10	RS	P, N, Am, I, Ac, G	15–40	0.5–10	6.2–9.0
T12	*B. megaterium* TEB4	EB	N, Am, I, Ac, G, T	20–40	0.5–10	6.2–8.2
T13	*Bacillus cereus* HEB8	EB	Am, I, Ac, C, G, T	15–40	0.5–6	5.2–9.0
T14	*Pseudomonas putida* ChSF180	RS	P, Am, S, I, Ac, T	4–35	0.5–4	5.2–8.2
T15	*P. putida* KnSF208	RS	P, Am, S, I, Ac	4–35	0.5–10	5.2–9.0
T16	*P. monteilii* ASF285	RS	P, Am, S, I, Ac, Ab, G,T	15–35	0.5–4	5.2–8.2
T17	*B. licheniformis* RSB14	RS	P, N, Am, S, Ac, C, G, T	25–50	0.5–12	7.2–8.2
T18	*B. cereus* KiEB25	EB	P, N, Am, Ac, C, G, T	20–40	0.5–6	6.2–8.2
T19	*P. plecoglossicida* PoSF314	RS	P, Am, S, I, Ac, Ab, G, T	4–35	0.5–4	5.2–8.2
T20	*P. monteilii* TSF7	RS	P, Am, S, I, Ac, Ab, G, T	4–35	0.5–4	5.2–8.2
T21	*B. subtilis* PoEB5	EB	H, P, N, Am, S, Ac, G, T	25–50	0.5–10	6.2–8.2
T22	Uninoculated control					

H HCN production, *N* nitrogen fixation, *P* phosphate solubilization, *Am* ammonia production, *S* siderophore production, *I* IAA production, *Ac* ACC deaminase activity, *C* chitinase activity, *Ab* antibiotic production, *G* antagonism against *Ganoderma applanatum*, *T* antagonism against *Thielaviopsis paradoxa*

8%, 10% and incubated at 30 °C. Agar–agar (bacteriological) @ 2% was used to maintain solid medium consistency. For both temperature and salinity tests, the growth observations were recorded every 24 h up to 7 days.

Phenotypic profiling of PGPR by Biolog Gen III microplate analysis

Phenotypic profile of the selected PGPR isolates was generated by Biolog (Gen-III) microbial identification system (Biolog Inc., Hayward, CA, USA). Cells grown for 24 h on Biolog Universal Growth (BUG) agar were collected and processed according to the manufacturer's instructions (http://www.biolog.com). Cultures were transferred to Inoculating Fluid A (IF A) and inoculum density was adjusted to 98% T using Biolog turbidimeter. Using multi channel pipette, cell suspension was inoculated into Biolog Gen III Microplates (100 µl/well) containing 96 wells that provide 94 phenotypic tests. Plates were incubated at 33 °C for 24 h. The optical density at 590 nm produced from the reduction of tetrazolium violet in each well was read after 24 h using a Biolog Microplate reader (version 5.1.1). Cluster analysis was done to compare patterns of substrate usage. Clustering was based on binary data (usage/non usage).

Molecular profiling of PGPR by RFLP analysis

Genomic DNA of the PGPR was isolated by GenElute Bacterial Genomic DNA Kit (Sigma, USA). Bacterial 16S rRNA gene were amplified by using the universal 16S rRNA gene primers F27 (AGAGTTTGATCMTGGCTCAG) and R1492 (TACGGYTACCTT GTTACGACTT). Amplification reactions contained 10×PCR buffer (2.5 µl), 2.5 mM dNTPs (2 µl), 25 mM $MgCl_2$ (1.5 µl), 5 U/µl Taq DNA polymerase (0.125 µl) and 20 µM primers F27 and R1492 (1.25 µl each). The amplification was performed using Veriti Thermal cycler (Applied Biosystems). The reaction conditions included an initial denaturation of 3 min at 94 °C followed by 30 cycles of 1 min at 94 °C, 30 s at 55 °C and 45 s at 72 °C with a final extension of 10 min at 72 °C and

then the samples were cooled to 4 °C. Amplified products were resolved by electrophoresis in 0.8% agarose gel and the expected band of ~ 1.5 kb was observed. PCR amplicons were purified with a PCR purification kit (DNA clean and concentrator-25 kit, Zymo Research, USA) to remove the contaminants. Aliquots of purified 16S rDNA PCR products were digested separately with four restriction endonucleases, HinfI, MspI, HaeIII and HhaI in 25 µl reaction volume by using the manufacturer's recommended buffer and temperature. Restricted DNA was analyzed by horizontal electrophoresis in 2% agarose gels. The gels were visualized and the gel images were digitized. Strong and clear bands were scored for similarity and clustering analysis using the numerical taxonomy analysis program package, Exeter software-2.02e package (NTSYS), USA. Similarity among the isolates was calculated by Jaccard's coefficient and a dendrogram was constructed using the UPGMA method.

Evaluation of PGPR for improving growth and vigour of coconut seedlings

Inoculation and growth observations

Evaluation of plant growth promotion (PGP) effect on coconut seedlings by the selected PGPR isolates was conducted in polybags in two separate trials (Trial 1 and 2) under field conditions, involving nine and eleven PGPR isolates, respectively. Just germinated coconut sprouts with roots (West Coast Tall var.) were obtained from the farm of ICAR-Central Plantation Crops Research Institute, Kerala, India. PGPR inoculum (10^8 cells ml^{-1}) was prepared by inoculating 20 l King's B/Nutrient Broth with respective isolates and incubated for 48–60 h. Root portion of the sprouts were dipped in culture broth for 12 h and were transplanted to polybags containing soil, vermicompost prepared from coconut leaves using *Eudrilus* sp. earthworm and sand in the ratio 3:1:1 (@ 25 kg $polybag^{-1}$. One litre of culture broth was added to each polybag. Seedlings without bacterial treatment served as control. Twenty replications of coconut seedlings were maintained for each treatment in a completely randomized design. All the routine cultural operations like weeding and irrigation (once in 5 days) were carried out. One litre of respective bacterial culture (10^8 cells ml^{-1}) was applied to each polybag as a booster dose after three months of planting. After 6 months of planting, the seedlings were uprooted and growth parameters like length (LL), width (LW) and number (L No.) of leaves, length of root (RL) and shoot (SL), collar girth of seedlings (GS), number of roots (R No.), fresh weight of shoot (FWS) and root (FWR), dry weight of shoot (DWS) and root (DWR) and total dry weight of seedlings (TDW) were determined. Leaf area (LA) of coconut seedling was calculated according to the formula given by Ramadasan et al. (1980).

Determination of major nutrients and beneficial microbial population in the rhizosphere soil

The colony forming units (cfu) of plant-beneficial group of microbes like fluorescent pseudomonads, *Bacillus* spp., free-living nitrogen fixers and phosphate solubilizers in the rhizosphere soil of coconut seedlings were enumerated by serial dilution and pour plating the aliquot on standard media for the organisms targeted. Colony forming units (cfu) were counted within 24–48, and the population data were log transformed and expressed as log cfu ml^{-1}. The pooled rhizosphere soil samples were sieved (2 mm pore size) and oven-dried and used for the estimation of total nitrogen (Kjedahl 1883), available phosphorus (Bray and Kurtz 1945) and available potassium (Hanway and Heidel 1952).

Determination of uptake of major nutrients in leaves of coconut seedlings

The coconut leaves were oven dried and ground into fine powder using sample mill (Cyclotec 1093, Sweden) and used for the estimation of total nitrogen (Kjedahl 1883), total phosphorus (Jackson 1967) and total potassium (Jackson 1967). The nutrient uptake was calculated using the formula, nutrient uptake (g) = (total nutrient content (%) in leaf sample/100) × dry weight of the plant (g).

Statistical analysis

The data were analysed by using SPSS statistical software (Ver.15) and one way analysis of variance was done with ANOVA procedure in SPSS. Comparison among treatments was achieved by using Duncan's Multiple Range Test (DMRT).

Results

From scores of PGPR isolated from rhizosphere and roots of healthy coconut palms, selected twenty (seven rhizospheric *Pseudomonas* spp. and 13 *Bacillus* spp., which included eight endophytes and five rhizospheric isolates) were characterized and tested for their ability to promote the growth of coconut seedlings. The treatment numbers, identities of the PGPR and their PGP properties are furnished in Table 1; Supplementary Figure S1.

Tolerance of PGPR to abiotic stress factors

In the current investigation, all the isolates showed very poor growth at pH 4.2 except for two isolates (*B. cereus* KnSB6 and *B. cereus* KiEB23). At pH 9.0, five isolates (*P. putida* KnSF208, *B. megaterium* KiSB10, *B. cereus* KnSB6, *B.*

cereus HEB8 and *B. cereus* HEB10) were able to survive as OD value recorded for them was > 0.100 at 600 nm. Maximum growth (OD = 1.0508) at pH 9.0 was observed for *B. cereus* HEB10. Fluorescent *Pseudomonas* spp. were able to tolerate wider range of pH from 5.2 to 9.0 (Table 1, Supplementary Table S1). In general, majority of the isolates exhibited luxuriant growth at temperatures ranging from 15 to 30 °C. Below 15 °C, *Bacillus* spp. could not grow but four fluorescent *Pseudomonas* spp. could grow till 4 °C. Above 35 °C, *Pseudomonas* spp. could not grow but *Bacillus* spp. grew well till 40–50 °C. Four of the *Bacillus* spp. showed moderate growth at 50 °C, and further increase in temperature led to decline in growth and at 60 °C, only one isolate (*B. cereus* ESB15) could grow (Table 1). Growth of PGPR decreased with increasing salt concentration. At 12% NaCl concentration, the growth could be observed for two isolates (*B. licheniformis* RSB14 and *B. cereus* ESB15). However, at least eight isolates could tolerate 10% salt concentration and their growth was observed after 3 days of incubation (Table 1). Between the rhizosphere and endophytic isolates, the former was found to possess diverse and wide ranging abiotic stress tolerance capabilities.

Phenotypic profiling of PGPR

Phenotypic profiling was carried out for all PGPR by using Biolog Gen III microplate. Each microplate consisted of 96 dehydrated wells of 71 carbon sources utilization tests and 23 chemical sensitivity assays. Cluster analysis of BIOLOG substrate usage data showed *Bacillus* spp. and fluorescent *Pseudomonas* spp. to form separate cluster at 0.43 similarity coefficient level (Fig. 1). All the PGPR were metabolically diverse as none of the PGPR showed 100% similarity, with positive tests ranging from 36 to 67 out of 94 tests (Supplementary Figure S2). All 13 *Bacillus* spp. grew at/utilized pH 6, D-raffinol, *N*-acetyl glucosamine, D-fructose, glycerol, lithium chloride, 1% NaCl, 1% sodium lactate, D-fructose-6-phosphate, L-alanine, L-serine, D-gluconic acid, L-lactic acid, L-mallic acid and potassium. None of them were positive for *N*-acetyl neuramic acid, Niaproof®4, vancomycin, tetrazolium violet and L-galactonic acid lactone tests. *Bacillus* spp. further formed two main phenons with six isolates each at 0.60 similarity coefficient level (Fig. 1) and one isolate was an outlier. The similarity coefficient range among the *Bacillus* spp. was 0.51 to 0.89. More than 89% similarity was showed by two isolates *B. megaterium* TSB16 and TEB4. While, all the coconut rhizospheric *Pseudomonas* spp. tested positive for 39 tests and negative for 31 tests in the Biolog Gen III microplate, they exhibited more than 80% similarity and formed two metabolically distinct groups at 0.82 similarity coefficient level. Phenon I and Phenon II of fluorescent *Pseudomonas* spp. consisted of four and three isolates, respectively. *Bacillus* spp. were more active in oxidizing carbon sources (46–76%), except one isolate, than *Pseudomonas* spp. (42–52%).

Molecular profiling of PGPR by RFLP analysis

RFLP analysis of the isolates resulted in different fingerprint patterns based on the restriction enzymes used. Using HinfI, HaeIII, or MspI, 4 to 7 fragments were recorded for all of the isolates. The patterns were digitized and analysed using NTSYS software. Digestion of the amplified 16S rRNA with HaeIII and HinfI was the most discriminatory, among the four restriction enzymes, for *Bacillus* spp. and fluorescent *Pseudomonas* spp., respectively (Fig. 2a–c). Restriction digestion with HhaI formed a monomorphic pattern and could not differentiate the isolates. At the similarity coefficient of 0.60, all the 13 *Bacillus* spp. divided into three groups on digestion with HaeIII. Group I had 2 isolates, *Bacillus* spp. RSB14 and KiEB23; Group II and III had five isolates each and one was an outlier. Three PGPR in cluster I of group II (*B. cereus* isolates ESB15, KnSB6 and HEB10) showed 100% similarity. *B. cereus*

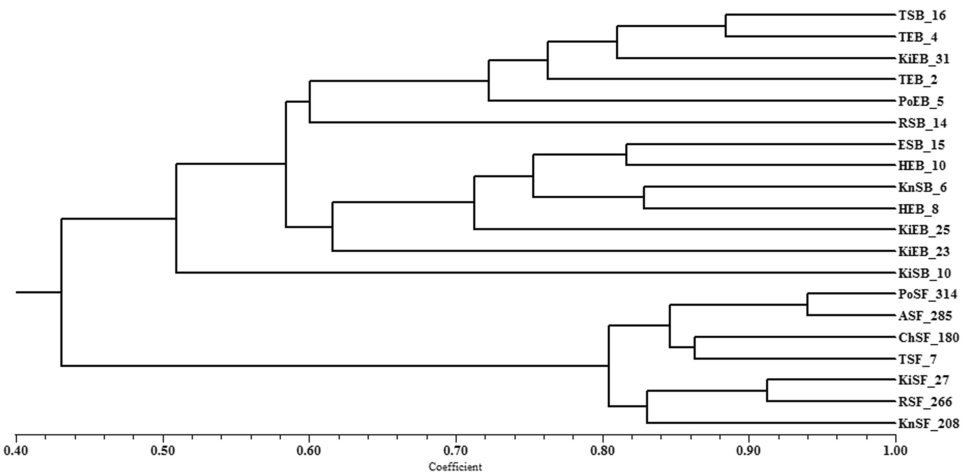

Fig. 1 Cluster analysis of BIOLOG substrate usage data by PGPR (the dendrogram shows the UPGMA algorithm and the similarity coefficient; distances calculated from utilization/non- utilization of 95 substrates)

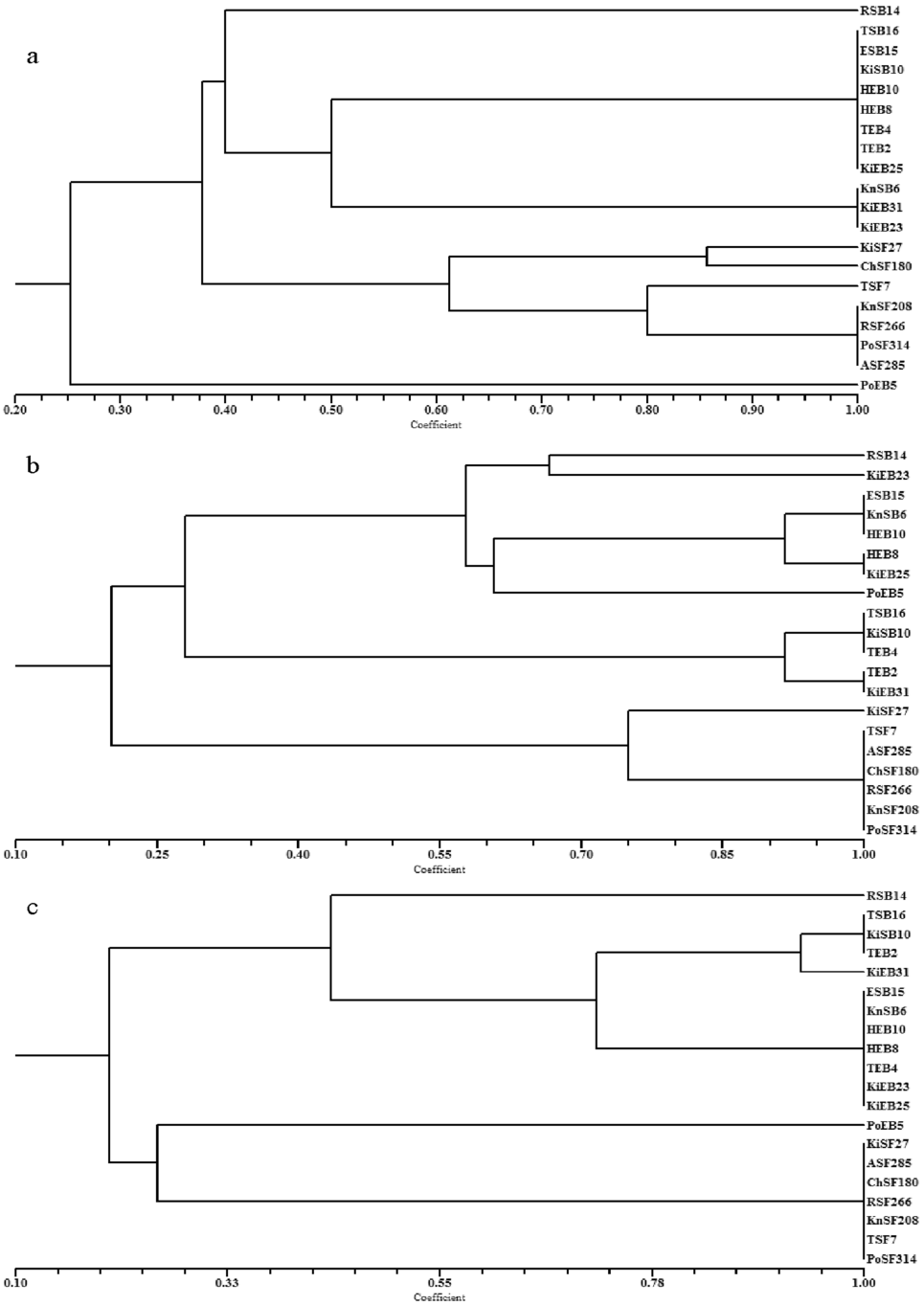

Fig. 2 Dendrogram showing the clustering of PGPR generated from RFLP analysis of 16S rDNA amplicon by restriction endonucleases **a** HinfI, **b** HaeIII and **c** MspI

HEB8 and KiEB25 in cluster II were found to be 100% similar with *B. subtilis* PoEB5 which existed as an outlier. Many of them in a single cluster showed 100% similarity. On digestion with HinfI, fluorescent *Pseudomonas* spp. could be grouped into two at 0.76 similarity coefficient level (Fig. 2a). *P. putida* ChSF180 and *P. plecoglossicida* KiSF27 formed group 1 and remaining five fluorescent *Pseudomonas* spp. formed second group. However, the restriction digestion with *Msp* could not differentiate the isolates.

Plant growth promoting ability of PGPR

Evaluation of PGPR for improving growth and vigour of coconut seedlings was conducted with twenty PGPR in two separate trials (trial 1 and 2). Each trial was statistically analyzed separately using Duncan Multiple Range Test (DMRT). Results of the studies are furnished in Figs. 3, 4, 5 and 6; Table 2; Supplementary Figure S3.

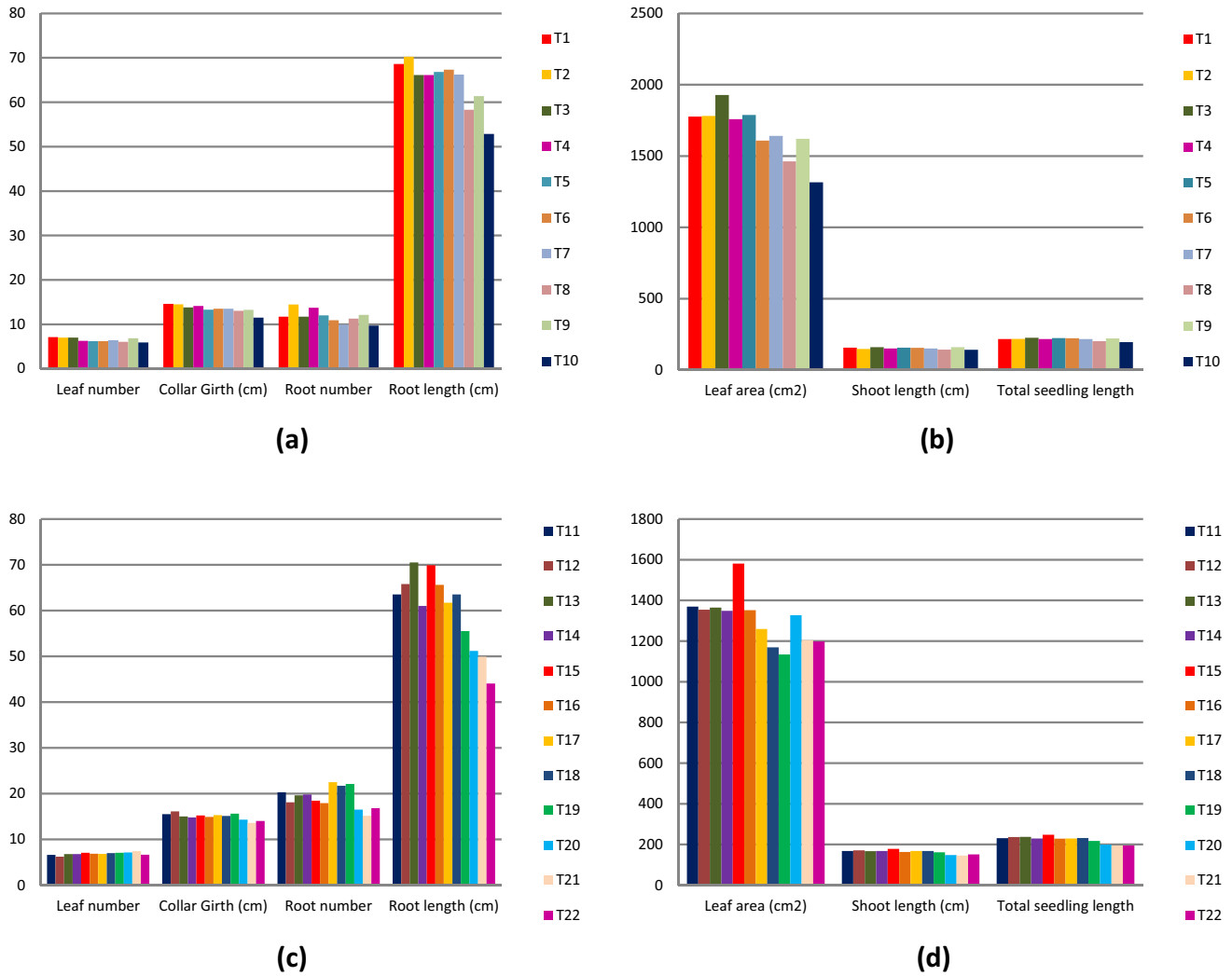

Fig. 3 Effect of PGPR treatments on different growth parameters of coconut seedlings [Trial 1 (**a**, **b**, T10 as control); Trial 2 (**c**, **d,** T21 as control); values are mean of twenty replications per treatment; refer Supplementary Table S1 for details]

Effect of bacterial inoculations on number of leaves, number of roots and girth of the coconut seedlings

Leaf number ranged from 5.9 to 7.4 with a maximum number of 7.1 (20% increase over control) by T1 (*B. megaterium* TSB16) and 7.4 (11% increase over control) by T21 (*B. subtilis* PoEB5) in trial 1 and trial 2, respectively (Fig. 3). Maximum number of roots (49% increase over control) were observed in T2 (*B. megaterium* TEB2) inoculated coconut seedlings in trial 1 and T17 (*B. licheniformis* RSB14) (32% increase over control) treatment in trial 2. Similarly, PGPR *B. megaterium* TEB2 and *B. megaterium* TSB16 were found to be most effective in increasing the area of the leaves (upto 26% compared to control). The highest collar girth was recorded in the seedlings inoculated with *B. megaterium* TEB4 in trial 2 (15% increase over control). Among the fluorescent *Pseudomonas* spp., the treatment T9 (*P. plecoglossicida* KiSF27) in trial 1 could produce significantly higher leaf numbers but not very significant improvement in collar girth compared with other microbial treatments. In trial 2, the treatment T15 (*P. putida* KnSF 208) had significant positive impact on production of leaves and the treatment T19 (*P. plecoglossicida* PoSF314) on collar girth of the seedlings.

Effect of PGPR inoculation on leaf area and length parameters of coconut seedlings

Most of the isolates significantly increased the leaf area and length parameters like shoot length, root length and total seedling length of coconut seedlings except some treatments (Fig. 3). The highest per cent increase in leaf area was recorded in coconut seedlings treated with T3 (*B. cereus* HEB10) (47% increase over uninoculated control); highest

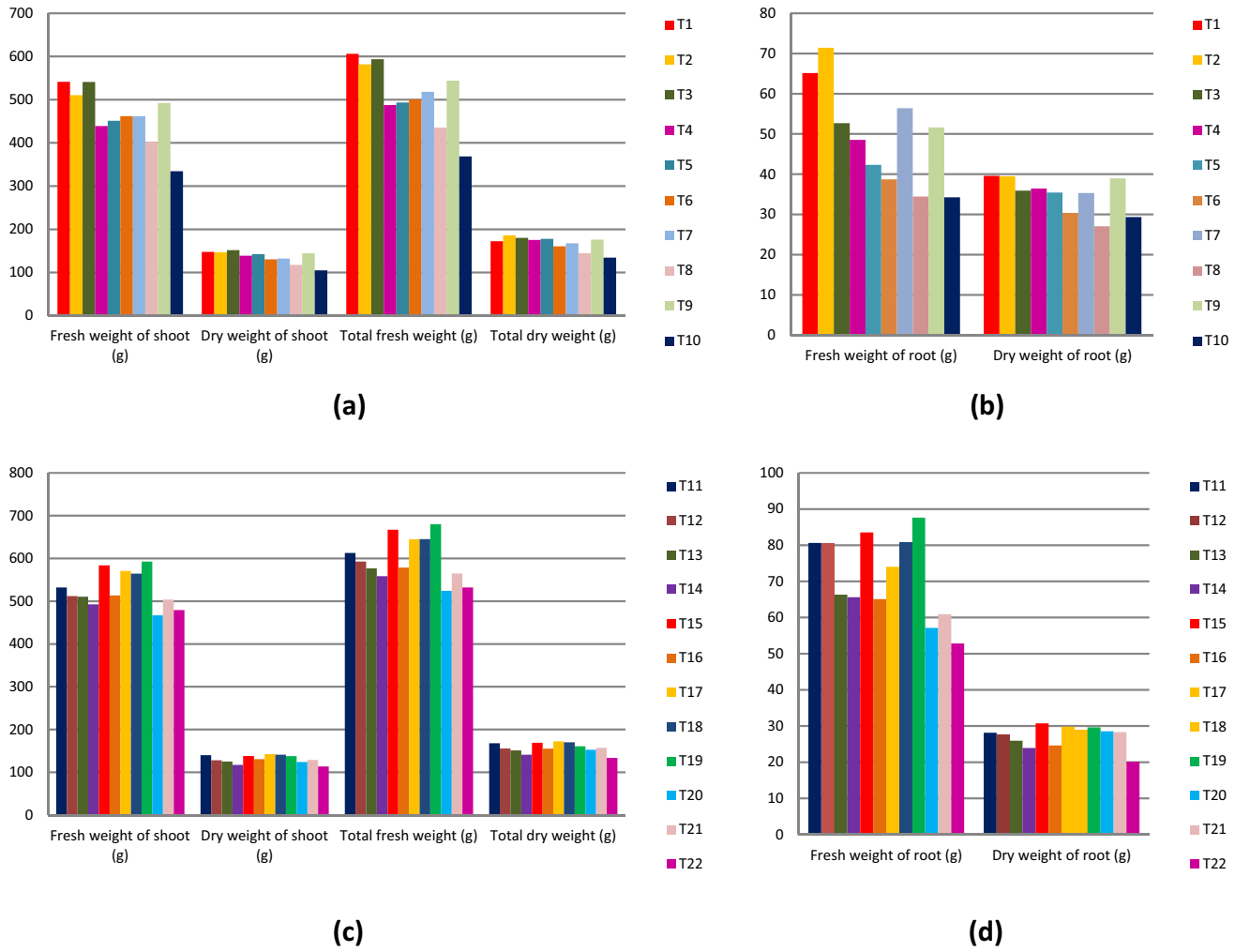

Fig. 4 Effect of PGPR treatments on biomass of coconut seedlings [Trial 1 (**a**, **b**, T10 as control); Trial 2 (**c**, **d,** T22 as control); values are mean of twenty replications per treatment; refer Supplementary Table S2 for details]

shoot (18% increase over control) length was recorded in the treatment T15 (*P. putida* KnSF208); root length was higher in T13 (*B. cereus* HEB8) (60% increase over control) and maximum per cent increase in total seedling length (27%) was recorded in coconut seedlings treated with *P. putida* KnSF208 (T15).

Effect of bacterial inoculations on biomass of coconut seedlings

Fresh weight of shoot was relatively superior in most of the inoculated treatments with 5 to 62% increase as compared to control (Fig. 4). The maximum fresh weight was recorded in T1 (*B. megaterium* TSB16) and T3 (*B. cereus* HEB10) (62% over control), followed by T2 (*B. megaterium* TEB2) (53% increase over control). Most of the isolates promoted root growth accompanied with higher root fresh weights compared to the control. It is evident from DMRT analysis that the fresh weight of roots of all the seedlings inoculated with PGPR was significantly higher than the control except one treatment in trial 1. Maximum increase in total seedling fresh weight was in response to inoculation of T1 (*B. megaterium* TSB16) (65% higher) followed by T3 (*B. cereus* HEB10) (61% higher).

Inoculation with PGPR influenced the dry weights (dry weight of shoot, root and total dry weight) positively for all PGPR applications compared to the control treatment (Fig. 4). Maximum total dry weight was observed in coconut seedlings inoculated with T2 (*B. megaterium* TEB2) (38% higher) followed by T3 (*B. cereus* HEB10) (34% higher) in trial 1 and *B. licheniformis* RSB14 in trial 2 that resulted in 29% increase over respective control. A comparison of the performance of the rhizospheric and endophytic PGPR on total dry biomass weight of coconut seedlings highlighted the better performance of the latter with six out of eight endophytic *Bacillus* spp. giving significantly higher

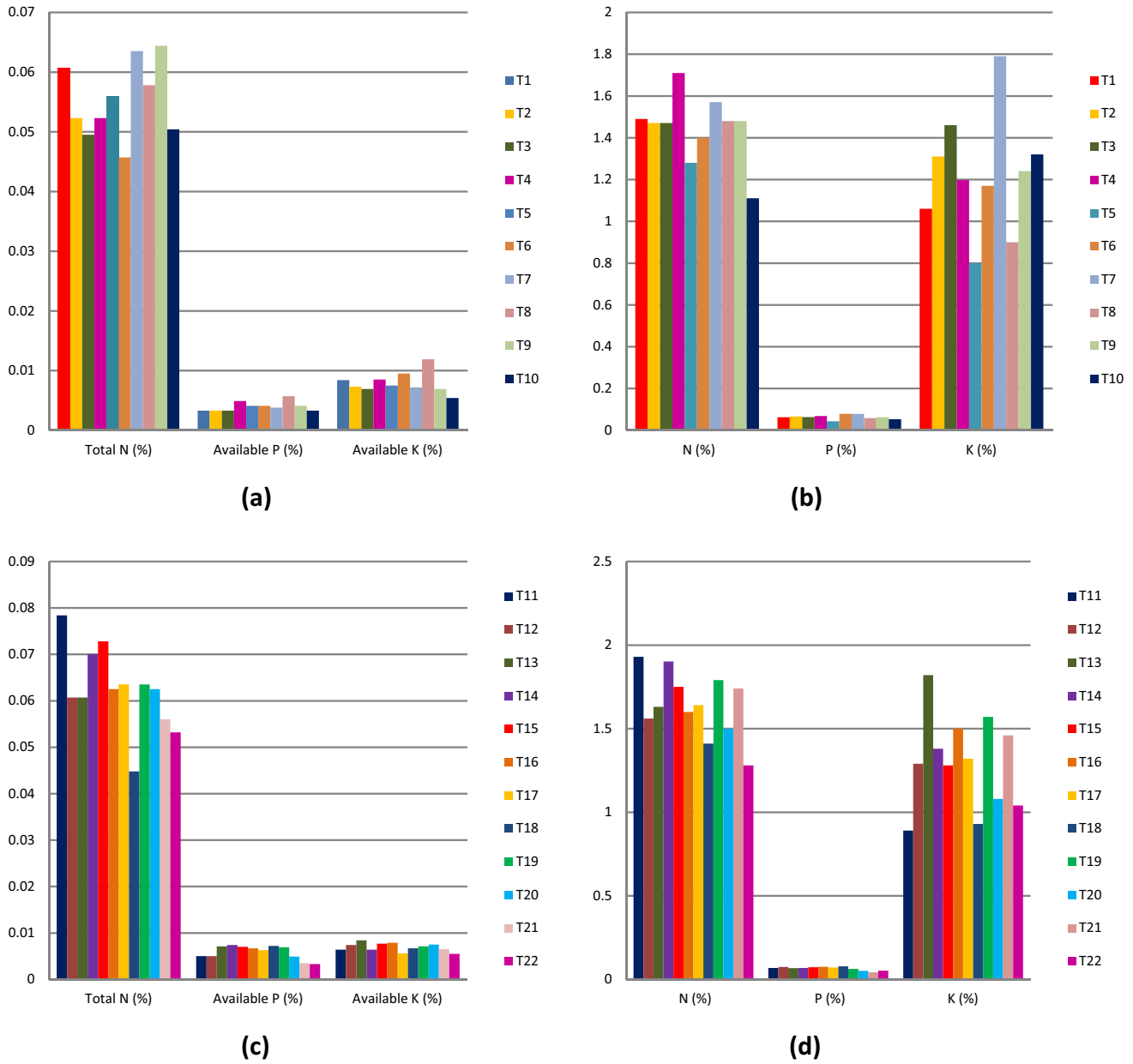

Fig. 5 Effect of PGPR treatments on major nutrients N, P and K in the rhizosphere soil and in the coconut seedlings [Trial 1 (**a** rhizosphere soil, **b** coconut seedlings, T10 as control); Trial 2 (**c** rhizosphere soil, **d** coconut seedlings, T22 as control); refer Supplementary Table S3 for details]

increments in dry weight compared to control in both the trials.

Effect of bacterial inoculations on soil nutrients, leaf nutrients and nutrient uptake of coconut seedlings

The data on N, P and K content in rhizospheric soil, plant parts (above ground level) and nutrient uptake by coconut seedlings are presented in Fig. 5; Table 2. The results indicated that most of the PGPR treatments significantly improved the nitrogen levels in rhizospheric soil, plant parts and its uptake by the seedlings compared to control. Statistically higher N content in rhizosphere soil was recorded with the application of *P. plecoglossicida* KiSF27 (T9), *Bacillus megaterium* KiEB31 (T7) in trial 1 and T11 (*Bacillus megaterium* KiSB10) in trial II. In case of available P and K in the rhizosphere soil, *P. plecoglossicida* RSF266 recorded maximum P (72% increase) and K content (120% increase) in trial 1, whereas, T14 (*P. putida* ChSF180) increased soil P and T13 (*B. cereus* HEB8) increased soil K in trial 2 (Fig. 5). A maximum of 47%, 124% and 53% increase in N, P and K content of

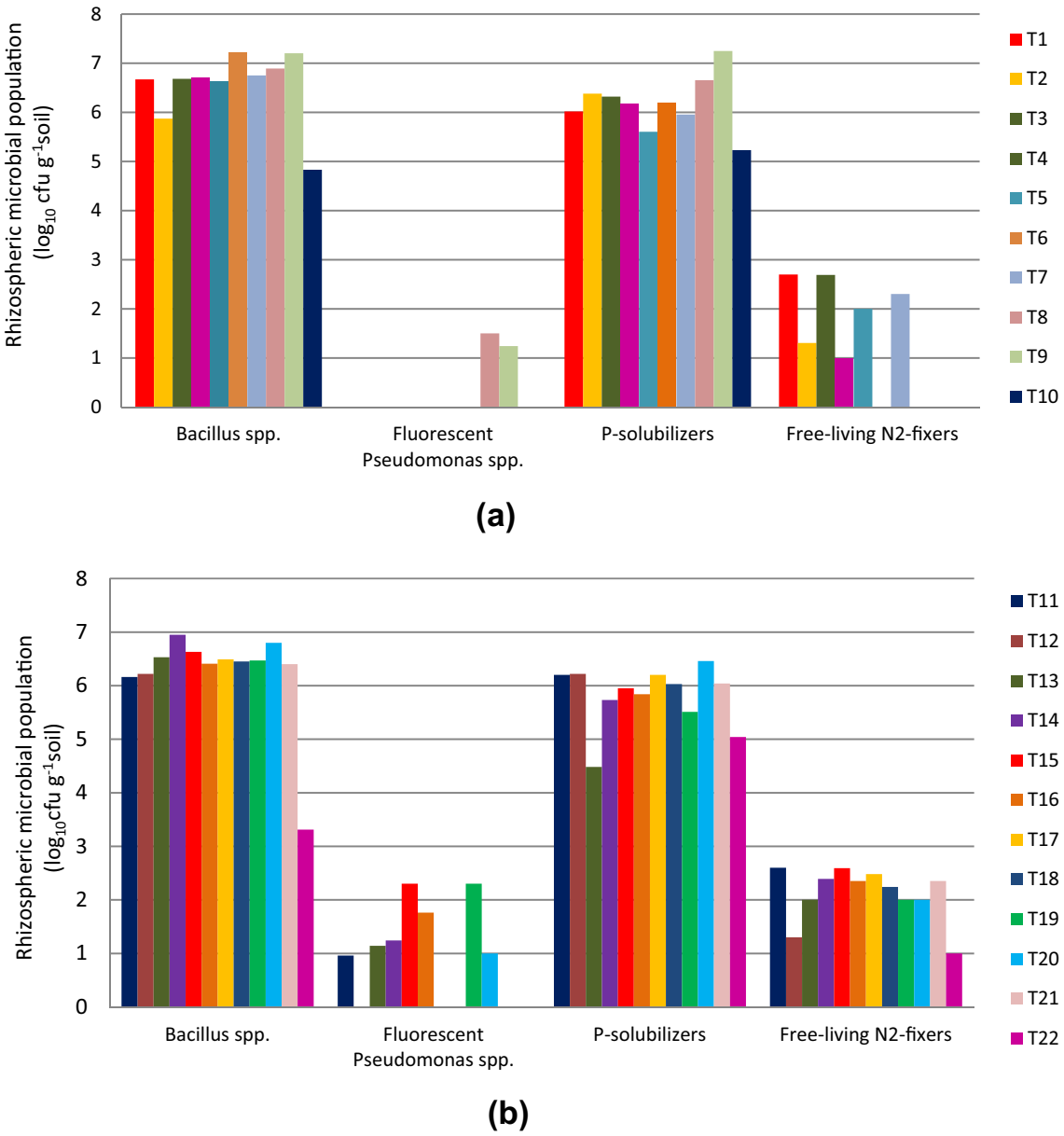

Fig. 6 Effect of PGPR inoculations on beneficial group of microbes in the rhizospheric region of coconut seedlings [Trial 1 (**a**, T10 as control); Trial 2 (**b**, T22 as control); refer Supplementary Table S4 for details]

rhizospheric soil, respectively, over control was recorded in second trial (Fig. 5).

In case of nutrient contents in the leaves of coconut seedlings, the bacterial inoculations increased the total N content from 10 to 54% over uninoculated control (Table 2). Maximum N content in leaf (54% increase) was shown by *B. cereus* ESB15 (T4) followed by *B. megaterium* KiSB10 (T11; 50% increase). In terms of the uptake, maximum N uptake (102% increase) was observed with the inoculation of *B. cereus* ESB15 (T4) over control. An increase of 88% in phosphorus uptake, over control, was recorded in seedlings treated with *B. cereus* KiEB25. The inoculation of *P. plecoglossicida* PoSF314 (T19) exhibited maximum increase (50%) in total K in the leaves while inoculation of *B. cereus* HEB10 (T3) recorded maximum K uptake (237%) in coconut seedlings over control (Table 2).

Effect on beneficial microbial population in the rhizosphere soil

Addition of the PGPR resulted in significant increase in rhizosphere microbial communities in the rhizosphere of coconut seedlings in both the set of field trials. The population of fluorescent pseudomonads was found to be higher in

Table 2 Effect of PGPR treatments on uptake of nutrients by coconut seedlings

Treatments	Nutrient uptake		
	N uptake (g/plant)	P uptake (mg/plant)	K uptake (g/plant)
Trial 1			
T1	2.20b	91.73f	1.77c
T2	2.16b	97.48c	2.63b
T3	2.24ab	95.52d	2.77a
T4	2.37a	94.32e	1.44e
T5	1.83c	61.35i	1.27f
T6	1.83c	103.30b	1.21f
T7	2.08b	103.88a	1.55d
T8	1.74c	68.32h	1.53de
T9	2.15b	89.92g	1.83c
T10 (control)	1.17d	61.07i	0.82g
Trial 2			
T11	2.70a	95.2d	1.60de
T12	2.00fg	95.6d	1.68cd
T13	2.04efg	83.9f	1.61de
T14	2.23cde	79.8g	1.62de
T15	2.41bc	101.7b	1.79c
T16	2.10def	98.8c	1.97b
T17	2.34bc	101.7b	1.51e
T18	1.99fg	111.3a	2.06ab
T19	2.46b	86.5e	2.17a
T20	1.87g	63.0h	1.35f
T21	2.23cde	60.8hi	1.71cd
T22 (control)	1.46h	59.3i	1.02g

All columns of each trial having the same letters are not different (P = 0.05) from each other by Duncan's multiple range test

trial 2 compared to the first (Fig. 6). A minimum increase by 2–3 factor $\log_{10}$ values in case of *Bacillus* spp. and one factor in case of P-solubilizer population was recorded in the PGPR- inoculated seedlings as compared to uninoculated ones. Significantly higher numbers of *Bacillus* and phosphate solubilizers were recorded in seedlings which were inoculated with *Pseudomonas* spp. (T8, T9) in trial 1 and (T14, T20) in trial 2.

Discussion

The twenty PGPR, comprising of fluorescent *Pseudomonas* spp. and *Bacillus* spp. possessing multiple PGP traits, were sequentially screened from the collection of 483 microbial isolates, which were originally isolated from the rhizosphere and roots of healthy and high yielding coconut palms growing in different states of India (George et al. 2018). From the genus angle, out of the 20 isolates, seven were *Pseudomonas* spp. and 13 were *Bacillus* spp. From the niche point, 12 were from rhizosphere and eight were of endophytic origin. While the rhizospheric isolates included both genera, all endophytes belonged to *Bacillus* genus. This hinted a preferential selection of gram positive *Bacillus* spp. by coconut as a close associative microsymbiont of the palm. Their plant growth promotion traits were tested and found to improve the growth of rice seedlings (George et al. 2018). Based on their PGP traits and performances in in vitro and green house studies, they were selected for testing for growth promotion efficacy with coconut seedlings. As performance of any potent PGPR in the field depends on its survival rate in the rhizosphere milieu, we additionally characterized the isolates for their tolerance towards abiotic stress factors like pH, salinity and temperature. The results indicated that most of the isolates grew well at a pH range 6.2–8.2, with one even at a wider range of 4.2–8.2. (*B. cereus* KnSB6). That said, five PGPR could tolerate pH up to 9 in the medium. In this study, most of the isolates survived up to 6% NaCl concentration in the medium and four *Bacillus* spp. showed growth at higher temperatures (50 °C). Overall, the analysis of PGP properties and abiotic stress tolerance traits indicated that rhizospheric isolates (both *Pseudomonas* spp. and *Bacillus* spp.) possessed more numbers of growth

promoting traits, tolerated wider range of temperature, NaCl and pH compared to endophytic isolates.

Coconut is mostly cultivated in soils that have low pH, so the spore-forming *Bacillus* spp. appeared to be more promising as bioinoculant in terms of their adaptability to wider pH range. Also, their ability to grow in higher salt and temperature conditions offers a scope for their use in coconut plantations in Karnataka, Tamil Nadu and Orissa States of India where large areas are salt-affected (Mandal et al. 2010) and encounter high temperatures during summer every year. A *Pseudomonas* strain isolated from the rhizosphere of date palm had earlier been found to give tolerance to maize grown in high salt and aluminium-stressed soils and promoted good root formation (Zerrouk et al. 2016). Thus, important traits for tolerating wide range of pH, temperature and salt concentrations expressed by PGPR in our study could help them perform in different soil conditions. The ability to withstand the adverse environmental conditions such as high salinity, high/low pH, and high temperature is significant not only for rhizobacterial survival in tropical agricultural soils but also for their use as biofertilizer (Banerjee et al. 2010) under the conditions of changing climate. The use of salinity- and drought-tolerant bacterial and fungal isolates was reported for amelioration of such abiotic stresses (Nadeem et al. 2014; Chakraborty et al. 2015).

An attempt was made to differentiate the PGPR based on their metabolic and genotypic diversity. BIOLOG metabolic profiling analysis had been demonstrated to be a highly effective method for differentiating among the species groups by cluster analysis (Dawson et al. 2002) and provided a large number of characters and produced robust data and more realistic representation of relationships among the strains (Yadav et al. 2011). Metabolically, all the 13 *Bacillus* spp. and 7 fluorescent *Pseudomonas* spp. formed separate clusters with greater than 0.43 similarity coefficient. *Pseudomonas* spp. formed three clusters at 0.84 similarity coefficient and maximum similarity between them was 93%. *Bacillus* spp. formed three separate clusters at a similarity coefficient of 0.76 based on the physiological and metabolic characters. Biolog system provided more accurate results where it analysed the ability of the cell to metabolize all major classes of biochemicals, in addition to determining other physiological properties such as pH, salt, reducing power and chemical sensitivity (http://www.biolog.com). Analysis of sugar utilization pattern of these PGPR via BIOLOG profiling was important because the survival of these isolates in the rhizosphere soil is dependent on their capability to use various organic compounds present in the root exudates. Additionally, the knowledge of the carbon utilizing pattern of each PGPR might prove helpful while making formulations for field applications. Though many of the PGPR were identified as same species of the *Bacillus* spp. or *Pseudomonas* spp., they were differing in their ability to utilize various carbon substrates. As these PGPR were originally isolated from the coconut rhizosphere and roots, obviously they could utilize those compounds in the root exudates of coconut and adapt to such environments easily (Bulgarelli et al. 2013), This might well be one of the reasons that isolation of PGPR from the native crop is preferred while going for the isolation of potent PGPR for a particular crop. The high utilization rates of various sugars, including those commonly present in coconut root exudates such as raffinose, lactose, glucose, fructose, serine, glutamine and alanine (Bopaiah et al. 1987) by these potent PGPR isolates, relate to the colonization of roots and enhancement of seedling growth (Alisi et al. 2005). Further detailed studies would be required to prove this aspect of application of PGPR in the field. Horemans et al. (2013) linked the use of carbon source utilization profile for studying the synergistic interactions in a bacterial consortium. Our results proved that the best performing PGPR in this study could utilize diverse sugars compared to the other PGPR. The potent PGPR *B. megaterium* TEB2 and TSB16 were found to be positive for maximum number of carbon utilization tests. They oxidized 74% and 76% of the carbon sources, respectively. Among *Pseudomonas* spp., *P. putida* KnSF208 showed maximum of 52% carbon utilization capability. The efficiency in promoting seedling growth might be due to the high metabolic activity associated with these isolates. The genotyping of rDNA-based fingerprints of PGPR using selective four restriction enzymes showed HaeIII and HinfI to be the most discriminative for *Bacillus* spp. and *Pseudomonas* spp., respectively. RFLP ribotyping is reported as an ideal method for differentiation among the members of well-defined species (Dawson et al. 2002).

Influence of PGPR on growth parameters of coconut seedlings was investigated by growing the inoculated seedlings in poly bags maintained under field conditions. Most of the growth parameters were positively influenced by PGPR and were significantly different from untreated control in DMRT analysis. Many fluorescent *Pseudomonas* and *Bacillus* spp. were reported as PGPR as they possessed multiple PGP traits that positively influenced plant growth (Patil et al 2016; Batista et al. 2016). All the elite strains used in our study were selected based on the strategic screening approaches in our previous research (George et al. 2018). This included in vitro testing for plant growth promotion attributes of microbial isolates and seedling bioassay under controlled and uncontrolled conditions with these isolates. These selected PGPR had performed better in all steps of selection process (George et al. 2018). As coconut is a perennial crop; for a good bearing garden, healthy and vigorous seedlings are sought by the farmers. Quality planting materials is, therefore, the first step towards better establishment of the crop, increased productivity and improved quality of coconut fruits for a considerable period of time (Chattopadhyay et al. 2004). Coconut seedlings respond positively and

develop faster on treatment with either chemical fertilizers (Almaden and Santiago 1980; Solangi and Iqbal 2012) or bioinoculants (Solangi and Iqbal 2012; George et al. 2013). We also found coconut seedlings to respond well to all the inoculated multi-trait PGPR and exhibit enhanced growth. *P. putida* KnSF208, *Bacillus licheniformis* RSB14, *B. megaterium* TEB2, *B. megaterium* TSB16, *B. cereus* KiEB25 and *B. cereus* HEB10 were found to be superior over uninoculated control and other PGPR treatments. They showed significant increase in most of the growth parameters over control as per DMRT analysis and also improved population of beneficial microbial populations in the rhizosphere of inoculated coconut seedlings. In earlier studies, we had reported production of multiple plant growth promotion traits like IAA production, capability to fix nitrogen as indicated by their ability to grow on N-free medium, P-solubilization, ACC deaminase activity and biocontrol activities like antagonism against coconut fungal pathogens, lytic enzymes production and antibiotic production by these bacteria (George et al. 2018). All these PGP traits directly or indirectly might have contributed to improvement of seedling growth and resulted in the production of vigorous coconut seedlings as compared to the uninoculated ones. The differential seedling growth in response to different PGPR might be due to their capability to utilize the root exudates and establish themselves in the rhizosphere region. This was illustrated by their distinct carbon utilization profiles. Hence, the multiple PGP traits possessed by these PGPR could have contributed to the enhancement of coconut seedling growth. The stimulatory effect might also be due to the fact that these treatments promoted and supported quite high population of beneficial microbes like fluorescent *Pseudomonas* spp., phosphate solubilizers and free-living nitrogen fixers in the rhizosphere of coconut seedlings. PGPR had been known to stimulate total microbial activity in the rhizosphere region in addition to increased shoot and root length, total dry weight in plants (Ahn et al. 2007). Similar effect of increased microbial population on PGPR inoculation was reported by Pandey et al. (2006) in maize seedlings on inoculation of *Pseudomonas putida* BO. A recent report of a single core taxa bacterium, *Variovorax*, regulating the growth of roots in complex interaction with other associated microbiome and the plant by fine tuning the auxin and ethylene metabolism (Finkel et al. 2020) is indicative of what might also be happening in our studies too.

Between the two genera, *Bacillus* and *Pseudomonas*, the former was able to utilize a wide array of substrates (carbon and others) and improve the dry weight content of the coconut seedlings than latter. In comparison, *Pseudomonas* spp. improved the nutrient uptake by coconut seedlings and promoted proliferation of the plant-beneficial microbiota in the rhizosphere region. Our findings reiterate the earlier work where application of several different species of *Pseudomonas* PGPR enhanced the NPK availability and uptake in tomatoes (Reyes-Castillo et al. 2019). Fan et al (2017) also reported that application of *Bacillus* spp. was able to improve N and P uptake in tomato plants. The evidence of better nutrient uptake at genomic level was reported, where application of consortium of *Bacillus* spp. was able to improve the NO_3 and NH_4 uptake in *Arabidopsis thaliana* by regulating the AtNRT1 and AtNRT2 gene families present in the plant (Calvo et al. 2019). Similar gene regulation activities might have resulted in better growth indices in coconut seedlings on application of *Bacillus* and *Pseudomonas* spp.

Numerous studies had reported that PGPR could be used as an effective supplement for promoting crop yields as well as soil health on sustainable basis. One of the critical parameters for evaluating the effect of PGPR on plant growth is the estimate of the total biomass dry weight, in contrast to fresh weights (Bashan and de-Bashan 2005). In our studies, the *Bacillus* and *Pseudomonas* spp. were observed to significantly increase the total dry biomass weight of the coconut seedlings. Stimulation of plant growth by the inoculation of PGPR had been reported in other crops too. Kuan et al. (2016) reported that inoculation of *Bacillus pumilus* S1r1, which was isolated from maize plants, increased the dry biomass, total N content as well as bacterial colonization in exo- and endo-rhizosphere in maize. Increased seedling biomass and nutrient uptake was recorded on inoculation of *B. licheniformis* MH48 in *Camellia japonica* seedlings (Park et al. 2017). *Serratia* strains isolated from the rhizosphere of rauli (*Nothofagus alpina*) in volcanic soils harboured PGPR mechanisms and increased the root collar diameter, height, relative chlorophyll content, biomass and nitrogen content of raulí plantlets (Martínez et al. 2018).

The detailed studies of the bacterial isolates from coconut rhizospheric and endophytic niches clearly showed PGPR capable of promoting growth of the coconut seedlings as influenced by nutrient uptake, positive plant–microbe feedback via stimulating rhizosphere microbial populations and activities resulting in better total dry biomass production compared to uninoculated seedlings. The endophytic *Bacillus* spp. were seen as better performers in coconut seedling trials than the rhizospheric isolates, even though they possessed less diverse PGP traits and abiotic stress tolerance limits. The possible reasons could be (i) endophytic bacteria have tighter association with the plants than rhizospheric isolates and this close association could have afforded them to exert a more significant impact on plant growth promotion, (ii) their ability to use diverse array of carbon sources would have allowed them to utilize the root exudates of coconut seedlings more efficiently and perform better PGP activities and (iii) eliciting positive plant–microbe feedback in terms of promotion of beneficial rhizospheric microbial community, particularly the free-living nitrogen fixers.

Conclusions

Our studies proved that the multi-trait PGPR of *Bacillus* and *Pseudomonas* genera, isolated from rhizosphere as well as root endophytic compartment of healthy and high yielding coconut palms, significantly improved the growth parameters of coconut seedlings. The endophytic *Bacillus megaterium* TEB2 and *B. cereus* HEB10, and rhizospheric *B. licheniformis* RSB14 were the most effective ones in promoting the growth of coconut. Their association resulted in production of vigorous and healthy coconut seedlings driven by better nutrient uptake, higher biomass production and stimulation of plant-beneficial rhizosphere microbiota. The PGPR had excellent adaptation to wide range of abiotic factors like pH, salinity and temperature as well as the organic chemicals released by the roots. The ability of the *Bacillus* spp. to withstand a good range of abiotic stresses, utilize wider array of carbon sources, be present in the rhizosphere and the endophytic niches along with their endospore-producing capability make these gram-positive bacteria an ideal choice compared to *Pseudomonas* spp. These native PGPR could be developed as bioinoculants for production of quality coconut seedlings as well as for adult coconut palms. The findings also offer chance to develop a MEMS from these elite PGPR for wider applications.

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Data availability All necessary data is available in the manuscript.

Declarations

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

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