Research Square

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Plant Growth-Promoting Bacteria Associated with the Nests of the Seed-Harvester Ant, *Trichomyrmex Scabriceps*

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Abstract

Colonies of seed harvester ants are commonly found in semiarid and arid areas of the world and have been studied for their seed dispersal behaviour. The present study focused on the bacteria associated with the nests of the harvester ant, *Trichomyrmex scabriceps*, and reveals that ant colonies link the aboveground resources with the belowground microbial communities as they accumulate organic debris in the close vicinity of their nests via their ecosystem engineering activities. Soil samples were collected from the nest chambers and the external debris piles of *T. scabriceps* colonies, located in managed ecosystems. The nest soil-associated bacteria were examined for their plant growth-promoting abilities via biochemical assays including phosphate solubilization, Indole acetic acid production, siderophore production and physiological assays including biocontrol potential against the soil pathogen, *Sclerotium rolfsii*. More than 60% of bacteria isolated from the ant nest-associated soil displayed plant-growth promoting ability. *Bacillus* sp., *Azotobacter* sp., *Klebsiella* sp., *Comamonas* sp., *Tsukamurella* sp., and *Pseudoxanthomonax* sp., demonstrated significantly high levels of gnotobiotic growth of the treated chickpea plants. The activities of phenylalanine ammonia-lyase and peroxidase enzymes were higher in plant growth-promoting bacteria treated and pathogen inoculated plants as compared to the control plants lacking the bacteria. Since *T. scabriceps* colonies often make their nests in the compact soil of unpaved paths of agroecosystems and gardens, these bacteria can act as highly effective biofertilizers and promote growth of the cultivated plants by increasing soil fertility and disease resistance attributes of the plant.

Introduction

Although recent studies have documented a high diversity of agroecosystem-inhabiting ants and reported the significant role of the ecosystem-engineering activities of ant colonies in soil fertility enhancement [1, 2], only scanty information is available on the association of ant colonies with plant growth-promoting bacteria (PGPB) [3]

The beneficial free-living bacteria present in soil that colonize the rhizosphere, the rhizoplane (root surface), or the plant root itself (within radicular tissues) and promote plant growth, are referred to as PGPB [4]. These PGPB enhance plant growth via production of phytohormones, such as indole acetic acid (IAA), gibberlin, and cytokinin [5]. The principal hormone IAA, produced by PGPB is responsible for physiological processes, such as cell division, root, and shoot development [6]. The insoluble phosphates in soil are also made available to the plants by phosphate-solubilizing microorganisms by the processes of acidification, chelation, and exchange reactions [7, 8]. Also, the secondary metabolites known as siderophores released from PGPB can chelate Fe^{3+} (ferric) ions from the soil with higher affinity and provide this complex to the plants [9]. Many plant growth-promoting (PGP) microbes also play an important role in suppressing phytopathogens, and this activity may be mediated via direct and/or indirect mechanisms [10, 11]. While the direct mechanisms involve the production of siderophores, antibiotic and lytic enzymes [12, 13], the indirect mechanisms involve induction of local and systemic resistance against phytopathogens [14] by production of phenols such as phenylalanine ammonia-lyase (PAL), and peroxidase (PO_X) which play an important role in plant disease resistance [15]. The use of these microbe-based biofertilizers helps in reducing the use of chemical fertilizers, improves soil fertility and contributes to enhanced plant productivity [16].

Enriched microbiomes documented to occur within ant nests and the surrounding areas are often found to be distinct from those found in bare soil, indicating the role of ant colonies in influencing soil microbial communities [17]. The richness and diversity of soil microbes are known to be affected by the differences in the organic amendments added to the soil [18]. Hence, the availability of substrates favored by PGPB within the nest chambers and in the external debris pile may be one possible reason for the reported PGP attributes of ant nest debris soil [1, 2]. Ant nests are characterized by the abundance of organic matter brought to the nests by the returning foragers as food for the colony members [19]. While the fecal matter is often deposited in fecal patches in specific locations within the nest [20], the uneaten matter is usually discarded at the external refuse piles. Under experimental field conditions, the yield of crop plants has been found to be significantly enhanced when grown in the nest debris pile soil of *Pheidole latinoda*, which preferentially colonizes cultivated areas [19, 2]. A meta-analysis by Offenberg and Damgaard, [21] indicates that ants may also be involved in the reduction of pathogen loads of the visited plants.

Many ground-nesting ant species, such as the seed harvester ants, construct extensively modified galleries in their nests, forage on the seeds of their preferred plant species and accumulate a huge amount of organic debris in the close vicinity of their nests [22]. Recent studies reveal that both the nest chambers as well as the external refuse piles of plant-visiting, *Camponotus compressus* colonies harbor many PGP microbes [3]. Diverse ant species are reported to inhabit highly disturbed, managed ecosystems, such as annual cropping systems [19]. Therefore, elucidating the influence of ant colonies on soil fertility and plant growth is of far-reaching significance. Thus, it is essential to ascertain whether PGPB are associated with ant nests and to find if they also affect plant growth via their effect on soil-borne pathogens.

Only scanty information is available regarding the influence of seed harvesting ant colonies on the microbial richness and these mainly pertain to the influence of the microbes on soil properties. *Trichomyrmex scabriceps* (Mayr 1865) is a seed harvester ant species widely distributed in many parts of India [23] but surprisingly, very little is known about its ecology. The nests of the seed-harvesting, *T. scabriceps* colonies are commonly found in hard, compact soil, such as that typically found on unpaved paths of agroecosystems, gardens and, roadsides (personal observations). Its nests are characterized by the presence of seed husk piles, a single pile being present at the nest entrance rim of a nest. Colonies of *T. scabriceps* have the potential to play a crucial role in linking the above-ground resources with the belowground biota, such as microbial communities.

In the present study, we have focused on the PGP microbiota associated with colonies of the Asian harvester ant, *T. scabriceps* (Mayr 1865), which is widely reported from a number of tropical and subtropical countries, including India and Sri Lanka [24]. Colonies of *T. scabriceps* commonly occur in arid and semiarid areas and the workers primarily forage on seeds. Our preliminary survey revealed the widespread occurrence of *T. scabriceps* colonies in

our study area. Moreover, our field observations (since April, 2018) suggest that the main nests (each main nest being accompanied by 1-3 satellite nests) are fairly long-lived (at least > 3 years). The PGP activities of ant nest associated microorganisms were further analyzed by the seed biopriming (SBP) method. For the SBP study, seeds of chickpea (*Cicer arietinum* L.) were preferred since this is one of the largest produced pulse crops in semi-arid tropical countries, including India, where it comprises about 40% of the total pulse crop [25]. India being the largest producer of chickpea contributes around 65% of the world's total production. Although a high number of plant pathogens have been reported to attack the chickpea crop, *Sclerotium rolfsii* is a well-known soil-borne fungal pathogen of chickpea crop in tropical and subtropical areas of the world [26]. It attacks the crop at the early seedling stage (resulting in the collar rot disease) and causes 55–95% mortality of chickpea seedlings under favorable environmental conditions [27]. The control of *S. rolfsii* in agriculture has been challenging, since the highly competitive saprophytic pathogen exhibits high survivability in dry climatic regions, occurs on a wide range of natural hosts and can persist in soil for prolonged periods even after several crop rotations [28]. Consequently, we examined the antagonistic (*in-vitro*) activities of isolated bacteria against *S. rolfsii.*

We hypothesize that soil fertility enhancement and the PGP effects of ant nest soil are due to the presence of associated PGPB. Due to the ecosystem engineering activities of ant colonies the ant nest soil with its high organic matter content provides suitable conditions for the growth of the PGPB, which could be directly involved in enhancing plant growth and possibly even in the reduction of soil-borne fungal pathogens such as, *S. rolfsii*. Therefore, in the present study, we addressed the following 4 inter-related questions: (1) Are the seed harvester ant colonies associated with PGPB? (2) Is there any difference between the abundance of PGPB in the debris pile and the nest chambers of *T. scabriceps*? (3) What is the direct impact of the PGPB isolates on plant growth? (4) Do these bacteria play any role in plant disease resistance?

Methods

Experimental Design

Soil Sample Collection. A total of 19 plots (area of each plot =10 ×10 m^2) were randomly selected in the botanical garden (covering an area of ~2 hectares) of Banaras Hindu University (BHU), Varanasi, U.P., India (25°18'N, 83° 03'E). The soil samples were collected (during August 2018), from the nest chambers and debris piles (located at the nest entrance rim) of *T. scabriceps* nests (n= 43) located in the field plots. The control garden soil samples were collected (from a depth of 10-20cm) from areas located at a distance of ≥ 5m from ant nests and vegetation. Ant nest soil sample collection was done only from those nests which were located at a distance of ≥ 2 m from the nearest vegetated area.

Isolation of PGP Bacteria

The collected soil samples were brought to the laboratory and placed into three groups: group 1 (control) contained garden soil, group 2 (experimental), which comprised soil collected (as per the method used by Shukla et al. [19]) from the external refuse piles of the seed harvester ant nests, and group 3 (experimental), from the ant nest chambers (from a depth ~9-20 cm). Each soil sample was serially diluted up to 10^{-8} dilution to obtain isolated colonies. Next, 0.1 ml of each serially diluted sample (10^{-1} -10^{-8}) was transferred to the respectively labeled Petri plates containing the nutrient broth (for bacterial culture) and incubated at 28° C for 24–48 h. The serial dilution was carried out in triplicate, on agar plates, where bacteria were isolated based on the colonial morphology (including size, shape, and other extracellular characteristics) of each. Next, each of these was isolated as a pure culture and stored on Nutrient Agar (NA) slants at 4° C.

Biochemical PGP Assay

For assessment of the plant growth-promotion abilities of the isolated bacteria, *in-vitro* phosphate solubilization, IAA production and siderophore production abilities were examined. Phosphate solubilization ability, estimated as per the method of Mehta and Nautiyal [29] involved inoculation of strains in National Botanical Research Institute's phosphate growth medium (NBRIP) [30] for 48 h at 28° C and 120 rpm. The reduction of the molybdo-phosphoric acid to stannous chloride, was recorded at 660 nm. IAA was estimated by using the method of Bric et al. [31]. The isolates were inoculated in Nutrient Broth (NB) containing tryptophan (5 mM), and then incubated for 48 h (28° C, 120 rpm). The NB cultures were further subjected to centrifugation (10,000 rpm for 15 min) and the supernatant was collected. One ml volume of the supernatant was mixed with 2 ml Salkowski reagent (1 mL of 0.5 M FeCl3 in 50 mL of 35% HClO4; [32]) and kept in the dark for 30 min. The chemical reaction resulting in a color change from yellow to pink/red showed a positive result for IAA production. The optical density (OD) was recorded at 535 nm by using a Thermo Scientific UV1 spectrophotometer. The siderophore production ability of isolates was assessed (both qualitatively and quantitatively) by the method of Bano and Musarrat [33].

Assessment of the biocontrol potential of the isolated bacteria

To investigate whether the bacterial isolates play any role in making the plant disease resistant, experiments were next conducted to find if they show any antagonistic activities against plant pathogens such as *S. rolfsii.*

Hence, further screening of the potential PGPB was done based on their effective antagonism against a well-documented [34], soil-borne plant pathogen, *S. rolfsii* (obtained from Mycology and Plant Pathology Dept., Institute of Agriculture, of BHU). Antagonistic activities of the isolated bacteria against *S. rolfsii* were evaluated *in-vitro* by using the dual-culture plate assay. In each case, a loopful of bacterial isolate was streaked, 2 cm away from the edge of each plate (n=3) containing NA. The pathogen was introduced on the plate by placing a plug (containing mycelia from the pure culture plates), 2 cm away from the other edge of the plate, on the side opposite to that inoculated by the isolated bacteria, maintaining a minimum distance of 5 cm between the two types of microbes. The plates were incubated at 27 ± 2° C and observed on the seventh day post-incubation where the percent inhibition of

pathogen was measured in terms of the distance (growth) traveled (in mm) by the pathogen and the bacterial colony towards each other on the same NA plate [35]. The experiment was set up in triplicate where the percent inhibition of the pathogen was calculated by using the following formula:

I = (C − T) × 100/ C

Where; I = percent (%) inhibition in mycelia growth; C = growth of pathogen in control plates (in mm); T = growth of the pathogen in dual culture plates (in mm).

Experimental setup

Inoculum preparation for seed biopriming

Pure culture of each bacterial isolate was inoculated in NB medium and placed in a shaker for ~24-48 h (to attain the stationary phase). Later the suspension was centrifuged at 10,000 rpm for 10 min at 4° C and the collected pellet was further washed with 10 ml of 0.85% sodium chloride solution to remove the residual NB. Finally, the pellets were suspended in 25 ml of 1% carboxymethyl cellulose (CMC) sodium salt suspension and vortexed vigorously to acquire a homogenous cell density of about 4×10^8 cfu/ml [36].

Seed biopriming with prepared bacterial suspension

Chickpea seeds were surface sterilized with 1% sodium hypochlorite (NaOCl) solution for ~60s, and then rinsed with sterilized distilled water (SDW) thrice, followed by brief drying under laminar flow, on pre-sterilized blotting paper. For seed biopriming experiments, 20 chickpea seeds (for each isolate) were placed on freshly sterilized blotting paper placed in a sterilized Petri plate and the prepared bacterial suspension (100µl) was poured on the cluster of 20 seeds. The excess amount of suspension was drained out. Only CMC treated seeds were treated as the control. These plates with seeds were incubated at 28±2˚C, with 98% relative humidity for 24 h.

In-vitro plant growth-promotion assessment

The growth-promoting ability of the bacterial isolates was assessed on chickpea seedlings by measuring its growth augmentation effect. The bioprimed seeds were placed in plastic pots, each containing a sterile sand-soil mixture. Each pot (treatment) contained 5 seedlings (single bacterial suspension) from a sterilized Petri plate. Also, each pot (treatment) had 5 replicates using a completely randomized design [37]. The pots were kept in the greenhouse chamber for 25 days at 28 ± 2°C and relative humidity (RH) of 76%. The control set contained only water-soaked seedlings. The isolate treated seeds expressing higher plant growth under gnotobiotic conditions were selected for phenylalanine ammonia-lyase (PAL), and peroxidase (PO_X) assessment.

Assessment of disease resistance in bioprimed seeds

The pathogen was multiplied by the method of Sarma et al. [38] and the resulting colonized culture was inoculated at the collar region of bioprimed chickpea plants. From each treatment (n=5) the nodal leaves from the bottom of the plant were collected after 24 h, 48 h, and 96 h without causing any damage to the plant. The collected leaves were washed, dried, and stored in a deep freezer (−80°C) for further use.

To assess phenylalanine ammonia-lyase (PAL) activity, the leaves of chickpea plants (0.1g) of control and experimental plants, were homogenized in 2 ml sodium borate buffer (0.1 M; pH 7.0) containing 1.4 mM mercaptoethanol. The resulting solution was centrifuged for 15 min at 16,000 (4°C). The reaction mixture containing 200 µl enzyme extract, 206 µl phosphate buffer (0.2M, pH 8.7), 1.3 ml distilled water and 1 ml L-phenylalanine (0.1M, pH 8.7) was incubated for 30 minutes at 32°C. The ongoing reaction was terminated by adding 500 µl trans-cinnamic acid (1 M) and the absorbance was recorded at 290 nm where PAL activity was expressed as µM TCA g^{-1} fresh weight (FW). To assess antioxidant activity, peroxidase (POx) activity was measured. Leaf sample (0.1g) was homogenized in 5.0 ml phosphate buffer (0.1 M; pH 7.0, ice cold), and further centrifuged at 16,000 rpm (4°C for 15 min). 50 µl of enzyme extract (supernatant) was added to the reaction mixture containing 1.5 ml of pyrogallol (0.05 M) and 500 µl of 1% H2O2. The reaction mixture without enzyme extract served as the control. The absorbance was recorded at 420 nm at intervals of 30 sec for 3 minutes until a constant change in absorbance value was attained. PO activity was expressed as U min^{-1} g^{-1} FW [39].

Genomic DNA isolation

Genomic DNA of the principal bacterial isolates was obtained by the method of Wilson [56] with a slight modification. The isolates were subjected to centrifugation and the culture pellets formed by the bacterial culture were subjected to lysozyme (0.5 mg ml-1), SDS (2.3%) and proteinase K (0.2 mg ml-1) treatments followed by heating at 55° C for 2–3 h. The suspension was again centrifuged. Genomic DNA from the supernatant was extracted using phenol–chloroform–isopropanol and precipitated using ethanol. For taxonomic identification, the genomic DNA (16S rDNA) of the isolated bacteria were submitted to Eze Diagon Healthcare Pvt, Ltd, Coimbatore, Tamil Nadu, India. The nucleotide sequences (PCR amplified) thus obtained were next submitted to NCBI GenBank to get the Accession Numbers. Further, a phylogenetic tree with isolates was constructed using MEGA 6 software to substantiate the classification.

Statistical Analyses

Data was statistically analyzed by ANOVA, and the treatment means were separated by Duncan's multiple range test (DMRT) at $P < 0.05$ using SPSS version 19.0 (SPSS Inc., USA).

Results

The control soil sample contained a total of 18 bacterial isolates, whereas the ant nest associated soil contained 57 and 27 bacterial isolates respectively obtained from the nest debris piles and nest chambers.

Table 1
Biochemical and physiological characterization of bacterial isolates from control soil (from 10-20cm depth, 5-10m away from ant nest and vegetation)

Bacterial isolate	Phosphate solubilization ($\mu g\ ml^{-1}$)	IAA production ($\mu g\ ml^{-1}$)	Siderophore production (%)	Biocontrol potential (%)
CB01	33.67 ± 0.251^{d}	27.86 ± 0.005^{d}	-	-
CB02	29.43 ± 0.252^{e}	11.36 ± 0.065^{m}	14.63 ± 0.374^{f}	-
CB03	35.97 ± 1.053^{c}	23.66 ± 0.005^{e}	18.48 ± 0.418^{d}	36.87 ± 0.821^{d}
CB04	38.63 ± 1.263^{a}	36.37 ± 0.009^{a}	31.08 ± 0.535^{a}	69.67 ± 1.062^{a}
CB05	39.03 ± 1.103^{a}	31.45 ± 0.106^{c}	27.13 ± 0.603^{b}	61.34 ± 1.528^{b}
CB06	22.73 ± 0.303^{h}	13.71 ± 0.068^{k}	-	-
CB07	28.63 ± 0.603^{f}	12.26 ± 0.009^{l}	11.40 ± 0.197^{g}	
CB08	31.53 ± 0.745^{jk}	17.02 ± 0.001^{i}	-	-
CB09	29.87 ± 0.702^{ef}	19.02 ± 0.006^{g}	16.63 ± 0.354^{e}	12.67 ± 2.082^{f}
† Values are the means of three replicates ± SE mean where – indicates no growth/no				
activity.				
‡ Values followed by different letters within a column are significantly different according to Duncan's multiple range test ($P < 0.05$).				

Biochemical and physiological screening

Microbial isolates demonstrating PGP characteristics were distinguished based on the preliminary biochemical screening involving the 3 types of *in vitro* plant growth-promotion assays: phosphate solubilization, IAA and siderophore production.

The nest debris soil harbored 63% (23 bacterial isolates) of isolates, with phosphate solubilization ability (ranging from 12 µg/ml to 108 µg/ml), whereas only 57% (21 bacterial isolates) of isolates were able to produce IAA, ranging from 10 µg/ml to 57 µg/ml. Further 10 isolates among them had the ability to produce siderophore (Table 2, SF 1). The highest (76%) phosphate solubilizing ability was demonstrated by the bacterial isolate MDB15 and the trend in the decreasing order was: MDB15 (76%) > MDB07 (63%) > MDB19 (57%) > MDB12 (54%) > MDB09 (38%). These isolates also expressed biocontrol potential against the plant pathogen, *S. rolfsii*. About 17% of total isolates showed positive results for all three characteristics (Table 3).

Table 2
Biochemical and physiological characterization of bacterial isolates: MDB (01- 23) from debris soil of Trichomyrmex scabriceps ant colonies

Bacterial isolate	Phosphate solubilization ($\mu g\ ml^{-1}$)	IAA production ($\mu g\ ml^{-1}$)	Siderophore production (%)	Biocontrol potential (%)
MDB01	14.22 ± 0.555^{c}	10.46 ± 0.025^{a}	-	-
MDB02	30.43 ± 0.252^{jk}	10.26 ± 0.055^{a}	-	-
MDB03	30.14 ± 1.749^{j}	16.18 ± 0.010^{e}	8.10 ± 0.448^{b}	-
MDB04	15.90 ± 0.100^{d}	17.50 ± 0.080^{fg}	10.08 ± 0.254^{c}	-
MDB05	23.03 ± 0.503^{g}	15.45 ± 0.106^{d}	7.13 ± 0.603^{a}	-
MDB06	12.73 ± 0.503^{b}	13.51 ± 0.068^{c}	11.60 ± 0.447^{d}	-
MDB07	108.63 ± 0.603^{p}	42.26 ± 0.007^{m}	51.40 ± 0.197^{k}	89 ± 5^{d}
MDB08	31.53 ± 0.945^{jk}	19.02 ± 0.001^{f}	-	-
MDB09	31.87 ± 0.702^{k}	29.02 ± 0.006^{j}	16.63 ± 0.354^{g}	12.67 ± 2.082^{a}
MDB10	66.53 ± 1.002^{n}	21.66 ± 0.013^{g}	13 ± 0.471^{e}	-
MDB11	19.20 ± 0.300^{f}	14.11 ± 0.005^{c}	7.84 ± 0.059^{b}	-
MDB12	76.27 ± 0.551^{m}	47.42 ± 0.006^{l}	32.58 ± 0.472^{i}	44.67 ± 3.055^{b}
MDB13	22.95 ± 0.852^{g}	19.77 ± 0.014^{h}	-	-
MDB14	26.40 ± 0.536^{i}	17.60 ± 0.006^{fg}	-	-
MDB15	41.83 ± 1.795^{o}	37.37 ± 0.009^{n}	47.08 ± 0.535^{j}	65.68 ± 2.517^{c}
MDB16	18.11 ± 0.800^{ef}	11.33 ± 0.006^{b}	-	-
MDB17	13.31 ± 0.302^{a}	10.12 ± 0.001^{a}	-	-
MDB18	13.18 ± 0.426^{a}	11.19 ± 0.001^{b}	-	-
MDB19	36.87 ± 0.351^{l}	21.86 ± 0.005^{k}	18.48 ± 0.428^{h}	12.33 ± 1.528^{a}
MDB20	24.71 ± 1.589^{h}	57.06 ± 0.001^{i}	14.60 ± 0.365^{f}	-
MDB21	11.76 ± 0.550^{b}	-	-	-
MDB22	$17.12\pm 0.694d^{e}$	12.85 ± 0.756^{g}	-	-
MDB23	18.107 ± 0.703^{ef}	-	-	-

† Values are the means of three replicates ± SE mean where – indicates no growth/no activity.

‡ Values followed by different letters within a column are significantly different according to Duncan's multiple range test ($P < 0.05$).

However, 78% of the total isolates present in the ant nest chamber soil demonstrated phosphate solubilization ability, ranging from 22 µg/ml to 293 µg/ml, and 52% of total isolates efficiently produced IAA (21 bacterial isolates), ranging from 12 µg/ml to 53 µg/ml. while 6 isolates showed efficient siderophore producing ability. Among these bacterial isolates: MNB05 (33%), MNB06 (78%), and MNB08 (69%) also indicated biocontrol potential (Table 3, SF 2). In contrast to the ant nest soil, the control soil contained fewer bacteria (45% phosphate solubilizing, 27% IAA producing, 11% siderophore producing and with biocontrol potential) with PGP activity (Table 1).

Table 3

Biochemical and physiological characterization of bacterial isolates: MNB (01- 17) from nest chamber soil of *Trichomyrmex scabriceps* ant colonies

Bacterial isolate	Phosphate solubilization (µg ml^{-1})	IAA production (µg ml^{-1})	Siderophore production (%)	Biocontrol potential (%)
MNB01	29.45 ± 0.12^{c}	-	-	-
MNB02	29.45 ± 0.12^{c}	-		-
MNB03	28.58 ± 0.25^{d}	27.55 ± 0.769^{e}	19.45 ± 0.239^{d}	-
MNB04	182.58 ± 0.41^{k}	31.15 ± 0.154^{f}	23.20 ± 0.307$^{e±}$	-
MNB05	208.46 ±0.24^{m}	43.47 ± 0.924^{h}	31.11 ± 0.864^{f}	68 ± 2.16^{b}
MNB06	271.20 ± 0.36^{p}	50.31 ± 0.153^{k}	67.43 ± 0.347^{g}	87 ± 2.49^{c}
MNB07	68.312 ± 0.52^{f}	12.02 ± 0.077^{a}	-	-
MNB08	292.69 ± 0.14^{q}	53.29 ± 0.153^{j}	71.33 ± 0.257^{i}	96 ± 1.63^{e}
MNB09	22.49 ± 0.251^{e}	-	18.15 ± 0.141^{c}	
MNB10	119.13 ± 0.24^{o}	-	10.86 ± 0.663^{a}	11 ± 0.82^{a}
MNB11	63.55 ± 0.14^{l}	39.73 ± 0.133^{g}	16.35 ± 0.12^{b}	-
MNB12	97.33 ± 0.74^{i}	6.98 ± 0.077^{c}	-	-
MNB13	31.85 ± 0.27^{a}	-	-	-
MNB14	104.62 ± 1.29^{j}	29.95 ± 0.671^{f}	-	-
MNB15	23.11 ± 0.74^{b}	-	-	-
MNB16	74.66 ± 0.27^{g}	15.91 ± 0.154^{c}	-	-
MNB17	76.86 ± 0.14^{h}	14.27 ± 0.133^{b}	-	-

† Values are the means of three replicates ± SE mean where – indicates no growth/no activity.

‡ Values followed by different letters within a column are significantly different according to Duncan's multiple range test ($P < 0.05$).

Effects of selected isolates on seed germination and growth of chickpea

After preliminary screening, a total of 6 isolates; MDB07, MDB09, MDB12, MDB15, MDB19, and MDB20 from nest debris piles and a total of 6 isolates; MNB03, MNB04, MNB05, MNB06, MNB08, and MNB10 from ant nest chambers were selected for seed biopriming, to assess their biofertilizer potential. All the isolates showed positive results for at least two of the biochemical assays (Table 2&3). The selected bacterial isolates when applied to chickpea seeds significantly enhanced seed germination, shoot length, root length and plant biomass (shoot fresh weight, root fresh weight, shoot dry weight, root dry weight) as compared to the negative control, in each case. Seed germination percentage increased up to 80% with debris pile soil isolates, and up to 89% in the case of nest chamber soil isolates. The shoot length was enhanced from 6.22–14.39% by the debris soil isolates and by 5.77–16.19% by the nest chamber soil isolates. The isolated bacteria effectively intensified root length up to 18.72% in the case of debris soil isolates and 14.45% by the nest soil isolates. In addition, bacterial isolates augmented total fresh weight (debris isolates: 28.65%-59.42%, nest isolates: 28.65%-59.42%) and total dry weight (debris soil isolates: 28.65%-59.42%, nest chamber soil isolates: 28.65%-59.42%) of chickpea seedlings (Table 4&5). Among 13 bacterial inoculants, treatment with MDB15, MDB12 from debris piles and MNB06, and MNB08 from nest chamber showed the highest PGP abilities for all growth parameters (SF 3). Hence, they were further analyzed for anti-oxidative enzyme activities.

Table 4
Effect of selected bacterial isolates: MDB (07, 09, 12, 15, 19 and 20), from nest debris soil of Trichomyrmex scabriceps ant colonies on plant growth promotion: Seed germination (SG), Shoot length (SL), Root length (RL), Shoot fresh weight (SFW), Root fresh Weight (RFW), Total fresh weight (TFW) and Total dry weight (TDW).

Treatment	Plant growth characteristics								
	SG (%)	SL (cm)	RL (cm)	SFW (gm)	SDW (gm)	RFW (gm)	RDW (gm)	TFW (gm)	TDW (gm)
Control	40± 1.58^{a}	6.29± 0.022^{a}	7.574± 0.021^{a}	0.147± 0.022^{a}	0.019± 0.006^{a}	0.127± 0.022^{a}	0.015± 0.004^{a}	0.273± 0.036^{a}	0.034± 0.006^{a}
MDB07	80± 1.58^{cd}	12.77± 0.033^{f}	14.66± 0.115^{f}	0.331± 0.013^{d}	0.057± 0.010^{c}	0.262± 0.024^{d}	0.033± 0.005^{bc}	0.592± 0.027^{d}	0.089± 0.009^{d}
MDB09	58± 1.48^{b}	10.16± 0.033^{c}	9.706± 0.017^{c}	0.273± 0.024^{c}	0.044± 0.013^{bc}	0.177± 0.014^{b}	0.030± 0.004^{b}	0.450± 0.045^{c}	0.074± 0.015^{cd}
MDB12	74± 3.6^{ef}	12.33± 0.022^{c}	16.35± 0.271^{e}	0.294± 0.015^{c}	0.051± 0.018^{c}	0.275± 0.030^{d}	0.034± 0.003^{bc}	0.569± 0.023^{d}	0.085± 0.016^{d}
MDB15	86± 1.14^{f}	14.39± 0.043^{g}	18.72± 0.018^{g}	0.378± 0.021^{e}	0.078± 0.014^{d}	0.327± 0.012^{e}	0.037± 0.002^{c}	0.705± 0.028^{e}	0.115± 0.015^{e}
MDB19	56± 1.52^{b}	10.55± 0.033^{d}	10.28± 0.066^{d}	0.239± 0.019^{b}	0.031± 0.011^{ab}	0.222± 0.026^{c}	0.031± 0.003^{b}	0.462± 0.022^{c}	0.062± 0.010^{bc}
MDB20	64± 1.14^{bc}	8.35± 0.031^{b}	8.810± 0.111^{b}	0.224± 0.015^{b}	0.033± 0.012^{ab}	0.151± 0.020^{ab}	0.018± 0.004^{a}	0.374± 0.044^{b}	0.051± 0.008^{b}

† Values are the means of 5 replicates with each isolate ± SE mean.

‡ Values followed by different letters within a column are significantly different according to Duncan's multiple range test ($P < 0.05$).

Table 5
Effect of selected bacterial isolates: MNB (03, 04, 05, 06, 08 and 10), from the nest chamber soil of Trichomyrmex scabriceps ant colonies on plant growth promotion. Seed germination (SG), Shoot length (SL), Root length (RL), Shoot fresh weight (SFW), Root fresh Weight (RFW), Total fresh weight (TFW) and Total dry weight (TDW).

Treatment	Plant growth characteristics								
	SG (%)	SL (cm)	RL (cm)	SFW (gm)	SDW (gm)	RFW (gm)	RDW (gm)	TFW (gm)	TDW (gm)
Control	41.7± 1.528[a]	5.77± 1.294[a]	5.23± 1.033[a]	0.28± 0.022[a]	0.042± 0.009[a]	0.218± 0.025[a]	0.022± 0.002[a]	0.496±0.034[a]	0.064± 0.007[a]
MNB 03	62.0± 1.732[d]	9.91± 1.208[b]	7.51± 1.208[bc]	0.38± 0.024[bc]	0.052± 0.009[a]	0.275±0.014[b]	0.034± 0.005[b]	0.660± 0.028[c]	0.086±0.010[b]
MNB 04	55.3± 1.155[c]	8.75± 1.134[c]	8.77± 1.136[cd]	0.41± 0.021[cd]	0.065± 0.009[b]	0.321± 0.026[c]	0.038±0.005[bcd]	0.727± 0.038[d]	0.104± 0.010[c]
MNB 05	80.7± 1.155[e]	11.91±1.136[d]	9.11± 1.108[d]	0.44± 0.059[d]	0.093± 0.008[c]	0.368±0.021[d]	0.041±0.006[c]	0.805± 0.063[e]	0.134±0.006[d]
MNB 06	86.0± 1.000[f]	16.19±0.853[f]	12.79±1.291[f]	0.69± 0.036[f]	0.122± 0.009[d]	0.469±0.025[e]	0.060± 0.007[d]	1.158± 0.020[g]	0.182±0.009[e]
MNB 08	88.7± 0.577[g]	14.02±0.762[g]	10.75± 1.025[e]	0.49± 0.049[e]	0.100±0.006[e]	0.390± 0.016[d]	0.044±0.005[c]	0.882± 0.035[f]	0.143±0.009[d]
MNB 10	44.3± 1.528[b]	10.77± 0.848[e]	6.35± 1.134[ab]	0.34± 0.031[b]	0.045± 0.007[a]	0.251±0.017[b]	0.025± 0.007[a]	0.587±0.037[b]	0.071± 0.009[a]

† Values are the means of 5 replicates with each isolate ± SE mean.

‡ Values followed by different letters within a column are significantly different according to

Duncan's multiple range test ($P < 0.05$).

Assessment of PAL and POx activities

Significantly higher PAL enzyme concentrations were recorded in the leaves of experimental seedlings as compared to those from control, the highest being in case of those treated by the isolate MNB08 (1430.18 U min -1 g-1 FW), the next in the decreasing order being treatments involving MNB06 (1262 U min -1 g-1 FW), MDB07 (1003 U min -1 g-1 FW) and MDB15 (1215 U min -1 g-1 FW) isolates (Fig. 1A). Also, the pattern of PO activity was found to be almost similar in treatments ranging from 6.45 U µl-1 min-1 for MNB08 to 3.91 U µl -1 min-1 for MNB15 (Fig. 1B). The highest activity of enzymes was observed at 72 h in all the treatments which gradually declined at 96 h (Fig. 1).

Finally, the foremost selected PGP bacterial isolates were identified at the molecular level with greater than 95% sequence similarity. The control soil contained bacterial isolates belonging to *Enterobacter* sp. (CB01), *Micrococcus* sp. (CB03), *Bacillus* sp. (CB04), *Serratia* sp. (CB05), and *Clostridium* sp. (CB09) while *T. scabriceps* refuse pile soil contained *Bacillus amyloliquifaciens* MDB07 (MT864742.1), *Bacillus* sp. MDB09 (MT864743.1), *Azotobacter chrococcum* MDB12 (MT864745.1), *Bacillus subtilis* MDB15(MT781408.1), *Bacillus* sp. MDB19 (MT864744.1) and *Klebsiellla pneumoniae* MDB20 (MT781409.1) (Fig. 2). The nest chamber soil supported six PGP bacterial isolates which were identified as *Comamonas zongliani* MNB03 (MT782285.1), *Comamonas* sp. MNB04 (MT864740.1), *Tsukamurella paurometabola* MNB05 (MT864741.1), *Bacillus tequilensis* MNB06 (MT782282.1), *Bacillus valezensis* MNB08 (MT782283.1) and *Psudoxanthomonas spadix* MNB10 (MT782284.1) (Fig. 2).

Discussion

The results of the present study reveal that the nests of the seed harvester, *T. scabriceps* ant colonies harbor a high diversity of PGPB which can directly enhance soil fertility and indirectly augment the growth of plants present in its vicinity. The present study provides clear evidence in support of the important ecological role of seed harvester ant colonies in serving as nutrient-enhancing links between aboveground plant resources and the belowground microbial communities. The control soil contained a lower diversity of PGPB than the debris pile and nest chamber soil of ant colonies (Table 2). The refuse pile soil of *T. scabriceps* colonies supported a higher number of phosphate solubilizing, IAA and siderophore producing bacteria as compared to those recorded in the ant nest chamber soil (Table 3). Among the bacterial isolates obtained from the debris soil, 68% of isolates demonstrated PGP potential either by phosphate solubilization or by IAA production, whereas 82% of the bacteria isolated from the nest chamber soil

exhibited the PGP potential. The siderophore producing bacteria were more abundant in the debris soil (Table 2). The enrichment of ant nest soil with certain bacterial taxa could be distinguished at the phylum level itself. For instance, the abundance of bacteria belonging to genus *Firmicutes* was evident in the refuse pile soil, while those belonging to *Actinobacteria* and *Proteobacteria* genera were prominent in the nest chamber soil. The experimental exposure of the chick pea plants to the pathogen *S. rolfsii* (by its inoculation in the potted soil) induced higher production of PAL and PO enzyme activities in the chick-pea plants (Fig. 1). The production of PAL and POx enzyme in the experimental increased progressively from 24 to 72 hours after pathogen inoculation. These results support earlier findings which show similar pattern of higher production of defensive chemical compounds such as lignin and phenol due to the effects of rhizobacteria on *S. rolfsii* challenged plants [40, 41]. The higher PO enzyme production may be related to the reduction in reactive oxygen species level that gets accumulated due to pathogen infection [42].

Our results also indicate that the abundance of *Bacillus* species in the nest pile soil could be contributing towards the nutrient richness of the refuse dumps reported in seed harvester ant nests [43]. Earlier studies reveal that bacterial species such as *B. amyloliquifaciens* and *B. subtilis* easily colonize plant roots [44, 45], and exert activity against many root or soil pathogens by secreting various metabolites [45, 43, 46]. *Azotobacter chrococcum*, also known as a nutrient indicator, is known to maintain plant health by fixing nitrogen [47]. Many of the bacterial species found to be associated with *T. scabriceps* ant colonies, in the present study, are documented to be widely used for soil bioremediation and as biofertilizer at the commercial level [48]. As far as we are aware this is the first report of the association of many PGPB with the nest soil of the seed harvesting, *T. scabriceps* colonies. Also, this is the first report of the antagonistic effect of ant nest associated PGPB on the soil pathogen, *S. rolfsii*.

While *Firmicutes, Bacillus, Azotobacter*, and *Comamonas* were the dominant bacterial genera associated with the nest soil of *T. scabriceps* ant colonies, as found in the present study, species belonging to *Ochrobactrum* and *Pantoea* genera were reported in the nest soil of the sugar-loving *Camponotus compressus* colonies [3]. It is likely that the composition of ant nest associated microbial communities is strongly influenced by the nature of available substrates which in turn would be strongly influenced by the type of food collected and consequently the foraging ecology of the ant species.

We suggest that the predominance of *Bacillus* species can be useful for both the *T scabriceps* colonies and the plant species. Since both *B. velezensis* and *B. tequelensis* reportedly exhibit biocontrol potential (Table 2) against seed pathogens [49, 50], it is highly likely that these bacteria help in the protection of seeds collected by seed harvester ant colonies. Earlier studies on fungus-growing, *Trachymyrmex* cf. *zeteki* ants have shown the symbiotic association of ants with actinomycetous bacteria which occur in the infrabuccal pocket of ants and produce antibiotics that inhibit the parasitic microfungus belonging to genus, *Escovopsis* [51]. Thus, the colonies of *T. scabriceps* ants may also have adaptedly maintained these soil bacteria to obtain protection against soil pathogens so as to safeguard the foraged seeds.

Our preliminary studies on *T. scabriceps* revealed that each main nest is associated with 2-4 satellite nests, each with 1-2 chambers functioning as granaries (pers. obs.). The diverse plant seeds present in these granaries are thus enriched with the beneficial bacteria. Further, these bacteria-protected seeds present in the satellite nests of *T. scabriceps* have the potential to be the future plants, particularly in the semi-arid areas, favoured by these species. Thus, colonies of seed harvesting ants concentrate organic matter and nutrients near their nests and can create biogeochemical hotspots in nutrient-poor areas. The other PGPB such as *C. zongliani, Comamonas* sp. [52], and *P. spadix* [53] are of significance from the perspective of soil bioremediation.

The plant-growth-promoting bacteria associated with ant nest soil provide phosphorus, along with other nutrients to the plants, while the antimicrobial metabolites from these bacteria can efficiently protect the plants from harmful microbes, such as the soil pathogen, *S. rolfsii*. Some of these bacteria (such as *Azotobacter* sp.) also possess the ability to fix atmospheric nitrogen. Besides solubilizing phosphorous, these ant nest associated bacteria contribute towards the release of phytohormones, and enzymes, all of which contribute towards plant growth and yield. In conclusion, our findings show that the colonies of seed harvester ant, *T scabriceps* are associated with various species of PGPB. The nutrient- and microbe-rich refuse piles of *T. scabriceps* colonies are not only of crucial importance for initiating habitat heterogeneity but the ant colony generated “hot spots” have implication for enhancing plant productivity in that area. Because the beneficial bacterial community of seed harvester refuse piles has immense potential to improve the plant growth-favouring attributes of the soil, they can promote seed protection and plant growth in arid and semi-arid areas, where they commonly occur as well as in many managed ecosystems, such as agricultural fields.

Conclusion

In conclusion, our findings show that the colonies of seed harvester ant, *T scabriceps* are associated with various species of PGPB. The nutrient- and microbe-rich refuse piles of *T. scabriceps* colonies are not only of crucial importance for initiating habitat heterogeneity but the ant colony generated “hot spots” have implication for enhancing plant productivity in that area. Because the beneficial bacterial community of seed harvester refuse piles has immense potential to improve the plant growth-favoring attributes of the soil, they can promote seed protection and plant growth in arid and semi-arid areas, where they commonly occur as well as in many managed ecosystems, such as agricultural fields.

Declarations

Ethics Approval Not applicable.

Competing interests-The authors declare that they have no conflict of interest.

Author Contributions-Rastogi NK designed the study and added comments in the drafts. Kumari P collected the soil sample and analysed the data. Rajput RS assisted in microbe isolation, and data analysis related to gnotobiotic plant growth promotion. Singh HB helped with the microbe identification.

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Figures

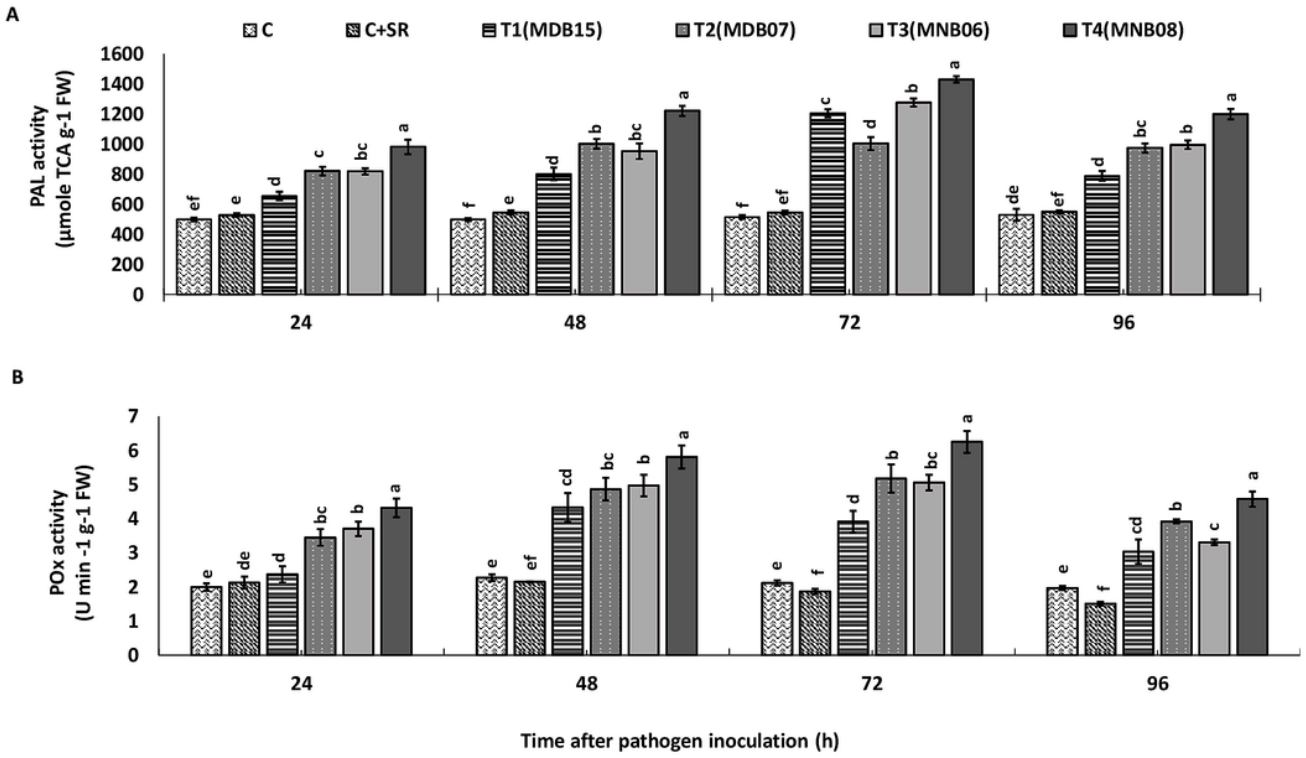

Figure 1

Activities of two enzymes: phenylalanine ammonia-lyase recorded as µM TCA (trans-cinnamic acid) g^{-1} fresh weight (FW) (A) and, of polyphenol-oxidase recorded as U min^{-1} g^{-1} FW (B) at intervals of 24h, 48h, 72h, and 96 hrs, in chickpea plants, germinated from bioprimed seeds with bacterial treatments (T): T1 (MDB15), T2 (MDB07), T3 (MNB06), and T4 (MNB08), and challenged with *Sclerotium rolfsii*. The two controls were: C (no treatment and *S. rolfsii*) and C + SR (control with *S. rolfsii*). Results are expressed as means of five replicates, and vertical bars indicate standard deviations of the means. Different letters indicate significant differences among treatment results taken at the same time interval according to Duncan's multiple range test at $p \leq 0.05$.

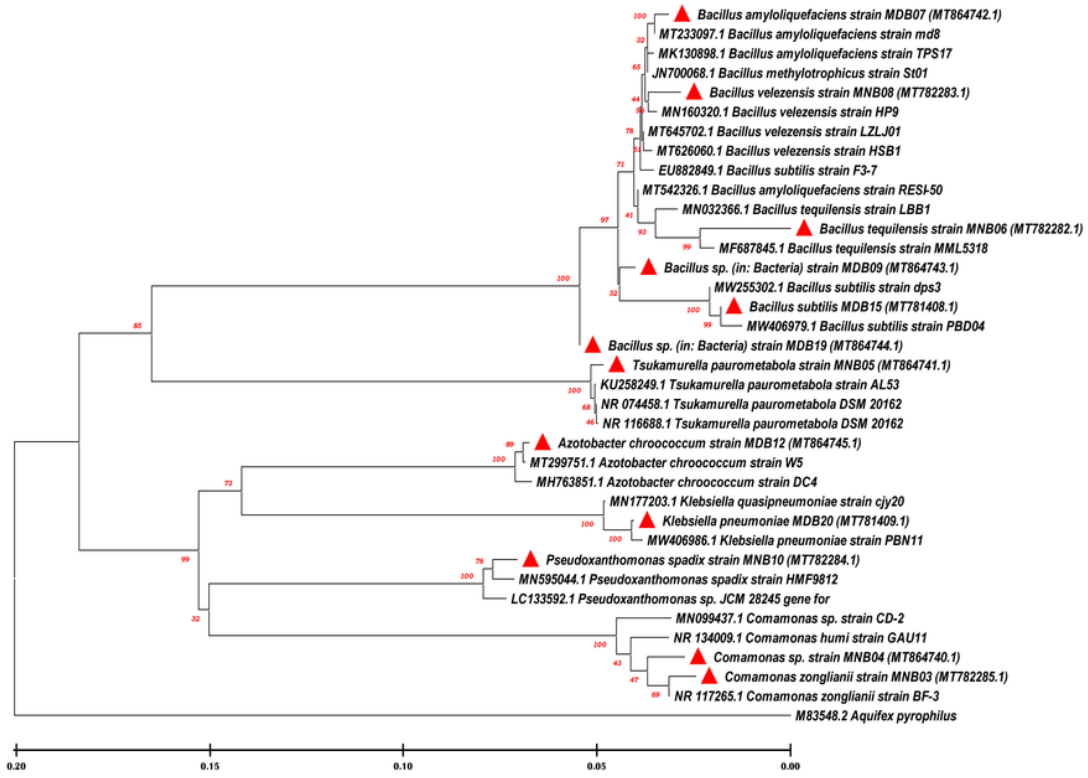

Figure 2

Phylogenetic analysis of 16s rRNA partial sequences for isolated PGPB, with neighbour joining method of phylogeny and 1000 bootstrap replications. The sequences isolated PGPB submitted to NCBI GenBank are highlighted with red color symbol, with their respective Accession number. The scale bar represents evolutionary distance.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Supplementarydata.docx