Journal of Pharmacognosy and Phytochemistry

Available online at www.phytojournal.com

E-ISSN: 2278-4136
P-ISSN: 2349-8234
JPP 2018; 7(2): 1681-1686
Received: 05-01-2018
Accepted: 06-02-2018

Rajni Devi
Department of Microbiology, COBS & H, CSKHPKV, Palampur, Himachal Pradesh, India

Richa Thakur
Department of Biochemistry, COBS & H, CSKHPKV, Palmapur, Himachal Pradesh, India

Correspondence
Rajni Devi
Department of Microbiology, COBS & H, CSKHPKV, Palampur, Himachal Pradesh, India

Screening and identification of bacteria for plant growth promoting traits from termite mound soil

Rajni Devi and Richa Thakur

Abstract
Background: Termites play an important role in soil ecology, transporting and mixing soil and organic material from different horizons. Diverse kinds of plant growth promoting bacteria (PGPB) have been reported in termite mound soil.
Objective: Here, an attempt has been made to screen and identify plant growth promoting traits in bacteria isolated and characterized from termite mound soil.
Material & Methods: Samples were collected from different regions of Himachal Pradesh and examined for bacterial, fungal and actinomycetes diversity. For in vitro screening of isolates for their PGP activities, tests include Indole Acetic Acid Test, production of ammonia (NH_3), production of hydrogen cyanide (HCN) and production of siderophores. Isolates were also screened for proteolytic, cellulolytic and pectinolytic activities.
Result: In total of 132 isolates comprising of 70 bacteria, 58 actinomycetes and 4 fungi were isolated from the different samples of termite mound soil. Among these microbial isolates, 21 produced IAA (0.6-47.56 µg/mL), 12 produced siderophores (9.27-65.48%SU), 13 produced ammonia in peptone broth and showed HCN production. Proteolytic activity was observed for 24 bacterial isolates, pectinolytic activity for 9 isolates while only 2 isolates showed cellulolytic activity on plate assay. All the isolates were identified on the basis of morphological, cultural and biochemical characteristics.
Conclusion: This study will facilitate accurate identification of the bacterial isolates that harbor PGP traits essential in controlling foliar fungal pathogens, thereby enhancing their capability to overcome fungal diseases.

Keywords: termite mound soil, Plant growth promoting bacteria, *Pseudomonas aeruginosa*

Introduction
Termites are recognized as one of the major ecosystem engineers in tropical soils (Dangerfield *et al.*, 1988) [6]. They promote soil transformation by disturbance processes; their effects on soil are caused mostly by their major construction activities of which their mounds are the most complex type. Termites play an important role in soil ecology, transporting and mixing soil and organic material from different horizons (Lavelle *et al.*, 1997) [13]. In some countries, termite mound soil has been used to enrich the crop field with available nitrogen, total phosphorous and an organic carbon than the adjacent soil (Breuning *et al.*, 2005) [2]. Although, usually considered as pests, termites can be valuable not only in forest ecosystem but also in organic farming. They are great decomposers of wood and plant debris, they aerate the soil and add nutrients to it. Plants also take up nutrients very easily from termite mound soil. Termite soil is providing a viable option to local farmers who can't afford to buy expensive inorganic fertilizers.
Diverse kinds of plant growth promoting bacteria (PGPB) have been reported in termite mound soil. Presence of these plant growth promoting bacteria in soil is of great importance as their bacterial community play a vital role in enhancing plant growth and hence yield in a sustainable fashion. Improving soil fertility by microbial inoculants is important for increasing crop yield.
Microorganisms with multiple plant growth promoting activity can aid in tailoring of plant production. PGPB influence the growth, yield and nutrient uptake by an array of mechanisms. They stimulate growth directly by solubilization of insoluble nutrients, production of growth hormones, nitrogen fixation and directly by antagonizing pathogenic organisms by the production of siderophore, lytic enzmes, antibiotics, fluorescent pigments and cyanides etc. (Flaishman *et al.*, 1996) [8].
Plant growth promoting bacteria produce physiologically active auxins i.e. indole acetic acid (IAA). It regulates several fundamental cellular processes including cell divisions, elongation and differentiation. Bacterial indole acetic acid also plays an important role in suppression of rot diseases in various plants (Khare and Arora, 2010) [10]. It has been reported that PGPB

enhance growth of plants by increasing available nutrients by degrading different types of complex substrates through production of different types of lytic enzymes. Antagonistic or biocontrol activity of bacterial isolates has been attributed to the production of different types of cell wall degrading enzymes like pectinase, cellulase and protease.

The large scale application of PGPB to crops as bioinoculants seems to be attractive as it may help in substantially reducing the use of chemical fertilizers and pesticides which often pollute the environment and reduce the fertility of soil. It has been reported that bacterial and microbial populations in termitaria soil are higher than the adjacent soil (Spain, 2004) [22]. Identification of more potent bacteria from termite mound has broadened the spectrum of PGPB that can survive and perform well in soil conditions.

Materials and Methods

Experimental Site

The present study was conducted in the Department of Microbiology, CSK HPKV, Palampur.

Sampling of termite mound soil

Samples for present study were collected from different regions of Himachal Pradesh as well as from Model Organic Farm, Department of Organic Agriculture, CSK HPKV, Palampur. Termite mound soil samples were collected in sterilized polythene bags from Baijnath, Nurpur and Model Organic Farm, CSKHPKV Palampur areas of Kangra district of Himachal Pradesh. These samples were brought to the laboratory and processesd immediately or kept at refrigerator conditions for further analysis.

Culturable diversity of termite mound soil

Microbial load was determined by using standard plate count technique of Wollun (1982) [26] by employing different media for different groups of microorganisms viz. nutrient agar, potato dextrose agar, actinomycetes isolation agar, Jensen's medium and Pikoskaya's medium for bacteria, fungi, actinomycetes, nitrogen fixing bacteria and P- solubilizers, respectively. Plates of nutrient agar, Jensen's Agar and Pikoskaya's agar were then incubated at 30-32°C for 48 hours; Potato Dextrose Agar at 28°C for 3-4 days; and Actinomycetes Isolation Agar at 28°C for 7-10 days. After the incubation period, the number of colonies on each plate were counted and expressed in terms of log CFU/g.

Standard strain for studying PGPR traits

A standard strain of *Pseudomonas aeruginosa* -MTCC-2588 was procured from the Institute of Microbial Technology (IMTECH), Chandigarh for studying different plant growth promoting traits. Standard strain was maintained on nutrient agar slants as well as in glycerol stocks.

Detection of plant growth promoting activities

Plant growth promoting traits in efficient isolates were detected by the following methods**.**

Isolation of free nitrogen fixers from termite mound soil samples

Nitrogen fixing microorganisms were isolated on nitrogen free Jensen's medium (Subbarao 1988) [23] and were then further tested for different PGPR traits.

Detection and Estimation of IAA

For screening of Indole acetic acid producing isolates, test culture was inoculated in Luri Bertani broth with tryptophan concentration of 0, 2 and 5 mg/mL and incubated at 28± 2°C for 7 days. Cultures were centrifuged at 3000 rpm for 30 min. Two milliliters of supernatant was mixed with 2 drops of orthophosphoric acid and 4mL of solawaski's reagent (50mL; 35% perchloric acid; 1mL 0.5M $FeCl_3$). Development of pink color indicated the production of Indole acetic acid. Optical density was recorded at 530 nm and the level of IAA produced was estimated from standard curve of IAA (Loper and Schroth, 1986) [14].

Standard Curve for IAA

Standard curve was prepared by taking standard IAA solution in 0-100 µg/mL concentrations in different test tubes. The volume was made upto 2 mL with distilled water and then 4 mL of Solawaski's reagent was added and tubes were incubated for 25 minutes at room temperature. Standard curve was prepared by plotting absorbance at 530 nm vs. concentration of IAA. (Fig.3.1)

Detection of siderophore production

The CAS assay (Schwyn and Neilands, 1987) [24] is a universal chemical assay for siderophore detection and is based on siderophores high affinity for ferric ion. When siderophore is present the following reaction occurs, which releases the free dye which is orange in color.

Fe^{3+} - dye (blue) + siderophore → Fe^{3+} - siderophore + dye (orange)

Qualitative estimation of siderophore production

Qualitative estimation of siderophore production was done by chrome azurol Sulfonate (CAS) assay with slight modification (Schwyn and Neilands, 1987) [24]. All the glassware's were washed in 6M HCl to remove traces of contaminating iron and then rinsed thoroughly with ddw. CAS agar plates were prepared and incubated for 24 hrs at 28°C for checking any contamination. Efficient isolates were spot inoculated on these plates and incubated at 28 ± 2°C for 3 days. The colonies producing color change of the medium from green to orange were considered as siderophore producers.

Quantitative estimation of siderophore production

Estimation of siderophore production was done by CAS liquid assay (Schwyn and Neilands, 1987) [24]. Culture supernatant (0.5 mL) was mixed with 0.5 mL CAS assay solution (1.5 mL of 1mM $FeCl_3.6H_2O$ in 10mM HCl + 7.5 mL of 2mM CAS stock solution dissolved in 50mM of HDTMA + 30mL piperazine (pH5.6) and final volume was made to 100 mL with ddw). Then 10 µL of shuttle solution (0.2 M 5-sulphosalicylic acid) was added and was allowed to stand for few minutes. Siderophore production was detected by the reduction in blue color of the solution. Color intensity of the solution was recorded at 630 nm against reference after 10 minutes at room temperature.

$$\% \text{ Siderophore units} = \frac{(\text{Absorbance of reference} - \text{Absorbance of sample})}{\text{Absorbance of reference}} \times 100$$

Qualitative assay for ammonia production

Bacterial isolates were tested for the production of ammonia in peptone water. Freshly grown cultures were inoculated in 10mL peptone water in each tube and incubated for 48-78 hours at 28 ± 2°C. Nesselar's reagent (0.5 mL) was added in each tube. Development of brown to yellow color indicated a

positive test for ammonia production (Cappuccino and Sherman, 1992) [4].

HCN production by isolates

All the isolates were screened for the production of HCN by using the method of Lorck (1948) [15]. Briefly, nutrient broth was amended with 4.4 g glycine/L and isolates were streaked on modified agar plate. A Whatmann filter paper No. 1 socked in 2% sodium carbonate in 0.5% picric acid solution was placed in the top of the plate. Plates were sealed with parafilm and incubated at 28 ± 2°C for 4 days. Development of orange to red color indicated HCN production.

Lytic enzyme production

Proteolytic activity

All the bacterial isolates were screened for proteolytic activity on skim milk agar plates (Kaur *et al*. 1988) [12]. Separately sterilized 1 % skim milk was added to nutrient agar before pouring the plates. Bacterial isolates were pin point inoculated on skim milk agar plates and plates were incubated for 3 days at 28 ± 2°C and observed for proteolysis i.e. clear zone produced around the bacterial colony.

Pectinolytic activity

Pectinolytic activity of organisms was carried out on solid medium. After 24-48 hours growth, plates were flooded with iodine solution (2 g KI and 1g I_2 crystals dissolved in 100 ml of water). A clear zone around the colony indicated the pectinolytic activity.

Characterization and identification of selected bacterial isolates

Bacterial isolates were identified on the basis of morphological (type, shape, staining, colony characteristics) and biochemical characteristics (Oxidase, Catalase, Utilization of Sugar, Indole, MR-VP, Citrate, Urease, etc.) according to the standard method descrived in Bergey's Manual of Systematic Bacteriology (Krieg and Holf, 1984) [11].

Results and Discussion

Culturable microbial diversity in termite mound soil

Microbial diversity in terms of population density of total culturable bacteria, fungi and actinomycetes was determined on nutrient agar, potato dextrose agar and actinomycetes isolation agar, respectively (Fig.1). A total of 132 isolates viz. 70 bacteria, 58 actinomycetes and 4 different fungal strains were isolated from termite mound soil samples of different areas of Himachal Pradesh. Table 1 depicts the microbial diversity in termite mound soil in terms of bacterial, fungal and actinomycetes population per gram of soil. It was observed that the bacterial, fungal and actinomycetes population varied from 6.34 to 7.05 log CFU/g, 2.29 to 4.26 log CFU/g and 4.47 to 6.45 log CFU/g. Data shown in table 1 reveals that termite mound soil was found to be rich in bacterial population in comparison to fungal and actinomycetes populations. These results are in conformation with the findings of Singh *et al.,* (1978) [20] and Fall *et al.*, (2004) [7]. Abundance of bacterial population in termite mound soil sample could be due to the fact that they can metabolize diverse kind of complex compounds, which are not attacked by other types of microorganisms (Wood, 1998).

Plant growth promoting traits

Isolation of free nitrogen fixing bacteria from termite mound soil

In present investigation, a total of 132 microbial isolates from termite mound soil were screened qualitatively for nitrogen fixing activity on Jensen's medium which is reported to be a better screening medium for free living nitrogen fixers. Among these 132 isolates, only 13 isolates were able to grow on Jensen medium. Boyer, (1955) and Meiklejohn, (1965) [17] also reported the limited number of free living nitrogen fixers in termite mound soil samples, thus supporting the observation of this study.

Detection and estimation of IAA production

All 132 isolates of termite mound soil were screened for the production of IAA along with strain of *Pseudomonas aeruginosa* (Table 2, Fig.2). Eighteen bacterial isolates indicated IAA production in Luria Bertani Broth medium containing 2 mg/mL of tryptophan in the range of 0.6-42.56 µg/mL, while 21 isolates exhibited an increased rate of in IAA production (1.6-47.56 µg/mL) when 5 mg/mL tryptophan was incorporated in the growth medium. Seven isolates *viz.* NTS 8, BTS 19, BTS 28, BTS 29, NTS 65, PTS 48 and NTS 18 showed IAA production of 15.2, 13.4, 14.52, 17.41 and 10.8 µg/mL, respectively, in the medium lacking tryptophan. Standard organism did not show any IAA production in medium lacking tryptophan, whereas in presence of 2 and 5 mg/mL of tryptophan, IAA production was found to be 12.24µg/mL and 21.41 µg/mL, respectively.

All the above 7 isolates produced much higher IAA in presence of 5 mg/ml of tryptophan as compared to standard strain, indicating that these are more efficient and might be playing an important role in organic farming when termite mound soil are directly used as inoculants. This observation is strengthened by the practice being followed in Africa where farmers are mixing termite mound soil directly with the field soil to achieve higher aid.

Detection and estimation of siderophore production

Siderophore production has been reported to be beneficial in growth promotion and disease suppression in plants. A total of 132 isolates from termite mound soil along with standard strain of *P.aeruginosa* were screened on CAS agar plates (qualitative estimation) and in CAS Liquid Media (quantitative estimation) for siderophore production, respectively. Twelve isolates were found to be positive for siderophore production both qualitatively and quantitatively (Table 3). Siderophore production varied among all isolates qualitatively in the range of 5-18 mm activity diameter, and quantitatively in the range of 9.27- 62.28 % SU. NTS 65 showed maximum siderophore production (62.28 % SU), whereas PTS 22 was the least siderophore producing isolate (9.27 % SU). The orange zone surrounding the colony indicated the excretion of siderophore and the diameter of the zone depicted the amount of siderophore excreted by bacterial isolates (Fig.3).

Qualitative assay for ammonia production

Twenty one isolates which were found positive for IAA and sidrophore production were further subjected for screening of ammonia production in peptone broth by using Nessler's

reagent. The change in colour from yellow to brown indicated production of ammonia. Out of 21 isolates, 13 isolates exhibited ammonia production in peptone broth (Fig.4). Maximum ammonia production was shown by three bacterial isolates *viz*. PTS 58, PTS 48 and NTS 78.

HCN production by bacterial isolates

Microbial cyanides seem to play a role in suppression of many plant diseases. A positive correlation between production of HCN and suppression of root rot by bacterial isolates has been reported (Defago *et al*, 1990) [5]. In present study, 21 isolates were tested for qualitative HCN production on nutrient agar plates supplemented with 0.14 per cent glycine. BTS 65, PTS 1, NTS 8, BTS 24 showed maximum HCN production (Fig.5). Several factors have been reported to influence the rate of HCN production. Glycine has been found to be the direct precursor of microbial cyanide production (Knowles, 1976; Voisard *et al*, 1989) [9, 25] and it has been found in root exudates (Bakker *et al*, 1989; Curl and Truelove, 1986) [1, 3]. Although the role of microbial HCN in disease suppression is not considered to be firmly established. It may be involved in the induction of plant resistance as reported by Schipper *et al.,* (1990) [21].

Qualitative screening of bacterial isolates for proteolytic activity

Microbial proteases are increasingly recognized as important virulence factors for a variety of pathogens. In present study, a total of 70 bacterial isolates from termite mound soil were screened for their proteolytic activity on nutrient agar plates supplemented with one percent skim milk. Out of 70 bacterial isolates, 24 were detected as positive for proteolytic activity and their activity ranged from 3 to 20mm (Table 4, Fig. 6). Isolates NTS 49, NTS 78 and BTS 17 exhibited maximum proteolytic activity with activity diameter of 20, 16 and 13mm, respectively. Several studies have demonstrated that the lytic enzymes of bacteria are involved in the control mechanisms against plant root pathogen including *Fusarium oxysporum* and *Rhizooctonia solani* (Nagrajkumar, 2004) [18].

Qualitaive screening of bacterial isolates for pectinolytic activity

Many plant-pathogenic bacteria and fungi are known to produce pectinolytic enzymes useful for invading host tissues. Moreover, theses enzymes are essential in the decay of dead plant materials by non pathogenic microorganisms and thus assist in recycling carbon compounds in the biosphere. All the isolates included in the study were screened on solid medium containing pectin and tested for pectinolytic activity. Only 5 isolates were found to be positive for pectinolytic activity on the basis of plate assay (Table 5, Fig. 7). BTS 16 and NTS 78 have shown maximum activity diameter of 15mm and 12mm respectively.

Marcia *et al.,* (1999) [16] isolated 168 bacterial strains from soil and screened them for pectinolytic activity. One hundred two were positive for pectinase depolymerization in assay plates as evidenced by clear hydrolization halos. Among them, 30% presented considerable pectinolytic activity.

Characterization and identification of selected bacterial isolates

A total of 31 bacterial isolates were identified on the basis of morphological and biochemical characteristics. Thirteen isolates were formed pellicles and sixteen isolates gave uniform growth while only two isolates formed sediments in nutrient broth. Six isolates (NTS 76, NTS 78, BTS 16, BTS 39, BTS 24, NTS 65 and BTS 21 produced colored pigments on nutrient agar and in broth. Most of the isolates formed circular colonies with entire margin, elevation of colony varied from raised, flat and umbonate to convex. Most of the bacterial population that was characterized on the basis of morphological and biochemical reactions consisted predominantly of Gram positive rods and cocci and only few were found to be Gram negative rods and cocci. Majority of isolates were oxidase, catalase and simmon citrate positive, while most of them were H_2S and MR-VP negative. Characterized isolates were found to be belong to the genera *Bacillus, Staphylococcus and Alcaligens.*

Table 1: Culturable microbial diversity in different samples of termite mound soil

Sample	Total bacterial count (log CFU/g)	Total fungal count(log CFU/g)	Total actinomycetes count(log CFU/g)
Organic farm	6.40	3.36	5.59
Palampur	7.05	4.26	6.45
Nurpur	6.34	3.37	5.72
Baijnath	6.98	2.29	4.47

Table 2: IAA production by bacterial isolates after seven days of incubation

Isolates	0 (mg/mL)	2 (mg/mL)	5 (mg/mL)
NTS 65	15.2	16.8	41.6
PTS 48	6.4	11.8	19
BTS 19	13.4	16.2	29.6
BTS 16	ND	ND	1.6
NTS 20	ND	0.6	4.6
BTS 28	14.52	28.12	34.5
NTS 8	17.43	42.56	47.56
BTS 50	ND	ND	7.66
P.aeruginosa	ND	12.24	21.41

Table 3: Screening of bacterial isolates for qualitative and quantitative siderophore production

Isolates	Diameter of colony (mm)	Diameter of orange zone (mm)	Activity diameter (mm)	% Siderophore units
NTS 65	7	25	18	65.48
NTS 78	12	24	12	48.36
NTS 24	8	24	16	57.19
PTS 22	13	18	6	9.27
PTS 48	9	20	11	48.96
NTS 76	10	15	5	15.62
P.aeruginosa	7	18	11	43.55

- Activity diameter (mm) = [Diameter of orange zone (mm) – Diameter of colony (mm)]
- % Siderphore units = $\frac{\text{(Absorbance of reference – Absorbance of sample)}}{\text{Absorbance of reference}} \times 100$

Table 4: Qualitative screening of bacterial isolates for their proteolytic activity

Isolates	Colony diameter (mm)	Total zone diameter (mm)	Activity diameter (mm)
NTS 49	10	30	20
BTS 17	7	20	13
NTS 78	15	31	16

Activity diameter (mm): Total zone diameter (mm) – Colony diameter (mm)

Table 5: Qualitative screening of bacterial isolates for their Pectinolytic activity

Isolates	Colony diameter (mm)	Total zone diameter (mm)	Activity Diameter (mm)
BTS 14	12	18	6
BTS 16	11	26	15
NTS 11	11	13	2
NTS 78	12	24	12
NTS 34	13	22	9

Activity diameter = Total zone diameter (mm) – Colony diameter (mm)

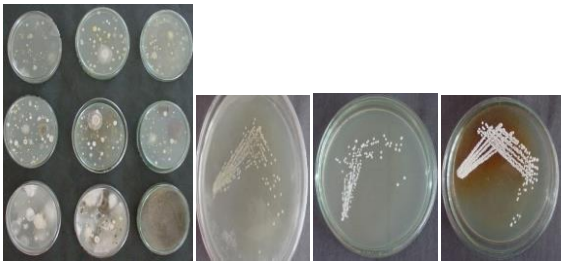


Fig 1: Culturable microbial diversity in different samples of Termite Mound Soil

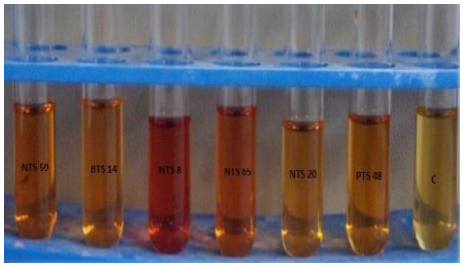
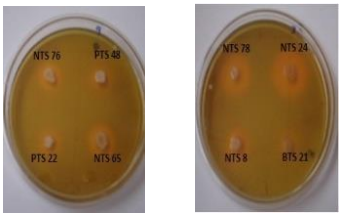


Fig 2: IAA production at different concentration of tryptophan



(a) **(b)**

Fig 3: Bacterial isolates showing siderophore production on CAS-agar plates (a-b)

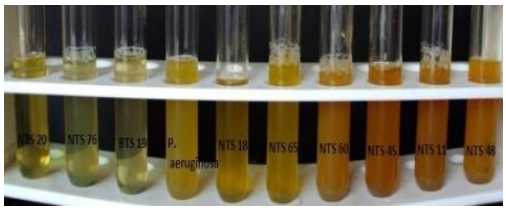
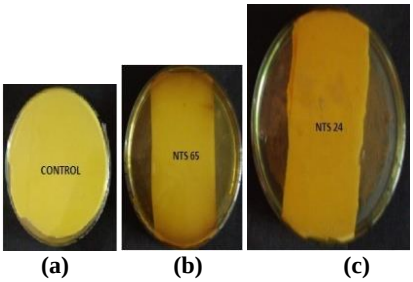
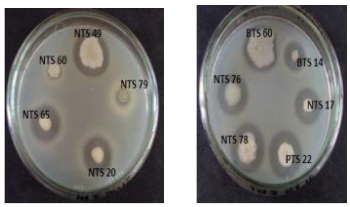


Fig 4: Ammonia production by bacterial isolates



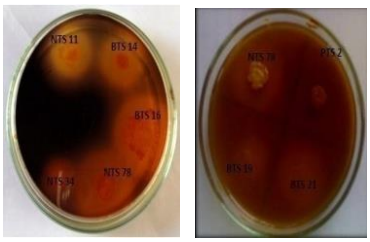
(a) **(b)** **(c)**

Fig 5: HCN production (b-c)



(a) **(b)**

Fig 6: Bacterial isolates showing Proteolytic activity (a-b)



(a) **(b)**

Fig 7: Bacterial isolates showing Pectinolytic activity (a-b)

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