

Isolation and Functional Characterization of Beneficial Bacteria Associated with Roots of *Thysanolaena Maxima* and Rhizospheric Soil Enzymatic Activities in *Jhum* Agriculture

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Abstract

The activity of amylase (AMY), arylsulphatase (ASA), β -glucosidase (GSA), dehydrogenase (DHA), acid-phosphomonoesterase (PHA) and protease (PRO) enzymes were analyzed in rhizospheric soils of broom grass, *Thysanolaena maxima* (TM) collected from fallow phases of 5 and 20 years *Jhum* cycles (F5 and F20, respectively) and their corresponding bulk soils. The activities of soil enzymes from rhizospheric soil of TMF5 were significantly higher relative to that of bulk soils and the rhizospheric soils from TMF20. The counts of rhizobacteria [$0.74 \pm 0.056 \times 10^7$ colony forming unit (cfu) g^{-1} soil] and root endophytic bacteria ($0.083 \pm 0.004 \times 10^4$ cfu g^{-1} roots) of TM from F20 fallow phase were higher compared to the counts of rhizobacteria and endophytic bacteria ($0.27 \pm 0.029 \times 10^7$ cfu g^{-1} soil and $0.05 \pm 0.008 \times 10^4$ cfu g^{-1} roots, respectively) of TM from F5 fallow phase. Altogether 63 isolates associated with TM were screened for multifaceted plant growth promoting (PGP) traits viz. production of pectinase and cellulase, IAA like substances, 1-aminocyclopropane-1-carboxylate deaminase (ACCD), N₂-fixation, solubilisation of inorganic phosphorus (iP) from Ca₃(PO₄)₂, AlPO₄ and FePO₄ and mineralization of organic phosphorus (Na-phytate). The PGP screening results indicated that the percent incidence of rhizobacteria and root endophytic bacteria for PGP traits was higher in F5 fallow phase as compared to F20 fallow phase. These results provided clear indication that TM plants play an important role in rejuvenating the biological activities (in terms of higher activities of enzymes in rhizospheric soils and greater population of beneficial rhizobacteria and root endophytes) in frequently burnt soils under shorter *Jhum* cycles.



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
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Introduction

Shifting cultivation or locally known as *Jhum* cultivation is an age old and dominant agricultural practice in the hill states of North East India.¹ Although it is considered to be well adapted to tropical climates and accessible to small farmers because of its low cost, degradation of land due to short *Jhum* cycle (earlier from a period of 15-20 years to now 2-3 years)² it has become a major constraint in the soil productivity.³

As per the practice of *Jhum* agroforestry system, after harvesting, the site is left as such so that it rejuvenates naturally with soil nutrient components which diminish after farming. It was observed that after burning of *Jhum* sites of Nagaland, some of the weed species for example, *Borreria hispida*, *Chromolaena odorata*, *Thysanolaena maxima* and *Ageratum conyzoides* showed abundance growth in the starting of secondary succession of the vegetation.⁴ Although weed infestation was carried out for agricultural practice, when *Jhum* cultivation sites were left for rebuilding of ecosystem, they again grew in abundance. In later stages of succession their dominance were replaced by shrubs or other woody plant varieties. As particularly these weed species were pioneering the secondary vegetation, the soil-plant-microbe interactions associated with these ESP can be considered as most potential interaction among the other members of the plant community for rebuilding of the *Jhum* soil ecology.

To study these interactions, enzyme activity of root associated soils of TM were analyzed and compared with bulk soil enzyme activity. Activities of soil enzymes such as arylsulphatase, dehydrogenase, phosphatase, β -glucosidase etc. were often used as indicators of soil quality after burning and anthropogenic disturbances⁵. Along with the assessment of soil enzymes, association of beneficial microbes with roots of TM was also studied, as enrichment of soil quality is further influenced by its associated microbial biomass.⁶

Materials and Methods

Selection of *Jhum* Fallow Site and Sample Collection

Considering the prevalence of the length of *Jhum* cycles, two fallows (secondary forest stands) of different ages viz. 5 years and 20 years (hereafter

referred as F5 and F20, respectively) in Mokokchung district of Nagaland (26°24.101'-26°24.599' N, 94°22.409'-94°22.539' E) were selected. This study area falls under wet, warm and humid tropical climate with annual rainfall from 1800 to 2600 mm and altitude ranges from 592 to 733 m above mean sea level. The *Jhum* practicing tribal farmers carried out the slash and burnt operations during the last week of November, 2014 and 1st week of February, 2015, respectively in F5 and F20 fallows. On completion of burning operation, following the onset of pre-monsoon shower in the month of April, various plant species started germinating on the burnt fields. Sampling of young TM (30-35 days old) was done from 50 days old burnt field (Fig. 1). TM samples were collected from F5 and F20 hereafter referred as TMF5 and TMF20 respectively.

To collect bulk soil and rhizospheric soil samples along with plant samples, each site was demarcated according to natural slope boundary of the hill i.e. summit, shoulder and back slope. Within a natural slope boundary, five random grids (each grid size was 25 m²) were considered.⁷ Soil samples from five random spots within each grid were collected and mixed them all to make one composite per grid. Thus, five composite soil samples were obtained along each natural slope boundary. Finally, each composite soil sample of summit, shoulder and backslope along the direction of slope gradient were mixed together to get overall five composite soil samples for each site.



Fig. 1.:The sampling of *Thysanolaena maxima* as early succession plant regenerating in after-burnt *jhum* field of Mokokchung district, Nagaland.

In doing so, the possible influence of slope gradient on soil attributes was confounded since, the present investigation aimed to study the effect of burning and the length of the fallow phases on soil biochemical attributes of rhizospheric soils of TM relative to bulk soils. Thus total ten composite grid samples for bulk soil (five samples for F5 hereafter referred as B1, B2, B3, B4 and B5; and five samples for F20 hereafter referred as B6, B7, B8, B9 and B10) were collected. Similarly total ten composite samples of TM for root and adhered soil were collected. Young TM (30-35 days old) were uprooted carefully using shovel. To separate rhizospheric soil, the loosely adhered soils were removed by jerking the whole root system and the tightly adhered soils along with fine roots of plant were collected in sterile zipped plastic bag. Thus from ten composite plant samples, ten composite samples for rhizospheric soil (five samples for TMF5 hereafter referred as RH1, RH2, RH3, RH4 and RH5; and five samples for TMF20 hereafter referred as RH6, RH7, RH8, RH9 and RH10) and total ten composite sample for roots (five samples for TMF5 and five samples for TMF20) were analyzed. After collection, both bulk soil samples and plant samples were stored at 4 °C until completion of analysis of biochemical and microbiological parameters.

Soil enzyme properties

Analysis of six soil enzymes was conducted on total ten composite grid samples for bulk soil and ten composite samples for rhizospheric soil keeping three replicates for each sample according to the standard procedures described by Page and his co-workers.⁸ In brief the procedures are described below. To determine AMY, soil supernatant was obtained after the incubation of soil with starch in citrate buffer at 37 °C for 2 hr.⁷ The presence of glucose was detected by Nelson-Somogyi method⁹ and intensity of blue colour developed was measured at 620 nm using microtiter plate reader (Multiskan, Thermo Scientific, USA). To determine ASA, soil was treated with p-nitrophenyl sulphate (PNS) in presence of toluene and acetate buffer and incubated at 37 °C for 1 hr. The hydrolytic release of sulphate from PNS was measured colorimetrically¹⁰. Determination of GSA was performed by incubating soil with p-nitrophenyl-β-D-glucoside (PNG) in presence of toluene and MUB (Modified Universal Buffer, pH 6.0) solution at 37 °C for 1 hr. Concentration of p-nitrophenol was measured colorimetrically in yellow coloured filtrate of incubated soil¹¹. For determination of DHA activity, soil was incubated with 2, 3, 5-triPhenyltetrazolium chloride (TTC) along with CaCO₃ at 37 °C for 24 hr.

Table 1: The comparative activities of soil enzymes in bulk soil between 5 and 20 years *Jhum* fallow phases

Fallow phase	AMY	ASA	GSA	DHA	PHA	PRO
F5B1	3.43±0.65	131±4.48	1.15±0.12	5.94±0.23	672±64.2	3.72±1.81
F5B2	3.42±0.67	95.0±2.05	0.84±0.10	2.53±1.11	628±32.3	4.47±1.35
F5B3	5.36±1.23	123±5.84	0.97±0.13	1.95±0.23	583±9.10	0.36±0.10
F5B4	2.39±1.08	91.1±6.91	1.01±0.19	1.87±0.17	877±78.9	2.91±1.58
F5B5	3.41±1.03	114.±10.6	1.02±0.16	4.14±0.60	363±26.0	1.18±0.31
F20B6	6.66±1.01	122±15.9	1.69±0.61	4.75±0.46	658±17.3	4.59±0.78
F20B7	3.76±0.65	153±13.0	0.59±0.19	6.11±0.63	951±11.5	2.31±0.85
F20B8	5.50±0.99	170±14.3	1.12±0.28	5.84±0.24	758±36.0	2.23±1.22
F20B9	7.97±0.50	147±11.5	1.51±0.31	3.33±0.46	858±15.4	5.55±0.77
F20B10	3.42±0.86	138±10.9	0.93±0.16	6.04±0.27	898±13.6	8.30±1.20

Values represent means ± SD (n = 3), F5, 5 year fallow phase; F20, 20 year fallow phase; AMY, amylase activity determined in terms of µg glucose soil g⁻¹ soil hr⁻¹; ASA, arylsulphatase activity determined in terms of pNP soil g⁻¹ soil hr⁻¹; GSA, β-glucosidase activity determined in terms of µg pNP soil g⁻¹ soil hr⁻¹; DHA, dehydrogenase activity determined in terms of TPF soil g⁻¹ soil hr⁻¹; PHA, acid-phosphomonoesterase activity determined in terms of µg pNP soil g⁻¹ soil hr⁻¹; PRO, protease activity determined in terms of µg tyrosine soil g⁻¹ soil hr⁻¹.

Filtered soil extractant was treated with methanol and formation of triphenyl formazan (TPF) was measured colorimetrically¹². For PHA determination, soil was incubated with p-NPP (p-nitrophenyl phosphate)

in presence of MUB and toluene at 37 °C for 1 hr. After incubation suspension was treated with CaCl₂ and NaOH. Released p-nitrophenol was measured colorimetrically.¹³ To determine PRO activity, soil

Table 2: The comparative activities of soil enzymes in bulk soil between 5 and 20 years *Jhum* fallow phases

Fallow phase	AMY	ASA	GSA	DHA	PHA	PRO
F5B1	12.1±1.53	182±3.18	2.25±1.13	9.27±0.50	976±22.5	5.36±0.08
F5B2	6.82±1.48	324±24.6	1.58±0.48	9.64±0.60	1559±18.2	9.96±0.13
F5B3	12.7±2.21	218±33.6	3.75±0.64	6.08±0.96	1041±47.9	12.5±0.28
F5B4	12.6±1.73	267±43.6	1.84±0.18	10.6±1.07	1737±10.3	7.79±0.31
F5B5	6.10±1.74	155±7.29	1.85±0.52	6.99±0.05	1169±18.5	6.41±0.38
F20B6	8.28±1.10	139±13.5	1.54±0.55	5.10±0.39	1200±91.5	8.78±0.51
F20B7	12.1±1.16	83.1±9.49	2.88±0.68	6.92±0.89	975±11.0	4.37±0.29
F20B8	8.52±1.20	164±26.3	1.57±0.16	6.90±0.22	835±25.8	7.03±0.40
F20B9	6.38±1.00	86.7±5.69	1.09±0.63	8.39±0.46	1082±44.2	8.93±0.46
F20B10	7.15±0.68	371±22.4	2.54±0.96	6.83±0.35	943±14.4	5.24±0.22

Values represent means ± SD (n = 3), F5, 5 year fallow phase; F20, 20 year fallow phase; AMY, amylase activity determined in terms of µg glucose soil g⁻¹ soil hr⁻¹; ASA, arylsulphatase activity determined in terms of pNP soil g⁻¹ soil hr⁻¹; GSA, β-glucosidase activity determined in terms of µg pNP soil g⁻¹ soil hr⁻¹; DHA, dehydrogenase activity determined in terms of TPF soil g⁻¹ soil hr⁻¹; PHA, acid-phosphomonoesterase activity determined in terms of µg pNP soil g⁻¹ soil hr⁻¹; PRO, protease activity determined in terms of µg tyrosine soil g⁻¹ soil hr⁻¹.

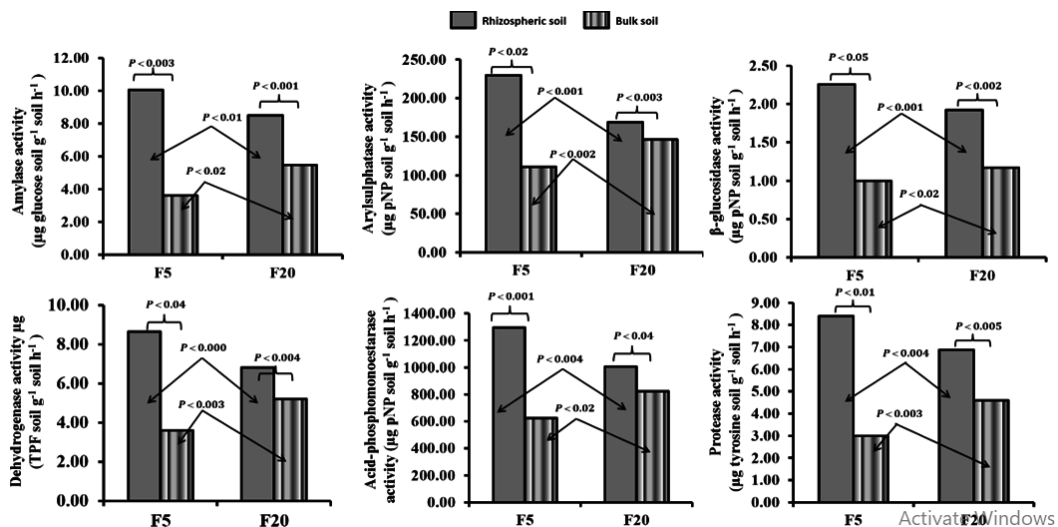


Fig. 2: The effect of length of fallow phase on the activities of soil enzymes. The comparison between F5 and F20 fallow phases or between rhizospheric and bulk soils within a fallow phase was determined by performing the paired t-test. The significance value (P ≤ 0.05) was indicated by paired arrows.

was incubated with sodium caseinate along with tris buffer at 50 °C for 2 hr in shaking water bath. Stopping the reaction with trichloroacetic acid supernatant was obtained and further treated with alkaline reagent and folin-ciocalteu reagent. After 1 hr, the concentration of tyrosine in the filtrate was determined colorimetrically.¹⁴

Isolation of Rhizospheric and Endophytic Bacteria

Isolation of rhizospheric bacteria was done from five composite rhizospheric soil samples from each fallow phase (sample RH1-RH5 from F5 and sample RH6-RH10 from F20). In similar way root samples were combined to isolate endophytic bacteria. The rhizosphere soil samples were processed as per the procedure described by Thokchom *et al.*,¹⁵ 1 g of rhizosphere soil was serially diluted and plated separately on Reasoner's 2 agar (R2A), Tryptic soy agar (TSA) and Potato dextrose agar (PDA). The morphologically different colonies were purified and stored in 30 % (m v⁻¹) glycerol at - 20 °C Ultra Deep Freezer. Prior to the isolation of root endophytes, roots were surface sterilized by washing the root bits (approximate 1 cm length) with successive dip in 70 % ethanol for 1 min - 2 % hypochlorite for 5 min - 70 % ethanol for 30 sec and 4 rinses with sterile distilled water. Root suspension was prepared by dissolving the grounded root sample in 1X PBS buffer in ratio 1:5. The suspension was precipitated at 4000 rpm for 20 min and supernatant was used to isolate endophytic bacteria in above mentioned three media.

Table 3: Root associated bacterial counts of *Thysanolaena maxima* from soils of different fallow phases

Fallow period	Population of bacteria (cfu x 10 ⁷ cells g ⁻¹ soil)	
	Rhizospheric soil	Root endophyte
F5	0.27 ± 0.029 ^a	0.05 ± 0.008 ^a
F20	0.74 ± 0.056 ^b	0.08 ± 0.004 ^b

Values are means ± SD, n = 5; Values that differed significantly are followed by different letters between F5 and F20; F5, 5 year fallow phase and F20, 20 year fallow phase.

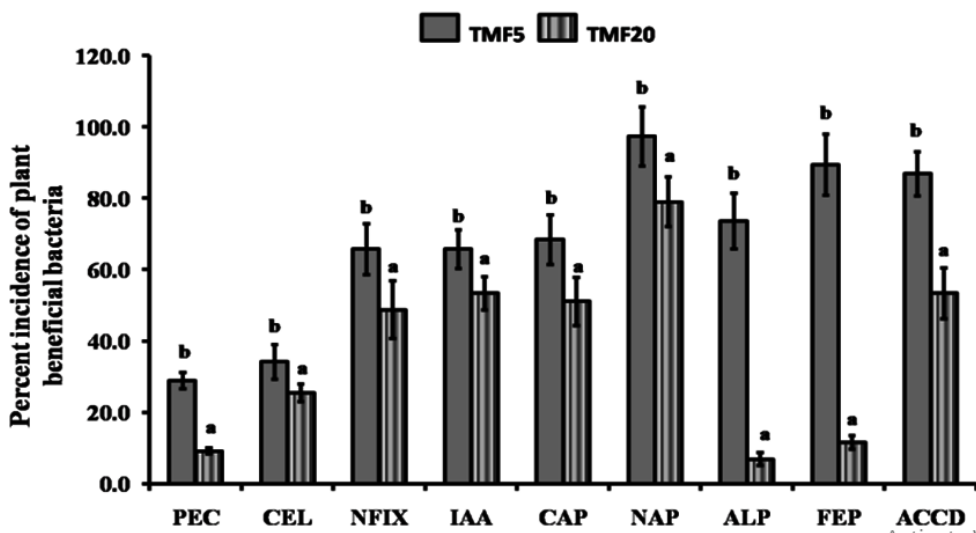


Fig. 3: The comparative incidence of beneficial bacteria associated with broom grass (*Thysanolaena maxima*) between 5 and 20 years *Jhum* fallows. The incidence was determined based on the plant growth promoting traits of bacteria. The comparison between F5 and F20 fallow phases was determined by performing the paired t-test (P ≤0.05)

The inoculum suspension of each pure isolate, prepared in nutrient broth (18-24 hr old, OD₆₀₀ 2.0), was subjected to the screening for various PGP assay. For each isolate 3 replicate analyses were performed.

***In-vitro* Assays for PGP Traits of Root Associated Bacteria**

Production of Pectinase

Pure isolates were spotted in nutrient agar plates supplemented with 0.5 % pectin. After five days of incubation the plates were flooded with 2 % CTAB solution for 20 min. Development of halo around the growth was considered as positive for pectinase production.¹⁶

Production of Cellulase

The isolates were grown on 0.5 % CMC (Carboxy Methyl Cellulose) agar plates. Two holes were made in the solid media one for sample and one for sterile distilled water.¹⁷ After five days of incubation the plates were flooded with 1 % congo red solution for 15 min. Destaining was done with 1 M NaCl solutions. The congo red clearing zone around the sample indicates cellulase activity of the bacteria.¹⁸

N₂ fixation

100 µl of pure bacterial culture was inoculated in nitrogen-free bromothymol broth along with uninoculated control and incubated for 3-5 days at 30 °C. The potential N₂ fixing bacteria were identified observing the blue colour development. Development of green to blue colour due to increase in pH of the medium on fixation of NH₄ by the bacterial isolate was a positive test for N₂ fixation.¹⁹

Production of Indoleacetic Acid (IAA)

Production of IAA was tested by growing the bacterium in Ashbys Mannitol broth supplemented with 1 % L-Tryptophan (9:1) from NB suspension of pure culture of each bacterium.²⁰ After 48 hr of incubation, cells were harvested at 4000 rpm for 20 min. The supernatant (2 ml) was mixed with 2 drops of orthophosphoric acid and 4 ml of Salkowski reagent (50 ml, 35 % of perchloric acid; 1 ml 0.5 M FeCl₃ solution). Development of pink colour indicated IAA production. Optical density was taken at 530 nm with the help of spectrophotometer (Spectrascan UV-2600, Thermo scientific, USA). Concentrations of IAA produced by cultures was measured with the

help of standard graph of IAA (Hi-media) obtained in the range of 5-30 µg/ml.²¹

Phosphate Solubilization

The isolates were tested for their ability to dissolve four different forms of insoluble iP viz. Pikovskaya broth²² amended with Ca₃(PO₄)₂, AlPO₄, FePO₄ and minimal media amended with sodium phytate (Na-P). After 72 hr of incubation, cells were harvested at 4000 rpm for 20 min. In 1 ml of clear supernatant 5 ml of extractant (0.03 N NH₄F, 0.025 N HCl) were added followed by addition of 5 ml of Brays reagent and 1 ml of stannous chloride to develop blue colour.²³ Optical density was taken at 660 nm with the help of spectrophotometer (Spectrascan UV-2600, Thermo scientific, USA). Concentrations of inorganic P was measured with the help of standard graph of P (Hi-media) obtained in the range of 0.08⁻¹ µg/ml.

ACCD Production

The activity of ACCD was assayed according to the modification of protocol of Honma and Shimomura,²⁴ which measures the amount of α-ketobutyrate produced when the enzyme ACCD cleaves ACC.²⁵ The enzyme activity was expressed as ηmol α-ketobutyrate mg⁻¹ protein hr⁻¹ and bacterial cell protein was determined by following Bradford protein assay.²⁶

Results

Soil Enzyme Properties of Rhizospheric and Bulk Soil

Activity of AMY, ASA, GSA, DHA, PHA and PRO was different in different soil samples. In five composite grid samples for bulk soil of F5 average activity for AMY ASA, GSA, DHA, PHA and PRO was found to be 3.60 µg glucose g⁻¹ soil hr⁻¹, 111.09 µg pNP g⁻¹ soil hr⁻¹, 1.0 µg pNP g⁻¹ soil hr⁻¹, 3.62 µg TPF g⁻¹ soil hr⁻¹, 624.82 µg pNP g⁻¹ soil hr⁻¹ and 3.0 µg tyrosine g⁻¹ soil hr⁻¹, respectively (Table. 1). The average activity for AMY ASA, GSA, DHA, PHA and PRO was found to be 5.46 µg glucose g⁻¹ soil hr⁻¹, 146.12 µg pNP g⁻¹ soil hr⁻¹, 1.17 µg pNP g⁻¹ soil hr⁻¹, 5.21 µg TPF g⁻¹ soil hr⁻¹, 824.91 µg pNP g⁻¹ soil hr⁻¹ and 4.59 µg tyrosine g⁻¹ soil hr⁻¹ in case of bulk soil from F20.

The rhizospheric soil from TMF5 showed average activity of 10.06 µg glucose g⁻¹ soil hr⁻¹, 229.64 µg pNP g⁻¹ soil hr⁻¹, 2.25 µg pNP g⁻¹ soil hr⁻¹, 8.67 TPF g⁻¹ soil hr⁻¹, 1296.97 µg pNP g⁻¹ soil hr⁻¹ and 8.39 µg

Table 4: The multifaceted PGP traits of root associated bacteria (Rhizospheric and endophytic) from TM collected from 5 years fallow *Jhum* cycle

Isolate	Pect. ^a	Cell. ^b	N ₂ -fix. ^c	IAA ^d	Dissolution/or mineralization of insoluble phosphates				ACCDi activity
					Ca ₃ (PO ₄) ₂ ^e	Na-phytate ^f	AlPO ₄ ^g	FePO ₄ ^h	
RR1	nd	16	+4	34.8±4.12	141±12.9	35.2±3.52	nd	15.9±0.32	43.1±2.13
RR2	3	nd	+3	nd	79.5±8.39	95.5±8.03	4.53±0.34	6.17±0.22	88.2±3.31
RR3	3	15	+5	64.2±9.35	54.2±5.93	85.7±9.43	14.3±0.52	18.6±1.47	24.4±0.64
RR4	nd	10	+3	73.2±7.47	98.7±14.7	91.0±9.58	20.0±0.29	21.5±0.67	36.3±0.94
RR5	nd	nd	+5	56.7±8.74	108±17.5	124±14.7	nd	19.7±1.46	nd
RR6	4	nd	+3	8.35±0.42	61.2±6.48	83.0±7.45	9.72±0.17	19.7±1.63	nd
RR7	nd	nd	+4	60.9±8.39	nd	92.0±6.93	4.09±0.26	nd	33.5±1.77
RR8	nd	nd	+3	nd	109±14.7	65.0±5.39	3.00±0.27	20.2±1.69	30.5±1.60
TR1	nd	nd	+1	nd	164±18.3	104±15.3	6.03±0.31	15.9±0.52	31.7±1.40
TR2	nd	nd	+5	19.6±0.73	106±13.7	147±17.0	25.5±0.21	nd	57.3±2.56
TR3	nd	nd	+1	nd	nd	90.5±8.33	3.09±0.1	7.00±0.58	38.0±2.52
TR4	5	17	+5	6.95±0.32	56±4.35	89.2±7.49	4.53±0.24	9.00±0.48	nd
TR5	nd	12	Nd	28.9±2.03	51.5±3.51	89.5±9.43	nd	9.50±0.73	46.5±1.92
TR6	5	nd	+1	nd	nd	91.0±10.3	5.75±0.37	28.7±2.48	73.6±4.93
TR7	nd	nd	Nd	17.3±1.91	40.2±4.52	87.2±8.57	74.5±0.33	10.3±1.17	29.4±1.63
TR8	2	10	+4	nd	92.2±11.5	89.5±9.75	45.4±0.42	nd	37.6±1.46
TR9	13	17	Nd	87.0±13.4	78.2±9.07	83±7.09	48.7±0.32	9.25±0.74	22.7±1.54
PR1	nd	nd	Nd	19.6±7.32	nd	104±16.0	60.0±0.39	27.9±3.89	41.5±2.73
PR2	nd	nd	+4	nd	31.5±4.32	95±14.07	27.9±0.41	28.2±1.07	29.4±0.97
PR3	nd	nd	+4	nd	nd	97.7±11.8	19.5±0.58	18.2±1.37	nd
PR4	nd	nd	+1	nd	nd	91.5±8.69	4.59±0.39	9.50±1.36	20.1±1.31
PR5	nd	nd	Nd	nd	nd	nd	8.25±0.63	11.7±1.81	47.0±3.47
PR6	nd	6	Nd	nd	37.5±2.63	121±17.0	4.66±0.33	9.00±1.32	13.5±0.77
PR7	4	7	+4	200±4.51	85.2±7.83	92.5±12.8	nd	9.25±0.94	90.5±7.60
PR8	nd	nd	Nd	80.1±8.93	nd	85.7±9.37	14.5±0.24	14.2±2.13	53.6±3.63
PR9	nd	nd	Nd	79.2±3.41	nd	81.5±8.72	nd	17.0±3.21	33.5±1.13
PR10	nd	nd	Nd	75.0±7.05	nd	98.7±11.6	25.7±0.53	14.6±4.34	61.3±2.52
PR11	nd	nd	Nd	42.7±4.74	nd	88.2±14.5	nd	nd	35.7±1.05
PR12	nd	nd	Nd	50.1±4.85	nd	107±17.3	33.6±0.35	31.2±0.47	nd
RE1	3	nd	+3	68.2±3.01	167±17.1	153±16.5	nd	5.5±0.27	32.1±1.07
RE2	nd	nd	Nd	83.7±7.32	116±14.2	110±10.4	5.56±0.23	45.2±4.54	47.2±2.67
RE3	nd	3	+3	nd	195±15.7	169±15.3	nd	14.8±2.07	26.2±2.94
RE4	nd	5	+4	73.1±5.06	269±13.5	208±14.9	5.16±0.46	9.92±2.35	13.5±0.73
RE5	2	nd	+2	9.29±0.41	180±12.1	187±15.4	3.91±0.17	27.9±5.86	39.3±1.05
TE1	nd	8	+3	130±12.3	134±12.4	130±12.9	nd	25.9±3.7	45.7±4.62
TE2	2	2	+3	nd	209±15.8	132±14.7	5.13±0.54	16.2±4.12	52.3±7.49
PE1	nd	nd	+3	49.8±4.91	246±13.2	138±12.4	nd	9.5±0.42	24.3±3.74
PE2	nd	nd	Nd	56.4±4.63	210±14.8	100±11.0	3.61±0.33	11.7±1.83	17.4±0.63

Values are means (±SE) (n=3); nd, not detected; ^a Pectinase activity determined in terms of solubilization zone diameter (mm); ^b Cellulase activity determined in terms of solubilization zone diameter (mm); ^c The qualitative N₂ fixation assay based on intensity of colour change in nitrogen-free bromothymol blue medium (+1 through +5, light blue to very dark blue); ^d Indole-acetic acid like substances production in terms of µg ml⁻¹ hr⁻¹; ^e Dissolution of Ca₃(PO₄)₂ in terms of presence of solubilized P in µg ml⁻¹ hr⁻¹; ^f Mineralization of Na-phytate in terms of presence of solubilized P in µg ml⁻¹ hr⁻¹; ^g Dissolution of AlPO₄ in terms of presence of solubilized P in µg ml⁻¹ hr⁻¹; ^h Dissolution of FePO₄ in terms of presence of solubilized P in µg ml⁻¹ hr⁻¹; ⁱ 1-aminocyclopropane-1-carboxylate deaminase (ACCD) production in terms of ηmol mg⁻¹ hr⁻¹.

Table 5: The multifaceted PGP traits of root associated bacteria (Rhizospheric and endophytic) from TM collected from 20 years fallow *Jhum* cycle

Isolate	Pect. ^a	Cell. ^b	N ₂ -fix. ^c	IAA ^d	Dissolution/or mineralization of insoluble phosphates				ACCDi activity
					Ca ₃ (PO ₄) ₂ ^e	Na-phytate _i	AlPO ₄ ^g	FePO ₄ ^h	
RR1	nd	5	+3	29.4±3.84	101±17.3	125±14.3	nd	nd	nd
RR2	nd	nd	+2	nd	74.0±8.66	nd	nd	nd	nd
RR3	nd	nd	+3	nd	nd	nd	nd	nd	24.7±1.90
RR4	nd	nd	+1	21.0±6.23	nd	nd	nd	nd	30.1±2.04
RR5	nd	7	+2	28.5±6.3	141±16.2	141±13.8	nd	nd	24.3±3.61
RR6	nd	11	nd	52.9±6.02	83.2±7.92	141±17.3	nd	nd	32.5±3.75
RR7	nd	10	nd	43.2±3.94	89.5±7.01	101±12.4	nd	nd	9.2±0.54
RR8	nd	nd	+2	nd	nd	nd	nd	nd	14.2±0.83
RR9	nd	8	+4	nd	nd	95±13.5	nd	nd	24.9±2.63
RR10	nd	8	+3	28.5±5.11	nd	nd	nd	nd	nd
RR11	nd	nd	nd	nd	nd	nd	nd	nd	39.5±3.89
RR12	nd	14	nd	nd	nd	78.2±9.46	nd	nd	nd
RR13	nd	nd	+3	185±14.1	nd	83.7±8.4	nd	nd	nd
RR14	nd	9	nd	nd	110±13.6	32.5±4.49	nd	nd	25.1±4.41
RR15	nd	nd	nd	52.2±10.9	nd	103±14.7	nd	nd	19.7±4.05
RR16	2	nd	+1	39.0±4.82	108±14.7	108±16.2	nd	nd	nd
RR17	nd	nd	+1	51.0±8.72	nd	nd	nd	nd	9.40±0.83
TR1	nd	nd	nd	nd	nd	92.0±7.35	14.7±0.93	nd	nd
TR2	nd	nd	nd	39.0±4.39	nd	110±11.8	nd	nd	15.8±1.54
TR3	nd	nd	+3	nd	nd	33.1±5.36	nd	nd	nd
TR4	nd	nd	+2	41.5±7.21	36.2±3.72	89.2±9.02	nd	nd	7.60±2.38
TR5	nd	nd	nd	42.6±5.83	35.2±6.57	83.7±8.4	nd	nd	nd
TR6	nd	nd	nd	nd	60.5±5.79	76.0±7.92	nd	nd	nd
TR7	nd	9	nd	nd	40.2±6.05	87.2±8.12	nd	nd	6.60±5.93
TR8	nd	nd	nd	nd	47.5±4.32	nd	nd	nd	nd
TR9	nd	nd	+2	37.9±7.64	nd	79.5±12.1	nd	nd	33.0±4.34
TR10	nd	nd	+1	73.2±11.2	48.5±7.31	60.5±7.9	nd	nd	nd
PR1	nd	nd	nd	nd	nd	73.7±5.07	nd	nd	nd
PR2	10	nd	nd	nd	44.5±3.88	72.2±6.49	nd	13.5±0.73	11.7±2.33
PR3	nd	nd	nd	41.4±7.3	36.2±3.18	38.7±5.65	nd	nd	8.70±1.03
PR4	2	nd	+1	nd	nd	31.5±5.2	5.22±0.43	nd	nd
PR5	nd	nd	+1	nd	nd	48.5±7.31	nd	nd	11.5±2.5
PR6	nd	nd	nd	nd	nd	27.8±3.92	nd	8.75±0.29	nd
PR7	nd	nd	nd	27.0±4.61	48.5±5.75	36.5±4.17	nd	3.00±0.21	7.30±3.1
RE1	nd	nd	nd	16.0±4.02	179±15.7	178±16.3	nd	nd	nd
RE2	nd	4	+1	22.9±6.21	41.7±6.93	62.9±11.4	7.07 ±1.34	40.2±6.32	27.1±4.23
RE3	nd	nd	+3	34.9±4.03	189±19.2	90.3±10.9	nd	nd	23.8±5.8
RE4	nd	nd	nd	77.2±10.2	nd	nd	nd	nd	nd
RE5	nd	nd	nd	nd	nd	76.2±8.32	nd	nd	nd
TE1	nd	2	+1	72.3±7.9	nd	58.1±4.79	nd	nd	11.5±3.09
TE2	nd	nd	nd	60.3±11.4	136±13.7	180±14.1	nd	nd	nd
TE3	nd	nd	nd	nd	180±13.1	65.3±4.22	nd	nd	12.1±5.73
PE1	1	nd	+2	nd	61.5±8.33	61.6±6.3	nd	32.0±2.04	nd

Values are means (±SE) (n=3); nd, not detected; ^a Pectinase activity determined in terms of solubilization zone diameter (mm); ^b Cellulase activity determined in terms of solubilization zone diameter (mm); ^c The qualitative N₂ fixation assay based on intensity of colour change in nitrogen-free bromothymol blue medium (+1 through +5, light blue to very dark blue); ^d Indole-acetic acid like substances production in terms of µg ml⁻¹ h⁻¹; ^e Dissolution of Ca₃(PO₄)₂ in terms of presence of solubilized P in µg ml⁻¹ hr⁻¹; ^f Mineralization of Na-phytate in terms of presence of solubilized P in µg ml⁻¹ hr⁻¹; ^g Dissolution of AlPO₄ in terms of presence of solubilized P in µg ml⁻¹ hr⁻¹; ^h Dissolution of FePO₄ in terms of presence of solubilized P in µg ml⁻¹ hr⁻¹; ⁱ i-aminocyclopropane-1-carboxylate deaminase (ACCD) production in terms of ηmol mg⁻¹ hr⁻¹.

tyrosine g^{-1} soil hr^{-1} for enzyme AMY, ASA, GSA, DHA, PHA and PRO, respectively (Table. 2). On the other hand, in five composite grid samples for rhizospheric soil of TMF20 gave average activity for AMY ASA, GSA, DHA, PHA and PRO as $8.49 \mu\text{g}$ glucose g^{-1} soil hr^{-1} , $169.02 \mu\text{g}$ pNP g^{-1} soil hr^{-1} , $1.92 \mu\text{g}$ pNP g^{-1} soil hr^{-1} , 6.83TPF g^{-1} soil hr^{-1} , $1006.97 \mu\text{g}$ pNP g^{-1} soil hr^{-1} and $6.87 \mu\text{g}$ tyrosine g^{-1} soil hr^{-1} , respectively.

The Population Incidence of Root Associated Bacteria of TM

The population of root associated (both rhizobacteria and endophytes) bacteria (counted in media R2A, TSA and PDA) of TM showed an increasing trend in the order of F20 > F5 and the bacterial population difference between F5 and F20 were significant (Table.3).

Out of 81 roots associated bacteria of TM, 29 and 34 rhizobacteria were obtained from F5 and F20 respectively; and 9 endophytes were obtained from each fallow cycle in three different media.

Incidence of Plant Beneficial Root Associated Bacteria of TM

Eighty one isolates were considered for in-vitro PGP traits (Table 4 and 5). Out of 29 rhizobacteria isolated from TMF5 production of pectinase, cellulase, IAA-like substances and ACCD were obtained in 8, 9, 18 and 24 strains, respectively; and out of 34 rhizobacteria from TMF20 these activity were found in 3, 9, 17 and 19 strains, respectively. Eighteen N_2 fixing rhizobacteria were isolated from TMF5 whereas 17 were isolated from TMF20. Dissolution of iP in media amended with $\text{Ca}_3(\text{PO}_4)_2$, Na-phytate, AlPO_4 and FePO_4 were observed in 17, 28, 23 and 25 isolates from TMF5, respectively; and 16, 26, 2 and 3 strains isolated from TMF20, respectively.

In case of 9 endophytic strains from TMF5, 3 isolates for pectinase activity, 4 for cellulolytic activity, 7 for both N fixation and IAA-like substances production and 5 for dissolution of iP in AlPO_4 were found to be positive. All isolates showed positive result while screening for iP dissolution in $\text{Ca}_3(\text{PO}_4)_2$, Na-phytate and FePO_4 ; and production of ACCD. For sample TMF20, out of 9 endophytes production of pectinase, cellulase, IAA-like substances and ACCD; fixation of N_2 ; and solubilization of iP in media amended

with $\text{Ca}_3(\text{PO}_4)_2$, Na-phytate, AlPO_4 and FePO_4 were observed in 1, 2, 4, 6, 6, 8, 1, 2 and 4 numbers of bacteria, respectively.

Discussion

The significance of TM as ESP in rejuvenating the biological activities (greater population of rhizobacteria and root endophytic bacteria and higher activities of soil enzymes) in burnt soils of shorter *Jhum* cycle has been demonstrated conclusively for the first time through this manuscript. In both fallow phases, activities of DHA, ASA, PHA, AMY, GSA and PRO were significantly higher ($p < 0.05$) in rhizosphere of TM than in bulk soils (Fig. 2). Higher activities of protease, invertase, cellulase, urease, and acid phosphatase in rhizospheric soils than bulk soils were also reported in earlier studies.²⁷ However, the activities of these enzymes in rhizospheric soil of TM collected from F5 fallow phase were significantly ($p < 0.05$) higher compared to that in F20 fallow phase (Fig. 2). Comparing bulk soils of two fallow phases, activities of DHA, ASA, PHA, AMY, GSA and PRO were found to be significantly higher ($p < 0.05$) for F20 fallow phase as compared to those of F5 fallow phase (Fig. 2). Availability of complex types of substrates in greater amount in longer fallow phase leads to secretion of mineralizing enzymes from increased soil microbes.²⁸ In a study conducted by Lungmuanaa *et al.*²⁹, activity of soil enzymes were found to be decreased after burning but gradually increased with time.

The root-associated bacterial population was more in TM from longer (F20) fallow phase (Table 3). Rhizobacteria and endophyte counts in rhizospheric soil and root sample of TMF20 were found to be 0.74×10^7 cfu and 0.083×10^4 cfu, respectively, whereas for TMF5 rhizobacteria and endophyte counts were found to be 0.27×10^7 cfu and 0.05×10^4 cfu, respectively. This is because, in longer *Jhum* cycle, the quantity of organic matter was high, so more number of macro- and microfaunal population exists to accomplish their decomposition.³⁰ Hence from a greater microbial reservoir higher number of bacteria colonizes the growing roots of TM in F20 fallow phase. However the percent incidence of plant beneficial bacteria isolated from TMF5 was higher than that from TMF20 (Fig 3). In TMF5 the incidence of root associated pectinase and

cellulase producers; N₂ fixers; IAA like substances producers; IP solubilizers in Ca₃(PO₄)₂, Na-phytae, AlPO₄ and FePO₄; and ACCD producers were found to 28.9, 34.2, 65.8, 65.8, 68.4, 97.4, 73.7, 89.5 and 86.8 %, respectively; whereas the same in TMF20 was found to be 9.3, 25.6, 48.4, 53.5, 51.2, 79.1, 7.0, 11.6 and 53.5 %, respectively. In fact, higher activities of enzymes in rhizospheric soils were indication of a greater functional diversity of the microbial community.³¹ Plant root exudates (basically the low molecular weight compounds) acts as microbial substrate or metabolic activators to stimulate activity of these enzymes.^{32,33} Again secretion of root exudates increases under the stress of nutrients and water.³⁴ As these stresses were more in soils from F5 than F20,³⁵ the higher activity of enzymes in rhizospheric soils could be linked with higher microbial secretion which was further influenced by release of more root exudates from TMF5.

In case of F5, soil is under more stressed condition compared to F20 soil. In F5 *Jhum* agroforestry, natural rebuilding process of ecosystem was destroyed due to frequent burning. Because of more nutrient availability in soil of F20 vegetation, the dependency of plant on soil microbes for their growth was minimized. In rhizosphere, the availability of nutrients was controlled by the combined effects of plant characteristics, soil properties and the interaction of roots with microorganisms.³⁶ But in soil of F5 *Jhum* field, the growth of new vegetation was much more dependent on efficient and positive root-microbe interactions. Incidence of more PGPB in rhizosphere of TMF5 indicated that under stressed situation the root system of plant TM accommodated only those bacteria which would be beneficial for their growth. According to earlier

reports, under stress conditions plant changes their root associated bacteria,³⁷ which is also mainly guided by the rhizodeposition from root.³⁸ To enhance nutrient availability in soil, more number of beneficial microbes is attracted to the rhizospheric soil.³⁹ As plants offer a suitable environment where only some selective microorganisms can exist,⁴⁰ thus filtering out the beneficial group of specific microbes only from the pool.

In conclusion, early generating plant species like TM plays important role in rejuvenating the biological activities in the burnt soils for shorter *Jhum* cycle and this process helps in re-establishing the linkages between above-ground and below-ground biota communities resulting gradual improvement in soil nutrient cycling processes.

Conclusion

The early generating plant species like TM plays important role in rejuvenating the biological activities in burnt soils of shorter *Jhum* cycle and this process facilitates in establishing the linkages between above-ground and below-ground biota communities leading to the gradual improvement in functioning of degraded *Jhum* lands.

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Conflict of Interest

Authors declare no conflict of interest.

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