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SEARCH FOR PLANT GROWTH PROMOTING ACTINOBACTERIA FROM A LIMESTONE MINING SPOIL SOIL IN MEGHALAYA

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ABSTRACT: Free-living actinobacteria were isolated from a limestone mining spoil soil located at Mawsmai in Meghalaya, India. The actinobacterial colonies obtained were screened for plant growth promoting properties based on their ability to produce indole-3-acetic acid and siderophores, their ability to solubilize phosphate and their ability to fix atmospheric nitrogen. Fifteen isolates thirteen of which were *Streptomyces* were found to produce indole-3-acetic acid above 15µg ml^{-1}. Eight isolates were found to solubilize phosphate above 350µg ml^{-1}. Out of these, three were *Streptomyces* and the remaining five were non-streptomycetes. Ten isolates which were all *Streptomyces* were found to produce siderophores with halo diameter greater than 25mm. 65% of the isolates, 93% of which were *Streptomyces* showed the ability to fix atmospheric nitrogen. Therefore, free-living limestone actinobacteria from Meghalaya more importantly *Streptomyces* showed plant growth promoting potential and some of them warrant further investigation through their interaction with plant systems and to find out if plants can benefit through such plant-actinobacteria interactions *in vivo*.

KEYWORDS: *Streptomyces,* Indole acetic acid, Phosphate solubilization, Siderophore, Diazotrophy, Sohra**.**

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1. INTRODUCTION

Actinobacteria are a diverse group of Gram-positive bacteria with high Guanine-Cytosine content in their DNA. They resemble fungi in morphology due to their branching aerial and substrate mycelium and bear chains of asexual spores. Actinobacteria are found in the soil as free living

bacteria in the rhizosphere as well as endophytes in roots and shoots of plants [1-3]. Actinobacteria along with other microorgansisms are actively involved in microbial mediated soil processes and makes them an important component in nutrient cycling and biogeochemical processes [4]. Numerous rhizospheric and endophytic actinobacterial species in association with plants have been reported to have various plant growth-promoting (PGP) properties [5]. They have also been shown to have antagonistic properties against many root-borne and disease-causing plant pathogens [6]. Plant growth-promoting actinobacteria (PGPA) have been reported to be mostly endophytic and the reported free-living ones in the soil are very closely associated with plant roots around the rhizosphere [7]. There have been numerous reports on isolation of rhizosphere competent free-living PGPA [8-10]. However, there have been few reports of free-living PGPA isolated from soils devoid of vegetation such as mining sites. Free-living PGPA from limestone mining site have been reported from Manipur in India [11,12] and showed that free-living PGPA from soils devoid of vegetation can also have PGP potential. It is therefore encouraging to search for more actinobacteria from limestone mining sites and similar environment for plant growth promotion. The state of Meghalaya in the North-Eastern part of India harbors a rich diversity of microorganisms [13] with numerous important functions and there have been numerous reports on microbial diversity and functions from this part of the country from various environments [13-18]. However, not much work on actinobacteria has been reported from this region and there has been no report on PGPA from limestone environment from here. Therefore, this work reports on the potential of free-living actinobacteria from a limestone mining site at Mawsmai, Meghalaya.

2. MATERIALS AND METHODS

2.1 Sampling site and sample collection

The sample collection site (Figure 1) was from a limestone mining site at Mawsmai, Meghalaya, India (N25°15.364' E91°43.885'). Mining spoil soil samples were aseptically collected in sterilized polythene bags and brought to the lab and stored at 4±1°C until further use.

2.2 Soil pretreatment and isolation of actinobacteria

The collected soil samples were air-dried at room temperature for one week and mixed together in equal proportions to get a composite sample. The composite sample was then separated into five parts and pretreated differently. Five different pretreatments were given viz. (i) dry heating at 120±1°C for 1 hour, (ii) wet heating at 70±1°C in a water bath for 15 minutes, (iii) 1.5% phenol treatment at 25±1°C for 30 minutes, (iv) 0.2% humic acid treatment at 25±1°C for 30 minutes and (v) a combination of dry heat and phenol treatment. 1g of each pretreated sample was suspended in 9ml sterilized distilled water and serially diluted up to 10^3 times. The serially diluted as well as undiluted sample suspensions were used as inoculum for isolation and 100µl of each sample suspension was spread on selective media plates. The selective media used were Actinomycete Isolation Agar (AIA), Bennett's Agar (BA), Starch Casein Agar (SCA), Streptomyces Agar (SA)

and Humic acid Vitamin Agar (HVA) [19]. The media were supplemented with nystatin (50µg ml^{-1}) and rifampicin (20µg ml^{-1}). The inoculated plates were incubated at 28±1ºC for four weeks. Based on colony morphology, the colonies were selected from the incubated plates, sub-cultured and maintained on Bennett's agar medium. The selected colonies were characterized and identified on the basis of micromorphology, biochemical and chemotaxonomic characteristics (data not shown here) [20].

Figure 1: Limestone mining site at Mawsmai, Sohra, Meghalaya

2.3 Indole-3-acetic acid production

Assessment of indole-3-acetic acid (IAA) production from the isolates was adapted from Dochhil et al. (2013) [21]. The isolates were cultured in 5ml Bennett's broth containing 0.2% L-tryptophan (membrane filtered) in a 15ml tube. Inoculated broth without L-tryptophan and broth with L-tryptophan but uninoculated were also prepared as controls. Following inoculation, the above broths were incubated at 28±1ºC with regular shaking for 14 days. Post incubation, the broth cultures were filtered using Whatman No.1 filter paper and 1ml of supernatant was taken and mixed with 2ml of Salkowski's reagent (2ml of 0.5M $FeCl_3$, 98ml of 35% $HClO_4$) and vortexed, followed by incubation at room temperature for 30 mins. The solution turned reddish-pink if IAA was present. The absorbance measurement was taken with a colorimeter at 530nm wavelength. Standard curve of IAA was prepared for the estimation of IAA concentration in µg ml^{-1}. All measurements were done in triplicates. Data was statistically analyzed using ANOVA and Duncan's test at $p < 0.05$ using XLSTAT software [22].

2.4 Phosphate solubilization

To determine the ability of the isolates to solubilize phosphate, the method of Chaiharn and Lumyong (2011) [23] was used. Cultures were grown in 4ml Pikovskaya broth containing tri-calcium phosphate in 15ml tubes at 28±1ºC for 14 days. Uninoculated broth was used as control. Post incubation period, the broth cultures were filtered using Whatman No.1 filter paper. 0.5ml of the supernatant was taken and mixed with 0.5ml of 10% trichloroacetic acid. To the mixture, 4ml of color reagent (1:1:1:2 ratio of 3M H_2SO_4: 2.5% ammonium molybdate : 10% ascorbic acid : distilled H_2O) was added and incubated at 26±1ºC for 15 mins. The absorbance of blue color developed was measured using a colorimeter at 820nm wavelength. The concentration of soluble

phosphate (µg ml^{-1}) was estimated from the standard curve of KH_2PO_4. All measurements were done in triplicates. Two-sample t-test at $p < 0.05$ was used to compare each pair of tested and control means. ANOVA with Duncan's test at $p < 0.05$ was used to compare all tested means.

2.5 Siderophore activity

Detection of siderophore production was performed according to the method of Khamna et al. (2009) [24]. Cultures were first grown in Bennett's Agar medium in Petri plates and incubated at 28±1ºC for 5 days. Actinobacterial discs (6mm) were then bored from the Bennett's agar plates using a 6mm cork borer and the discs were placed on Chrome Azurol S (CAS) agar medium and incubated at 28±1ºC for 10 days. CAS was prepared by mixing 60.5mg of Chrome Azurol S dissolved in 50ml of distilled water with 72.9mg of CTAB dissolved in 40ml of distilled water, after which it was mixed with 10ml of 1mM $FeCl_3.6H_2O$ in 10mM HCl. CAS agar was then made by mixing the CAS with 900ml of King's Medium base (Proteose peptone 20g, K_2HPO_4 1.5g, $MgSO_4.7H_2O$ 1.5g, Agar 20g) and the final pH adjusted to 6.8. Siderophore-producing isolates produced orange halos around the discs. Siderophore activity was assessed by measuring halo diameter in mm. All measurements were done in triplicates. The sample means were statistically compared using ANOVA with Duncan's test at $p < 0.05$.

2.6 Diazotrophy

Detection of the ability to fix atmospheric nitrogen (diazotrophy) among the isolates was performed according to Franco-Correa et al. (2010) [4]. The isolates were inoculated in nitrogen-free medium (NFB), nitrogen-low medium (Ashby) and nitrogen standard medium (ISP2) to assess their ability to grow in these media. The NFB medium was supplemented with 0.01% bromothymol blue, a pH indicator which appears green below pH 7.0 and blue above pH 7.0. Upon inoculation, the cultures were incubated at 28±1ºC for 3 weeks. The ability of the isolates to grow in NFB and Ashby media and their ability to change the color of NFB medium from green to blue was observed and recorded. All measurements were done in triplicates.

3. RESULTS AND DISCUSSION

3.1 IAA production

The range of IAA production by the isolates was from 6.21±0.22 µg ml^{-1} to 32.95±0.53 µg ml^{-1} and fifteen isolates mostly *Streptomyces* produced IAA above 15µg ml^{-1} which is slightly above the moderate production range of 0.2-15µg ml^{-1} (Jog et al. 2016). *Streptomyces* LD-37, *Actinomadura* LD-35 and *Streptomyces* LD-28 produced the highest level of IAA of 32.95±0.53, 31.84±0.75 and 31.12±0.71 respectively which were not significantly different from each other at $p<0.05$ (Table 1). However, 95% of the isolates showed the ability to produce IAA significantly greater than the controls at $p < 0.05$. The production of IAA even though was slightly above the moderate production range, was however, narrow and comparatively very low as compared to reports from other workers because 127µg ml^{-1} to 197 µg ml^{-1} of IAA production by actinobacteria have been reported [21,24-

26]. However, the high IAA-producing actinobacteria (which were mainly *Streptomyces*) reported by the above workers were endophytic and rhizosphere-competent isolates. The IAA-producing isolates from this work were from a limestone mining site devoid of vegetation due to mining activities. These isolates therefore have lost the expoure to enough levels of plant exudated L-Tryptophan, a precursor for IAA production to trigger high production of IAA. Hence, there is a possibility of retriggering enhanced production of IAA in them if they are inoculated around plant root rhizosphere.

Table 1: Isolates producing IAA above 15µg ml^{-1} in descending order in the culture supernatant

Isolates	IAA production (µg ml^{-1})		
	Tested$^{\$}$	Control 1	Control 2
Streptomyces LD-37	32.95±0.53^{*a}	7.61±0.12	5.98±0.14
Actinomadura LD-35	31.84±0.75^{*a}	5.81±0.59	
Streptomyces LD-28	31.12±0.71^{*a}	6.40±0.12	
Streptomyces LD-24	27.04±0.47^{*b}	4.96±0.33	
Streptomyces LD-25	25.88±0.86^{*bc}	6.53±0.22	
Streptomyces LD-42	25.04±0.59^{*cd}	9.11±0.18	
Streptomyces LD-32	24.04±0.20^{*de}	8.67±0.33	
Streptomyces LD-15	23.28±0.62^{*de}	7.21±0.40	
Streptomyces LD-43	22.91±0.93^{*e}	11.53±0.51	
Streptomyces LD-14	22.75±0.61^{*e}	7.14±0.31	
Streptomyces LD-45	22.33±0.23^{*e}	7.81±0.04	
Nocardia LD-44	22.21±0.86^{*ef}	3.49±0.11	
Streptomyces LD-40	20.49±0.35^{*fg}	10.75±0.30	
Streptomyces LD-26	19.09±0.50^{*g}	5.88±0.40	
Streptomyces LD-41	15.37±0.42^{*h}	3.47±0.09	

$^{\$}**Tested**, Medium with L-tryptophan and inoculated; **Control 1**, Medium without L-tryptophan and inoculated; **Control 2**, Medium with L-tryptophan but uninoculated

Values followed after ± are standard errors of their sample means

* denotes tested is significantly higher than control(s) at $p<0.05$

Values followed by same superscript letters are not significantly different from each other at $p<0.05$

3.2 Phosphate solubilization

The range of phosphate solubilization was from 5.67±0.88 µg ml^{-1} to 462.67±21.46 µg ml^{-1} and eight isolates solubilized phosphate above 350µg ml^{-1} (Table 2) with a non-streptomycete isolate LD-16 showing the highest phosphate solubilizing ability of 462.67±21.46 µg ml^{-1}. 36% of the isolates showed phosphate solubilizing ability significantly higher than their controls at $p < 0.05$.

The phosphate solubilizing ability of the best phosphate solubilizers from this work were comparatively lower than the highly cited PGP rhizobacteria *Bacillus* (957µg ml^{-1}) and *Pseudomonas* (1500µg ml^{-1}) [26]. There was also a report on phosphate solubilizing *Streptomyces* with phosphate solubilization as high as 1916µg ml^{-1} [26]. Nevertheless, some other workers have reported on actinobacteria with phosphate solubilizing ability comparable to the isolates from this work ranging from 100µg ml^{-1} to 680µg ml^{-1} [27-34]. The phosphate-solubilizing actinobacteria reported by above workers were from various sources viz. endophytic, rhizospheric, marine sediments and fungal fruiting bodies. It has been reported that there is a strong negative correlation between pH values and soluble phosphate concentration in the presence of phosphate solubilizing bacteria [35] and the soil pH from the sample collection site being slightly alkaline (7.12±0.9) might explain why the phosphate solubilizing isolates from this work are not very strong phosphate solubilizers.

Table 2: Isolates which could solubilize phosphate above 350µg ml^{-1} in descending order in the culture supernatant

Isolates	Phosphate solubilisation (µg ml^{-1})	
	Tested[$]	Control
LD-16 (Non-Streptomyces)	462.67±21.46[*a]	130.67±5.81
LD-09 (Rhodochrous group)	410.67±2.91[*b]	
Nocardia LD-11	396.00±3.06[*bc]	
Streptomyces LD-04	377.00±6.51[*cd]	
Nocardia LD-10	365.67±0.33[*d]	
Streptomyces LD-33	363.67±14.86[*d]	
Nocardia LD-44	362.00±14.19[*d]	
Streptomyces LD-30	353.67±9.91[*d]	

[$]**Tested**, Medium containing tri-calcium phosphate and inoculated; **Control**, Medium containing tri-calcium phosphate but uninoculated

Values followed after ± are standard errors of their sample means

* denotes tested is significantly higher than control at $p<0.05$

Sample means followed by same superscript letters are not significantly different from each other at $p<0.05$

3.3 Siderophore activity

Siderophore activity (halo diameter in mm) of the siderophore-producing isolates ranged from 9.33±0.33 mm to 37.33±1.20 mm with four *Streptomyces* isolates viz. LD-31, LD-27, LD-36 and LD-03 showing the highest activities (Table 3) and the photographs depicting their activity is shown in Figure 2. Ten isolates showed siderophore activity above 25mm of halo diameter which is the usual range reported by other workers [24,36-40]. All the high siderophore-producing isolates were

Streptomyces. Similarly, the above workers also reported *Streptomyces* as the most common siderophore producers which were recovered from different niches such as rhizosphere, vermicompost soil and plant tissues as endophytes. Usually, iron-deficient conditions trigger siderophore production in microorganisms [41] but the study site is a limestone mining site which is expected to be contaminated with various heavy metals including iron due to mining activities [42]. But, maybe due to low iron bioavailability [43], the isolates were triggered for siderophore production.

Table 3: Isolates showing siderophore activity in descending order above halo diameter of 25mm

Isolates	Halo diameter (mm)
Streptomyces LD-31	37.33±1.20[a]
Streptomyces LD-27	37.00±0.58[a]
Streptomyces LD-36	36.00±0.58[a]
Streptomyces LD-03	35.00±1.00[ab]
Streptomyces LD-02	33.33±0.88[bc]
Streptomyces LD-19	32.33±0.33[c]
Streptomyces LD-18	32.00±0.58[c]
Streptomyces LD-30	31.67±0.67[c]
Streptomyces LD-05	31.33±0.67[c]
Streptomyces LD-01	28.33±0.67[d]

Values followed after ± are standard errors of their sample means

Sample means followed by same superscript letters are not significantly different from each other at $p<0.05$

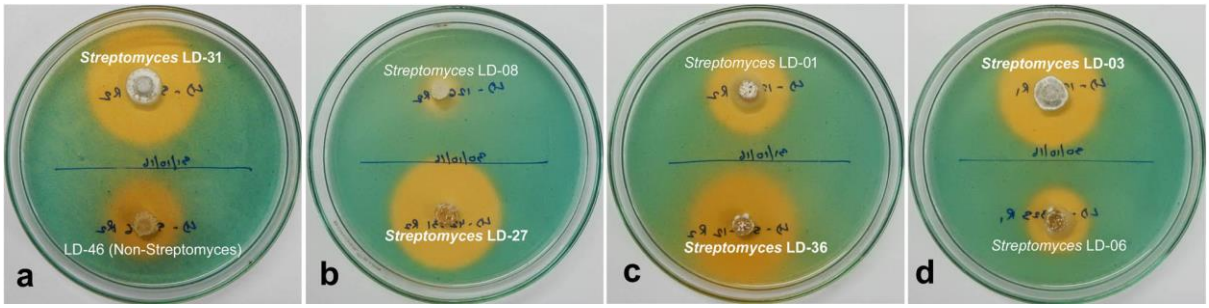

Figure 2: Photographs of CAS agar plates showing siderophore activity of the best siderophore producers whose names are boldfaced on the photographs. a, *Streptomyces* LD-31; b, *Streptomyces* LD-27; c, *Streptomyces* LD-36; d, *Streptomyces* LD-03

3.4 Diazotrophy

93% of the isolates had good growth in nitrogen-rich (ISP2) medium and the remaining 7% grew moderately. On the nitrogen-low (Ashby) medium, none of the isolates could show good growth. However, 42% of them showed moderate growth and the remaining 58% showed poor growth. On the nitrogen-free (NFB) medium, none of the isolates had good growth and only 10% of them all

Streptomyces could grow moderately and increased the pH of the medium (detected through change in color of the medium from green to blue). 55% of the isolates showed poor growth, out of which 88% showed visible increase in pH of the medium. Overall, 65% of the isolates were found to be able to fix atmospheric nitrogen out of which 93% were *Streptomyces*. The increase in pH of the medium inoculated with diazotrophic isolates were most probably due to conversion of free atmospheric di-nitrogen into ammonia. Photograph depicting the experimental setup for diazotrophy *in vitro* is shown in Figure 3. Most of the diazotrophic isolates in this work were *Streptomyces*. Other workers have also reported *Streptomyces* as one of the commonly occurring diazotrophs from rhizosphere and root tissues of plants [44-46]. Many other actinobacterial genera have also been reported to have diazotrophic property [5]. *Frankia* is a well-known nitrogen-fixer under free-living as well under endosymbiotic conditions [5]. Trujillo et al. (2010) [47] reported the genus *Micromonospora* to be widespread in the root nodules of *Lupinus angustifolius* and were found to be able to fix atmospheric nitrogen. Qin et al. (2015) [45] have also reported *Nonomuraea* and *Micrococcus* as nitrogen-fixers. However, *Streptomyces* being an abundant genus and due to its versatility in using various types of nutrients, it is easily and abundantly isolated in the laboratory and hence more diazotrophic *Streptomyces* isolates are recovered from different niches as compared to the other rarer genera.

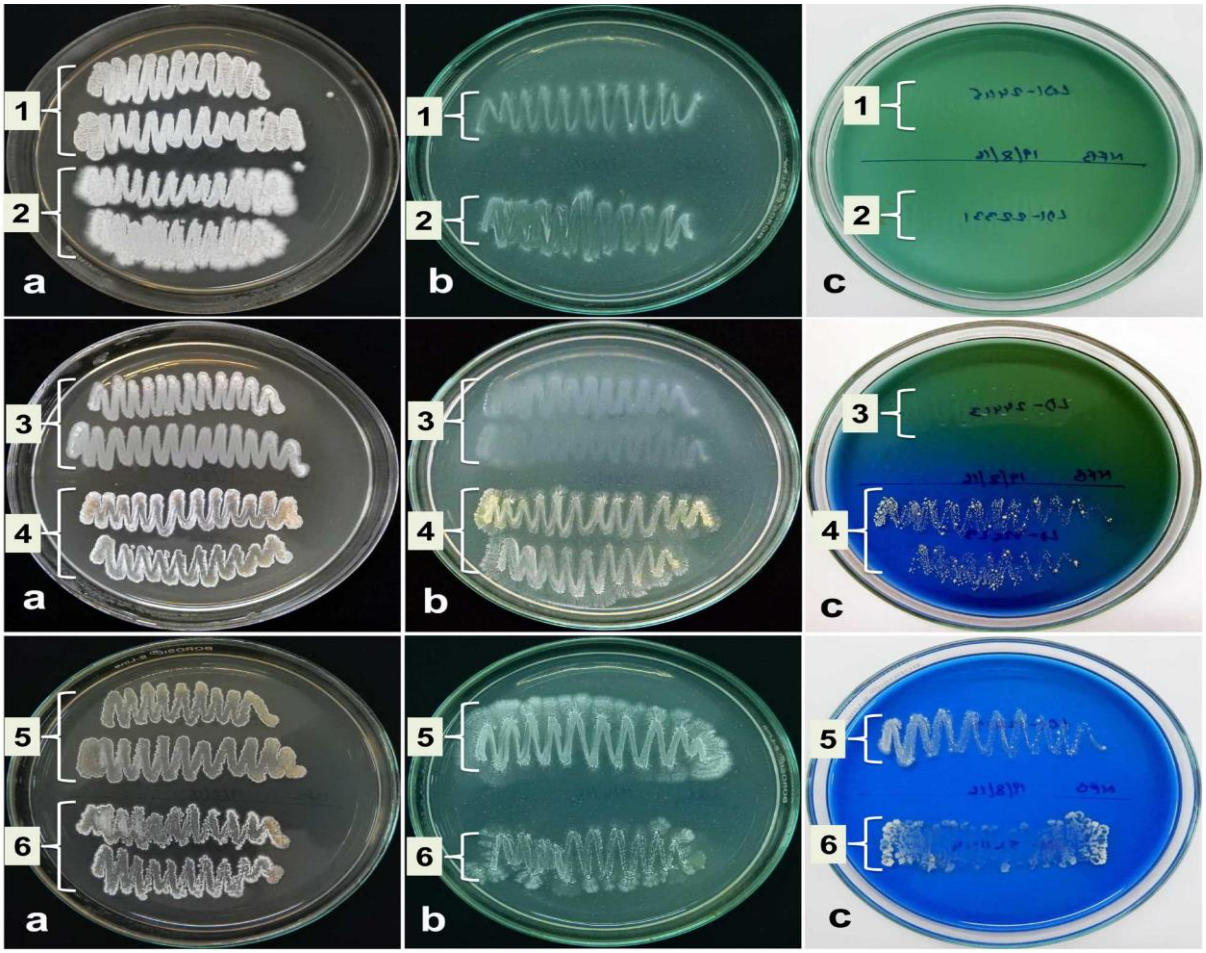

Figure 3: Photographs of isolates grown on (a) nitrogen-rich (ISP2) medium, (b) nitrogen-low

(Ashby) medium and (c) nitrogen-free (NFB) medium.

1, LD-16 (Non-Streptomyces); 2, LD-13 (Non-Streptomyces); 3, LD-20 (Non-Streptomyces); 4, *Streptomyces* LD-22; 5, *Streptomyces* LD-08; 6, *Streptomyces* LD-38

4. CONCLUSION

Among the projected PGP isolates, thirty-five isolates were found to have at least one potential PGP property and three isolates had two out of the four PGP properties tested. *Streptomyces* was the dominant PGPA with twenty-nine isolates out of the thirty-five isolates projected. The other PGP isolates were three *Nocardia* isolates, one *Actinomadura* isolate, one isolate belonging to the Rhodochrous group and one unidentified non-Streptomycete isolate. The projected PGPA isolates reported in this work were comparable in their PGP ability to the PGPA reported by other workers. Some of the best performing PGPA isolates from this work warrant further investigations *in vivo* in a plant-actinobacterium interaction set-up. The limestone mining site at Mawsmai from where the soil samples were collected was having soil physico-chemical properties not conducive for plant growth the reason being due to removal of vegetation due to mining activities. The soil moisture content, organic carbon and the common nutrients required by plants viz. available nitrogen, phosphorus and potassium were all found to be very low (data not shown). Yet, the actinobacteria isolated from the site were found to have PGP potential. This only tells us that such degraded lands can be reclaimed by re-vegetating them and the potential microorganisms in the soil are already there to establish a positive interaction with plants and vice versa. Moreover, the resourceful microbes from such environments also have the potential to be developed as microbial inoculants and biofertilizers in agriculture for better crop production and better plant health.

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CONFLICT OF INTEREST

The authors declare that they do not have any conflict of interests.

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