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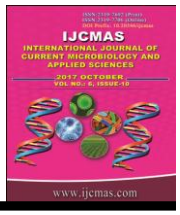

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Media Comparison for Enumeration of Actinobacteria and their Catabolic Diversity in the Crop Rhizosphere of Arid, Semi-Arid and Humid Regions (AER 3 and AER 6) of India

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A B S T R A C T

Rhizosphere soils of sorghum, pearl millet, pigeon pea, finger millet and groundnut from sub-tropical arid and semi-arid regions (Karnataka, Andhra Pradesh and Rajasthan) and from humid pristine forest soils of Karnataka were used to enumerate actinobacterial populations and their catabolic diversity. The pH of the soils ranged from 5.7 to 9.1 and organic carbon content from 0.075 to 4.3%. Of five different cultural media used, Humic acid vitamin agar was found to be superior for enumeration of Actinobacteria. Forty one (41) Actinobacterial isolates were characterised for morphological and biochemical attributes and tentatively assigned to four genera - *Streptomyces* (26 no.), *Nocardia* (12), *Micromonospora* (1) and *Saccharopolyspora* (2). The catabolic diversity of Actinobacterial isolates (21 carbon compounds) showed that Shannon-Weaver diversity index (H) was high (3.54). Dendrogram of Actinobacterial isolates showed some degree of catabolic relatedness among arid and semi-arid zone isolates. All the isolates had 18% similarity. At 66% level of similarity there were 18 clusters and this diversity in carbon utilization was much more in the alkaline arid and semi-arid soils (2.81) than in the acidic humid soils (1.94). The species evenness in humid zone isolates (1.00) was slightly higher to that of arid and semi-arid zone (0.993) isolates. The results show that arid and semi-arid regions represent extremely rich reservoir for the isolation of a significant diversity of Actinobacteria and that Humic acid- Vitamin agar is best for recovering greater numbers and diversity.

Introduction

Actinobacteria are the most widely distributed group of microorganisms and occur as saprophytes in soil (Takizawa *et al.*, 1993). They are Gram-positive, prokaryotic microorganisms; their growth and proliferation in soil is influenced by number of factors such as soil type, organic matter content, salinity, relative moisture content, temperature and vegetation (Waksman, 1967). Most Actinobacteria in soil belong to the genus *Streptomyces* (Goodfellow and Simpson, 1987, Suzuki *et al.*, 2000) and 60 per cent of the biologically active compounds such as antifungal and antibacterial compounds or plant growth promoting substances have originated from this genus

(Ilic *et al.*, 2007). *Streptomyces* and other Actinobacteria are major contributors to biological buffering of soils and play an important role in organic matter decomposition (Dhingra and Sinclair, 1995). They are able to produce spores, which help in dissemination and resistance to many adverse conditions (Goodfellow and Williams, 1983; Chater, 1993).

For isolation of soil Actinobacteria, selection of media is important for understanding their ecological properties and for discovery of novel strains which can produce useful bioactive secondary metabolites. Numerous media have been described for the isolation of Actinobacteria from soil and other natural materials. Use of isolation media with high carbon to nitrogen ratio and resistant complex carbon and nitrogen sources, *e.g.*, starch, casein, chitin, humic acid *etc.*, is suitable for isolation and enumeration of Actinobacteria (Gray and Williams, 1971). The increasing emergence of new diseases and pathogens, and the antibiotic resistance in recent years have caused an interest in searching for new biologically active compounds, thus the studies on distribution and diversity of Actinobacteria are crucial in discovering new strains and to explore ecological niches in different regions worldwide. The aim of this study was to compare different media for enumeration of Actinobacteria and to study the catabolic diversity of Actinobacteria in arid, semi-arid and humid regions of India.

Materials and Methods

Soils

Soil samples were collected from the rhizosphere of Sorghum (*Sorghum vulgare*), Pearl millet (*Pennisetum typhoideum*), Pigeon pea (*Cajanus cajan*), Finger millet (*Eleusine coracana*) and Groundnut (*Arachis hypogea*) grown in arid region of Jaisalmer in Rajasthan; arid and semi-arid regions of Belgaum, Hubli, Bijapur and Tumkur districts of Karnataka and Anantapur in Andhra Pradesh. Two soil samples from pristine forest in humid region of Karnataka (Kegdal, Vibuthi). Soil samples were air dried and analyzed for their physico-chemical properties by standard procedures (Hesse, 1971).

Isolation and comparison of media for enumeration

Isolation of Actinobacteria was done by standard plate count methods after serial dilution of soils. Five different media were used for isolation and enumeration of Actinobacteria *viz.*, actinomycetes isolation agar, starch casein agar, arginine glycerol salts medium, humic acid vitamin agar and Kuster's agar.

Forty one morphotypes were short listed and were characterized in detail for morphological and biochemical attributes using the Bergey's manual of determinative bacteriology 9th edition (Holt *et al.*, 1994). Morphologically distinct Actinobacterial isolates were selected on the basis of spore color, mycelia formation, pigment production and reverse side color formation (data not shown) and these isolates were tentatively assigned to four genera - *Streptomyces* (26 no.), *Nocardia* (12), *Micromonospora* (1) and *Saccharopolyspora* (2) and tested for utilization of different carbon compounds.

Carbohydrates utilization

Actinobacteria were tested for utilization of twenty one different carbon compounds like monosaccharides (adonitol, arabinose, dextrose, dulcitol, fructose, galactose, inositol, mannitol, mannose, rhamnose, sorbitol, xylose), disaccharides (cellobiose, lactose, maltose, melibiose, salicin, sucrose,

trehalose), trisaccharides (raffinose) and polysaccharides (inulin) as per procedure of Shirling and Gottlieb (1966) using carbohydrate impregnated discs instead of sugar solutions.

Actinobacteria were inoculated into 50 ml tryptone yeast extract broth in Erlenmeyer flasks and incubated for 48 hours at 28^0 C in an orbital shaker at 125 rpm. Sterile glass beads were added and subjected to vigorous agitation to break the mycelia. Homogenized culture was centrifuged at 8000 rpm for 5 minutes to pelletize the culture. The supernatant was decanted and the culture was re-suspended in 1.5 ml of sterile distilled water and centrifuged to pelletize the culture. The procedure was repeated 4-5 times. Finally the pellet was used for inoculating the plates. Approximately 50 µl of washed inoculum (test culture) was placed on one edge of the phenol red agar medium and streaked across the Petri dish. The carbohydrate impregnated discs (25 mg carbohydrate/disc) were placed and pressed gently on the surface of the plate at sufficient distance (2 cm) from each other. Plates were incubated at 36 ± 1.0^0 C for 48 hours and results were recorded, carbon utilization was visualized by change in colour around the disc from red to yellow.

Diversity analysis

The diversity of Actinobacteria based on carbon utilization was calculated by using the formula (Saeki *et al.,* 2008).

$H^1 = -\Sigma^s (P_i \ln P_i)$

P_i = Fraction of individuals belonging to i^{th} species

$E=H/H_{max}$

Where, E=Evenness, H= diversity, H_{max}= maximum diversity possible

Cluster analysis of catabolic diversity

The carbon utilization (21 sources) pattern of the Actinobacteria was used for cluster analysis. A binary matrix was constructed where 0 denotes inability and 1 denotes ability to catabolize a particular source. The data was analyzed using the SIMQUAL (Similarity for Qualitative data) of NTSYS- (Numerical taxonomic and multivariate analysis system) PC2 package. The isolates were grouped as per Jaccard's similarity index by the unweighted paired group method using arithmetic means (UPGMA) and depicted as a dendrogram.

Results and Discussion

Isolation and media comparison

The pH of the soils varied from 5.7 to 9.1 in semi-arid and arid soils with organic carbon content of 0.075 to 0.70. In the humid soils the pH averaged 6.0 while org C content was 2.9 % (Table 1). Among the different media tested, higher population of Actinobacteria was recorded on Humic acid vitamin agar (29 x 10^4cfu/g soil) while the lowest Actinobacterial population was recorded in argnine glycerol salts medium (2.3 x 10^4 cfu/g soil). Humic acid vitamin agar was the best medium for enumeration of Actinobacteria from most of the soils. These results are in conformity with Hayakawa and Nonomura (1987) who demonstrated the superiority of Humic acid vitamin (HV) agar over colloidal chitin agar, glycerol-arginine agar and starch-casein-nitrate agar, for the isolation and enumeration of soil Actinobacteria. Similarly, Coombs and Franco (2003) observed that the nutrient poor media such as HV agar, TWYE (Tap water yeast extract) and YECH (Yeast extract- casein hydrolysate) agar are most effective for isolation of endophytic actinobacteria from wheat roots. Organic media with low nutrient concentrations like WYE (water yeast extract) and CAYEG

(casamino acids-yeast extract-glucose) agar were found best for isolating diverse Actinobacteria by avoiding contamination and overgrowth by eubacteria and fungi (Crawford *et al.*, 1993). When the average counts of Actinobacteria obtained from different type of soils grown on five different media were compared, the highest Actinobacterial population was observed in rhizosphere soils of sorghum and pigeon pea in black soils which was twofold higher than that of red soils of sorghum, finger millet and groundnut which may be due to soil type (higher clay content and organic carbon) (Table 2).

Varalakshmi *et al.,* (2010) also observed that black soils harboured more population of Actinobacteria in the rhizosphere of fox tail millet as compared to red soil. Actinobacteria make up 10-15 per cent of the total microbial population in both virgin and cultivated soils (Alexander, 1961). In alkaline soils, especially when dry, the relative abundance is spectacularly high. However, in comparison with true bacteria, Actinobacteria are less common in wet than in dry soils. The population is likewise greater in grassland and pastured soil than in cultivated fields (Alexander, 1961). In this study, comparison of Actinobacterial population of forest soil (Kegdal forest, black soil) was made with adjacent cultivated soil (Koundal, Sorghum, black soil) and found that the Actinobacterial population was more in cultivated soils (by 37.5 %), even though the pH was almost similar (6.1-6.2) but the organic carbon was higher in forest soil (3.60%) than in cultivated soils (0.81%) (Table 1). This may be due to the high wetness of forest soil (virgin) as compared to the cultivated sorghum soil. The findings of the study supports the similar observation made earlier by Alexander (1961).

Table.1 Details of crop, location and physico-chemical properties of collected soil samples

Soil sample no	Crop	Type of soil	Place	District and State	pH	Org. C (%)
1	Forest	Pristine(Black)	Kegdal	Uttarakannada, Karnataka	6.1	3.60
2	Forest	Pristine(Laterite)	Vibuthi	Uttarakannada, Karnataka	5.9	4.30
3	Sorghum	Hilly(Black)	Koundal	Belgaum, Karnataka	6.2	0.81
4	Sorghum	Plains(Black)	Hubli	Hubli, Karnataka	8.1	0.66
5	Pigeon pea	Plains(Black)	Honaganahalli	Bijapur, Karnataka	9.0	0.70
6	Pearl millet	Plains(Black)	Honaganahalli railway gate	Bijapur, Karnataka	8.7	0.63
7	Sorghum	Plains(Red)	Timmapur	Gadag, Karnataka	8.4	0.65
8	Finger millet	Plains(Red)	Ballenahalli	Tumkur, Karnataka	5.7	0.46
9	Groundnut	Plains(Red)	Anantapur	Anantapur, Andhrapradesh	7.0	0.27
10	Pearl millet	Desert(loamy sand)	Jaisalmer	Jaisalmer, Rajasthan	9.1	0.17
11	Desert soil	Desert(Sand dune)	Sam	Sam, Rajasthan	9.0	0.075

Table.2 Enumeration of Actinobacteria (No. X 10^4cfu/g soil) in different growth media

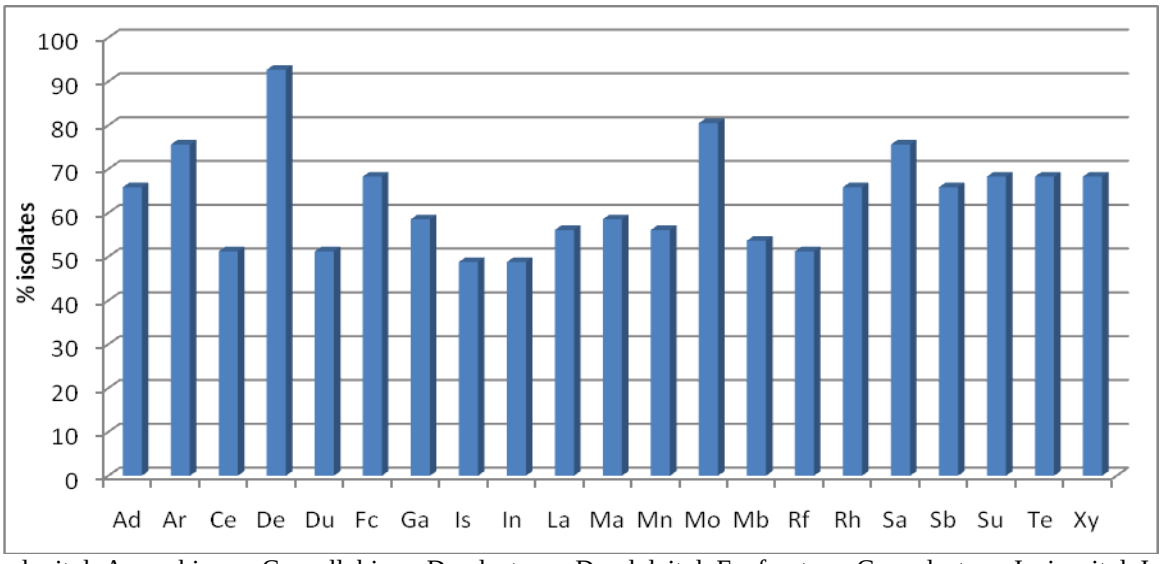
Location	Crop	AIA	SCA	HVA	AGS	KA
Kegdal (Karnataka)	Forest soil	8.5	8.0	14.7	3.1	8.6
Vibuthi (Karnataka)	Forest soil	7.5	7.2	9.0	3.0	6.7
Koundal (Karnataka)	Sorghum	8.0	7.5	27.7	4.0	11.8
Hubli (Karnataka)	Sorghum	8.8	7.3	28.0	4.2	12.1
Honaganahalli (Karnataka)	Pigeon pea	9.0	7.7	29.0	5.0	12.7
Honaganahallirailway gate (Karnataka)	Pearl millet	7.8	6.7	16.2	3.3	8.5
Timmapur (Karnataka)	Sorghum	5.2	4.6	10.0	2.8	5.7
Ballenahalli (Karnataka)	Finger millet	5.5	4.1	10.5	2.3	5.6
Anantapur(Andhra Pradesh)	Groundnut	5.0	5.0	12.2	2.9	6.3
Jaisalmer (Rajasthan)	Pearl millet	7.8	7.0	6.4	6.0	6.8
Sam (Rajasthan)	Desert soil	5.6	5.7	5.2	4.0	5.1
Average counts (Population)		7.15	6.44	15.35	3.69	8.2
S.Em ±		0.04	0.02	0.02	0.01	0.05
CD @ 1%		0.14	0.08	0.06	0.04	0.18

*AIA: Actinomycetes Isolation Agar, SCA: starch Casein Agar, HVA: Humic acid Vitamin Agar, AGS: Arginine Glycerol Salts medium, KA: Kuster's agar

Table.3 Shannon diversity index and evenness of Actinobacterial isolates

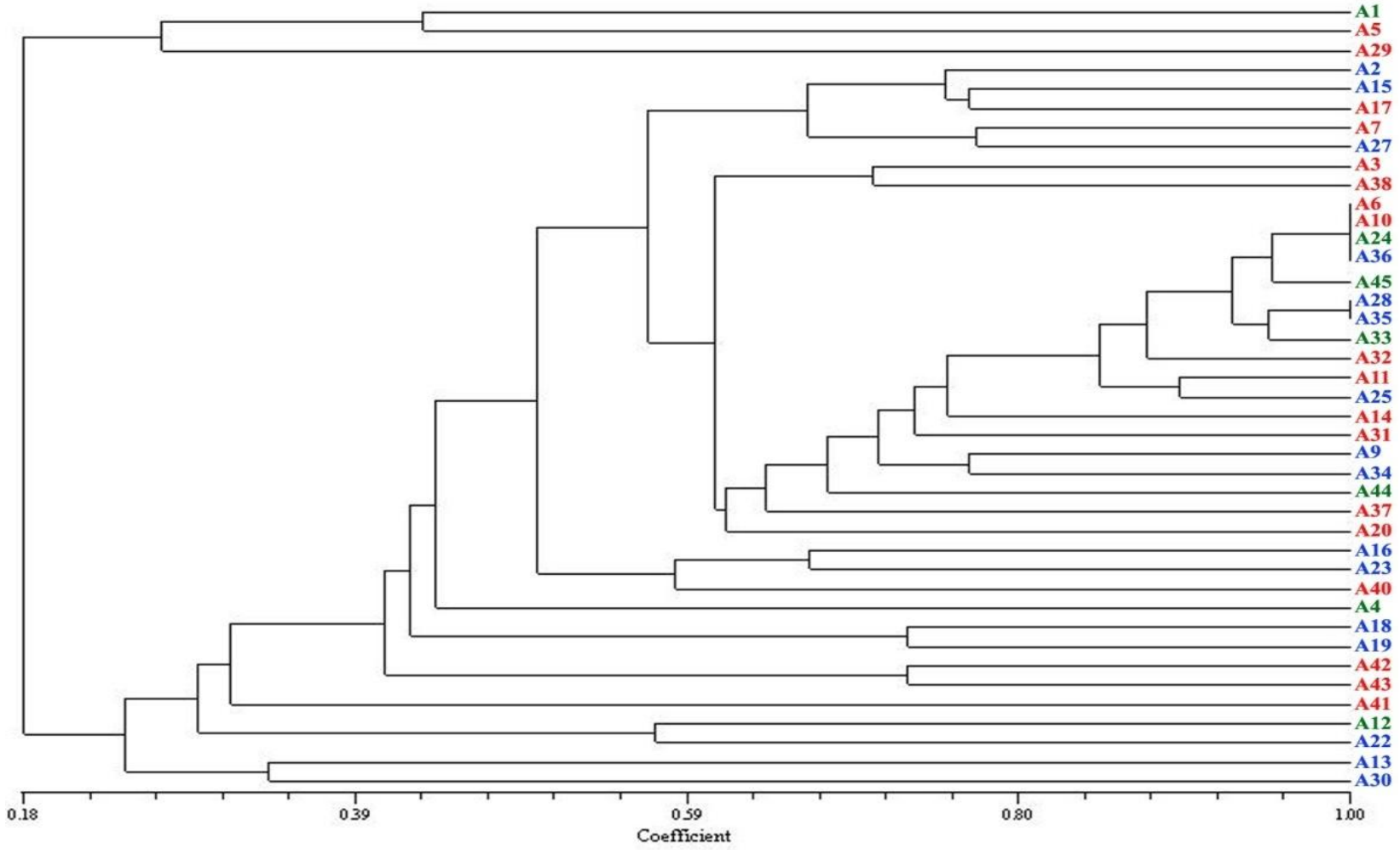
Climatic region	No. of isolates	Shannon and Weaver diversity index (H)	Evenness (E) of the species
Arid	18	2.81	0.993
Semi-arid	16	2.81	0.993
Humid	7	1.94	1.000
All	41	3.54	0.982

Fig.1 Carbohydrates utilization by Actinobacterial isolates



Ad: adonitol, Ar: arabinose, Ce: cellobiose, De: dextrose, Du: dulcitol, Fc: fructose, Ga: galactose, Is: inositol, In: inulin, La: lactose, Ma: maltose, Mn: mannitol, Mo: mannose, Mb: melibiose, Rf: raffinose, Rh: rhamnose, Sa: salicin, Sb sorbitol, Su: sucrose, Te:trehalose, Xy: xylose

Fig.2 Dendrogram of Actinobacterial isolates (41) based on carbohydrate utilization (% similarity)



A1: *Streptomyces*, A5: *Streptomyces*, A29: *Nocardia*, A2: *Streptomyces*, A15: *Streptomyces*, A17: *Streptomyces*, A7: *Streptomyces*, A27: *Streptomyces*, A3: *Streptomyces*, A38: *Streptomyces*, A6: *Streptomyces*, A10: *Streptomyces*, A24: *Nocardia*, A36: *Streptomyces*, A45: *Streptomyces*, A28: *Nocardia*, A35; *Nocardia*, A33: *Nocardia*, A32: *Streptomyces*, A11: *Streptomyces*, A25: *Nocardia*, A14: *Streptomyces*, A31: *Nocardia*, A9: *Streptomyces*, A34: *Nocardia*, A44: *Streptomyces*, A37: *Nocardia*, A20: *Micromonospora*, A16: *Streptomyces*, A23: *Nocardia*, A40: *Streptomyces*, A4: *Saccharopolyspora*, A18: *Streptomyces*, A19: *Streptomyces*, A42: *Streptomyces*, A43: *Nocardia*, A41: *Saccharopolyspora*, A12: *Streptomyces*, A22: *Nocardia*, A13: *Streptomyces*, A30: *Streptomyces*

Carbohydrates utilization

Actinobacteria are heterotrophs and utilize a variety of carbon sources ranging from simple (organic acids and sugars) to highly complex molecules (polysaccharides, lipids, proteins and aliphatic hydrocarbons). Cellulose is decomposed by many Actinobacterial species. It is also reported that many strains are capable of degrading starch, inulin and chitin (Alexander 1961). Chitin hydrolysis is especially characteristic of Actinobacteria. The metabolism of unusual organic molecules such as paraffins, phenols, steroids and pyrimidines is well documented for *Nocardia* spp. whereas *Micromonospora strains* decompose chitin, cellulose, glucosides, pentosans and possibly lignin.

In this study all the 21 carbon sources were utilized by one or the other Actinobacterial isolates. The highest number of isolates utilized monosaccharides followed by disaccharides, trisaccharides and polysaccharides. The best carbon source was found to be dextrose which was utilized by maximum number of isolates (90%) followed by mannose (80%) and arabinose (75%). The least used carbon sources were inositol and inulin (49%) (Fig. 1). These results indicate that Actinobacteria are highly non-specific, in their carbon requirements. Strzelczyk and Pokojska-Burdziej (1984) also reported the non-specificity of Actinobacteria for carbon requirements.

Shannon diversity index and cluster analysis

Bsed on the utilization of 21 carbon sources the Shannon-Weaver diversity index (H) of the Actinobacteria in arid and semi-arid soils is 2.81 and the species evenness is 0.993. In humid soil, the diversity is lesser (1.94) but species are more evenly distributed (1.000) (Table 3).

The dendrogram of Actinobacterial isolates showed that they were quite diverse in their utilization of carbohydrates. All the Actinobacterial isolates had only 18 per cent level of similarity. The isolates were thus very diverse and had lot of differences in carbon utilization (Diversity index H=3.54). The high extent of diversity was also apparent from the high number of clusters (6 clusters at 33 per cent, 11 clusters at 50 per cent and 18 clusters at 66% level of similarity, Fig. 2). There was one mega cluster (cluster 6 with 20 isolates) which had most of the isolates having representation of all the three regions. Cluster 7 had 5 isolates belonging to arid and semi-arid regions. All other clusters had 2 or 3 isolates. The data showed that isolates from arid and semi-arid regions were more diverse (H=2.81) compared to humid region (H= 1.94). Higher diversity in arid and semi-arid regions can be attributed to soil diversity, host crop and the ecological niche (drought stress and low substrate availability) which resulted in greater speciation (Odum *et al.,* 1971). The higher acidity of forest soils (pH ~ 6.0) probably contributed to the lower diversity compared to arid and semi-arid soils (pH ranged from 7.0-9.1). Thus the results from our study confirm that both arid and semi-arid regions represent extremely rich reservoir for the isolation of a significant diversity of Actinobacteria.

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