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Phylogenetic Analysis of Tolerant Bacteria from *Parthenium hysterophorus* (L.) Amended Soil by Bootstrap Approach

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Abstract: Experiments were done to construct phylogenetic tree of tolerant bacteria of *Parthenium hysterophorus* amended soil and to analys its phylogeny on the basis of bootstrap value of evolutionary tree. Different proportion (5, 10, 15 and 20%) of shed dried powdered *Parthenium* (whole plant) was mixed with the soil collected from agroecosystem and bacterial population was analyzed over a period of 45 days at an interval of 15 days, using dilution plate method. Results obtained on bacterial population have been compared with those of control. The population ranged from $44.6\pm1.9798\times10^9$ to $53.10\pm1.2727\times10^9$ in control showing increasing trend while in *Parthenium* amended soil it decreased significantly corresponding to the increasing concentration of *Parthenium* at different intervals. Morphological details of different bacterial colonies showed increase in population of irregular – undulate colony, while other showed decrease in amended soil. Genomic analysis and phylogenetic tree of this bacterial colony revealed that it is in close association with EF031071.1 and have bootstrap value 99 which implies that sample analyzed is *Bacillus sp.* BFF - 3. Dendrogram of analyzed sample have 11 closely related strains of which 8 are distributed in 4 distinct groups (I, II, III and IV) and three are remained ungrouped.

Key words: *Bacillus* sp. BFF-3 · PCR · BLAST · Probes

INTRODUCTION

Soil rich in minerals and regenerative microorganisms forms a dynamic living system that works in concert with plants and the atmosphere to create the web of life in which humans are also a strand. The microbial flora and fauna of soil is an important constituent since soil fertility, plant growth performance and ultimately agriculture productivity depends on it [1, 2]. The microbial population of soil is made up of five major groups including bacteria, actinomycetes, fungi, algae and protozoa. Among these, bacteria comprise the most abundant and important group for decomposition of waste [3]. Bacteria use waste for their own metabolism and finally they produce some simple and useful compounds which are important for soil health, agriculture and overall to balance the natural ecosystem. Soil microbes also secrete some acid which is an important factor in converting the waste to compost [4]. However the microbial population, microbial biomass and their activities in soil may fluctuates due to different soil management practices [5, 6], amendment of soil by weeds [7, 8], green manure and rice straw [9-12], sewage sludge and application of chemical fertilizers and pesticides etc.

Soil community analysis has been limited in the past because only a minor proportion of the microbial population is cultivable and very little information is available about changes in the composition of soil microbial communities [13-15]. Recent applications of molecular biology have provided tools to determine microbial presence and diversity in the environment [16, 17]. A number of molecular genetics techniques, such as total DNA isolation and characterization, G+C composition, rRNA sequence, PCR amplification of rDNA, PCR amplification of functional genes and *in situ* hybridization of rRNA oligonucleotide probes are being used to study microbial communities [18]. Presently, the rRNA genes in DNA taken directly from soil can be amplified using PCR, the products cloned and the nucleotide sequence determined. A number of researches have begun by using these techniques, to examine the biodiversity of soil microbial communities [19-21] but no

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work has been done to determine the phylogenetic position of bacteria from *Parthenium hysterophorus* amended soil.

In this experiment, 16S rDNA was used and amplified by PCR to construct phylogenetic tree of tolerant bacteria of *Parthenium hysterophorus* amended soil and phylogenetic analysis was done on the basis of bootstrap value of evolutionary tree.

MATERIALS AND METHODS

Soil Sampling and Experimental Setup: Soil was collected with the help of sterilized equipment from a depth of 10-15cm from the agro ecosystem around Department of Zoology, Ranchi University, Ranchi, India. The characteristics of soil analyzed are given in table 1.

In the experiment, powder of *Parthenium hysterophorus* which was prepared by mechanical chopping of whole plant followed by shade drying and grinding was mixed in soil collected from agro ecosystem in the concentration of 5, 10, 15 and 20%. Whole setup was prepared in plastic container and was kept in moist condition. One container without *Parthenium hysterophorus* was kept as control. Bacterial colonies appearing in *Parthenium* containing soil at every interval were taken as *Parthenium* tolerant forms.

Soil Bacteria Culture: Bacterial culture was done from *Parthenium hysterophorus* amended and control soil, over a period of 45 days at an interval of 15 days using dilution plate method [22, 23]. 1 mL inoculums of the primary suspension were taken & Czapek Dox agar media (peptone -10g / L, NaCl-5g / L, beef extract- 10g / L, agar-15g / L, pH-7) was used for culture. The petriplates (diameter 100mm) were incubated at 37°C for 48 h. Colonies so cultured were isolated and retained for subsequent screening like pure culture, gram staining and genomic analysis, on the basis of which their phylogenetic tree was constructed.

Genomic Analysis: For genomic analysis, DNA was isolated from the pure culture of *Parthenium hysterophorus* tolerant bacterial colony. Its quality was evaluated on 1.2% Agarose Gel, a single band of high-molecular weight DNA has been observed. Fragment of 16S rDNA gene was amplified by PCR from the above isolated DNA. The PCR amplicon was purified to remove contaminants. Forward and reverse DNA sequencing reaction of PCR amplicon was carried out with 8F and 1492R primers using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer. Reverse sequence of 989 bp of 16S rDNA gene was used to carry out BLAST with the nrdatabase of NCBI gene bank database [24, 25]. Based on maximum identity score first ten sequences were selected and aligned using multiple alignment software program and the phylogenetic tree was constructed using molecular evolutionary genetics analysis software version 4.0 (MEGA 4) [26]. The bootstrap consensus tree inferred from 500 replicates [27] was taken to represent the evolutionary history of the taxa analysed which was used to analyze its phylogeny.

Table 1: Edaphic profile of experimental soil

Characteristics	Value (M SD)
pH	6.37±0.21
Organic Carbon(mg / g soil)	8.31±1.92
Nitrogen(mg / g soil)	0.58±0.11
Phosphorous(Kg / hec.)	31.93±2.97
Potassium(Kg / hec.)	158.4±8.57

RESULTS

Qualitative Analysis of Bacterial Colony: It was done by studying the colony morphology. The developed bacterial colonies on the nutrient agar plates with respect to their shape and margin were of 4 types *i.e*. circular-entire, irregular-undulate, punctiform and filamentous (Table 2). In control, 50% of the colonies were circular-entire, 46% were punctiform and 4% of the colonies were irregular-undulate. Colony with filamentous shape and margin was absent in control. The elevation of circular-entire colonies were either flat (20%), raised (75%) or convex (5%) and with white (40%) or green (60%) colour. The elevation of punctiform colonies were only flat, with green (50%) and white (50%) colour. While the elevation and colour of irregular-undulate colonies was only flat and white respectively. In 5% *Parthenium* amended soil, the developed colonies were circular-entire (26%), punctiform (64%), irregular-undulate (8%) and filamentous (2%). The elevation and colour of all the four colonies were same as control except filamentous colony. The filamentous colony had only flat elevation and white colour. Morphological details of bacterial colonies obtained in 5, 10, 15 and 20% *Parthenium hysterophorus* amended soil (Table 2) showed that the population of different colonies obtained in these samples was either less than the control or they showed little variation. Only colony with irregular shape and undulate margin showed a significant increase from 4% (control) to 20% as concentration of *Parthenium hysterophorus* in soil increased. The bacteria constituting irregular-undulate colonies were observed to be bacilli and their response to gram's stain was positive (Fig. 1).

Table 2: Morphological details of bacterial colonies in culture condition

% of *Parthenium*	Shape	Margin	Elevation	Colour
Control	46% punctiform	46% entire	100% flat	50% green
				50% white
	50% circular	50% entire	20% flat	40% white
			75% raised	60% green
			5% convex	
	4% irregular	4% undulate	100% flat	100% white
5% *Parthenium*	64% punctiform	64% entire	100% flat	50% green
				50% white
	26% circular	26% entire	20% flat	40% white
			75% raised	60% green
			5% convex	
	8% irregular	8% undulate	100% flat	100% white
	2% filamentous		100% flat	100% white
10% *Parthenium*	45% punctiform	45% entire	100% flat	50% green
				50% white
	41% circular	41% entire	20% flat	40% white
			75% raised	60% green
			5% convex	
	12% irregular	12% undulate	100% flat	100% white
	2% filamentous		100% flat	100% white
15% *Parthenium*	36% punctiform	36% entire	100% flat	50% green
				50% white
	47% circular	47% entire	20% flat	40% white
			75% raised	60% green
			5% convex	
	15% irregular	15% undulate	100% flat	100% white
	2% filamentous		100% flat	100% white
20% *Parthenium*	25% punctiform	25% entire	100% flat	50% green
				50% white
	52% circular	52% entire	20% flat	40% white
			75% raised	60% green
			5% convex	
	20% irregular	20% undulate	100% flat	100% white
	3% filamentous		100% flat	100% white

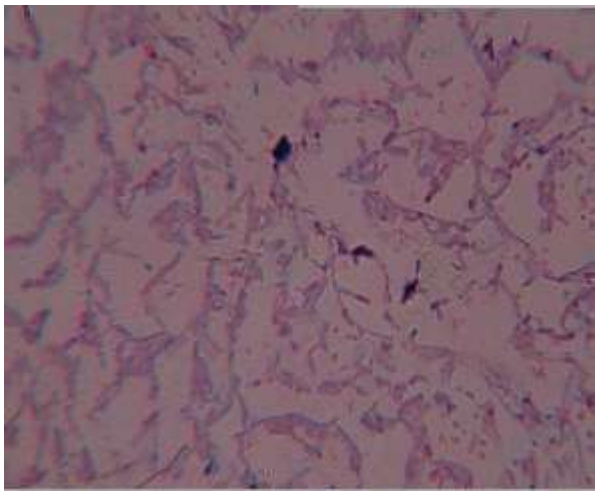

Fig. 1: Gram staining of *Bacillus sp*. BFF-3

Quantitative Analysis of Bacterial Colony: The bacterial colonies were enumerated and represented as number of colony forming units (cfu) per g of the soil sample (Fig. 2). The bacterial population data were further subjected to 2 – way analysis of variance. In control, the bacterial population was $44.6\pm1.9798 \times 10^9$, which significantly decreased to $33.4\pm1.5556 \times 10^9$, $28.0\pm0.8485 \times 10^9$, $21.2\pm0.4242 \times 10^9$ and $9.85\pm0.4949 \times 10^9$ respectively in 5, 10, 15 and 20% of *Parthenium hysterophorus* amended soil on 15th day of experiment. On 30th day, the bacterial population observed was $49.0\pm3.2526 \times 10^9$, $39.6\pm3.6769 \times 10^9$ and $32.40\pm3.5355 \times 10^9$, $25.60\pm1.6971 \times 10^9$ and $17.50\pm0.9899 \times 10^9$ in control, 5, 10, 15 and 20% of *Parthenium* amended soil respectively. On 45th day, a similar trend of decrease in bacterial population was observed viz. $53.10\pm1.2727 \times 10^9$ (control), $37.3\pm1.8384 \times 10^9$ (5%),

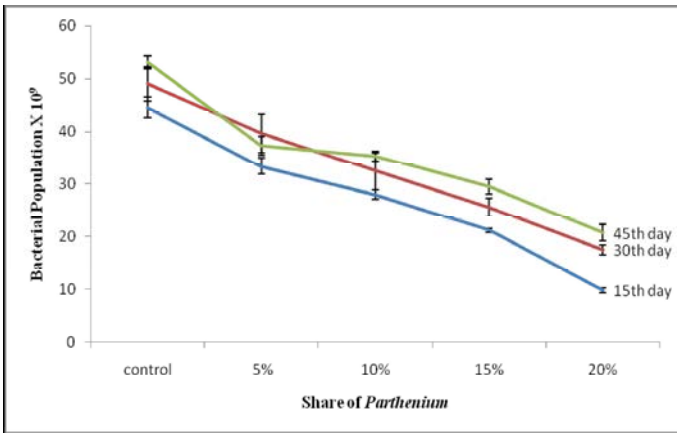

Fig. 2: Bacterial population (values are per g of soil sample

Table 3: Analysis of variance (Two way)

Variation	SS	df	MS	F	Significance
Between conc.	1820.20733	4	455.05183	175.2671	$p < 0.001$
Between days	158.684333	2	79.342167	30.55931	$p < 0.001$
Error	20.7706667	8	2.5963333		

Table 4: Close homologs of *Bacillus sp.* BFF - 3

Accession	Description	Max. score	Total score	Query coverage	E value	Max. ident.
EF031071.1	*Bacillus sp*. BFF-3	1827	1827	100%	0.0	100%
HM003212.1	*Bacillus cereus* strain WYLW1-7	1784	1784	99%	0.0	99%
FJ644692.1	*Bacillus cereus* strain MX3	1784	1784	99%	0.0	99%
FJ263046.1	*Bacillus cereus* strain ZD19	1784	1784	99%	0.0	99%
HM003208.1	*Bacillus cereus* strain WYLW1-1	1783	1783	99%	0.0	99%
FJ959367.1	*Bacillus subtilis* strain 0-2	1783	1783	99%	0.0	99%
GU085229.1	*Bacillus sp.* BD-31	1783	1783	99%	0.0	99%
FJ763650.1	*Bacillus cereus* strain S72	1783	1783	99%	0.0	99%
EU857430.1	*Bacillus cereus* strain B1	1783	1783	99%	0.0	99%
GQ199727.1	*Bacillus sp*. 210_25	1783	1783	99%	0.0	99%

Max. score = maximum score; E value = expected value; Max. ident. = Maximum identification

35.30±0.9899 $\times 10^9$ (10%), 29.5±1.4142 $\times 10^9$ (15%), and 20.70±1.5556 $\times 10^9$ (20% *Parthenium* amended soil). 2 - way analysis of variance revealed that bacterial population has been significantly affected by both the concentration and duration of amendment (treatment) of soil by *Parthenium hysterophorus* (Table 3).

Phylogenetic Analysis of *Parthenium* Tolerant Bacterial Colony: Genomic analysis of *Parthenium hysterophorus* resistant irregular – undulate bacterial colony was done. The sample was found to be *Bacillus sp.* BFF-3 (Gene bank accession No.- EF031071.1) based on nucleotide homology and phylogenetic analysis. PCR of fragment of 16s rDNA gene from the isolated DNA of bacterium shows amplicon band of 1500bp when resolved on agarose gel. (Fig. 3). Other close homologs

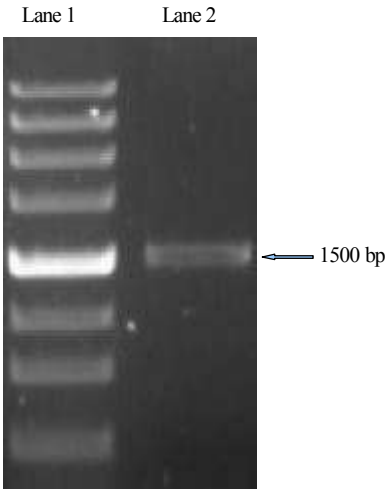

Fig. 3: Gel image of 16S rDNA amplicon

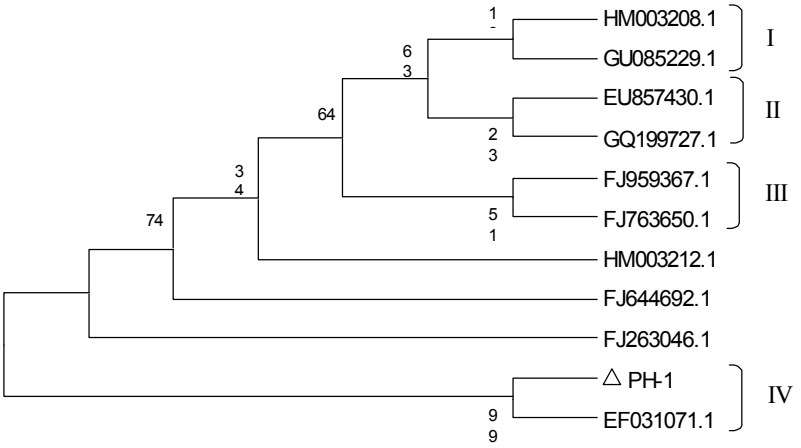

Fig. 4: Phylogenetic tree showing position of Δ PH – 1 (*Bacillus sp.* BFF-3)

for *Bacillus sp.* BFF-3 based on BLAST data are represented in Table 4 and its phylogenetic tree is shown in Fig.-2. It was observed that *Bacillus sp.* BFF-3 is closely related to *Bacillus cereus* strain WYLW1-7, MX3 and ZD19 whose maximum score is 1784 which is equal to their total score. They all show 99% sequence similarity in query coverage of amino acids. Other close homologs of *Bacillus sp.* BFF-3 also scored maximum score equals to total score. The expected (E) value of all these *Bacillus* strains are 0 and it depicts that all the 10 close homologs of *Bacillus* strain are homogenous in comparison to *Bacillus sp.* BFF-3.

Phylogenetic tree shows that sample (Δ PH-1) is in close association with EF031071.1 which has bootstrap value of 99 (at node), and it implies that sample analysed is *Bacillus sp.* BFF-3 (Fig. 4). This monophyletic group of sample and *Bacillus sp.* BFF-3 further show close relation with FJ644692.1 (*Bacillus cereus* strain MX3) and have bootstrap value 74. This entire group has bootstrap value 34 with HM003212.1 (*Bacillus cereus* strain WYLW1-7). Again the value for HM003208.1, GU085229.1 group, EU857430.1, GQ199727.1 group and FJ959367.1, FJ763650.1 monophyletic group is 18, 23 and 51 respectively. All these strains together have bootstrap value 64 and are also other descendants of ancestor of *Bacillus sp.* BFF-3 strain but they are distant members of Δ PH-1 and *Bacillus sp.* BFF-3 (EF031071.1) monophyletic group.

DISCUSSION

Parthenium hysterophorus L. is an obnoxious weed and is considered to be one of the worst weed for agriculture, environment and human health in the world. Quantitative analysis revealed that bacterial population decreases significantly as concentration of *Parthenium* in soil increases on 15th, 30th, and 45th day of experiment (Fig. 2). This depreciation could be because of the difficulty in survival and multiplication of bacteria in soil containing decomposed material of weed or accumulation of phenolic compounds and toxins of plant viz. sesquiterpene lactone (parthenin), caffic acid and ansic acid etc. Batish *et al*. [28] explained such significant alterations due to problem in the availability of major as well as micronutrients and also amounts of phenolics from *Parthenium* amended soils. It is also observed that phenolic compounds may alter the accumulation and availability of nutrients in soil [29, 30]. Ahmed and Ahmad [31] found that insecticide, chlorpyrifos causes significant reduction in number of soil bacteria. Jeyalakshmi and Valluvaparidasan [32] also worked to assess the distribution of soil microorganisms in the *Parthenium hysterophorus* infested soils of Tamil Nadu and revealed that a total of 13 fungi were isolated with different per cent distribution.

The sample identified was *Bacillus sp*. BFF-3 strain, is an aerobic, endospore forming and mobile gram positive rod shaped bacteria. Felsenstein [27] proposed that bootstrap value of 95% or greater be considered statistically significant and indicate support for a clade; alternative nodes can be rejected if they occur in less than 5% of the bootstrap estimates. Hillis and Bull [33] also stated that bootstrap values of 50% or more may be overestimates of accuracy. Here in this experiment, bootstrap value between sample (ΔPH-1) and EF031071.1 (*Bacillus sp.* BFF-3) is 99 and it implies that both are sister species and thus a clade (monophyletic group). In the dendrogram shown here 11 strains are present of which 8 strains are distributed in 4 distinct groups (I, II, III and IV)

and three are remained ungrouped (Fig. 4). All the ungrouped strains of *Bacillus sp.* BFF-3 monophyletic group are members of the *Bacillus cereus* (Bc) group and 50% of the grouped members are also of Bc group. However, all the strains are members of genus *Bacillus* and family Bacillaceae. *Bacillus cereus* is an opportunistic pathogen causing food poisoning manifested by diarrhoeal or emetic syndromes [34]. It is closely related to the animal and human pathogen *Bacillus anthracis* and *Bacillus thuringiensis*. Xu and Cote [35] stated that *Bacillus anthracis*, *Bacillus cereus*, *Bacillus mycoides* and *Bacillus thuringiensis* belongs to the same group from 40 Bacillaceae studied species. The phenotypic and genotypic similarities between all four species have been well documented [36 -38]. Recently Helagson *et al*. [39] proposed to regroup *B. anthracis, B. cereus* and *B. thuringiensis* in a single species on the basis of genetic evidence. Since the genus *Bacillus* was first described [40], the number of *Bacillus* species has fluctuated widely. Rossler *et al*. [41] grouped nine *Bacillus* species into four clusters. In particular, 16s rRNA gene sequence analysis by Ash *et al*. [38] revealed five phylogenetically distinct clusters of species and three ungrouped species from 51 *Bacillus* sp. Studied. Many *Bacillus* species that belonged to these phylogenetic groups have been reclassified as members of novel genera or have been transferred to other genera [42, 43]. Despite the reduction in the number of species in the genus *Bacillus*, the genus is considered as one of the largest genera and additional *Bacillus* species from diverse habitats have been described recently [44, 45, 46].

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REFERENCES

1. Ayanaba, A., S. B. Tuckwell and D.S. Jenkinson, 1976. The effects of clearing and cropping on organic reserves and biomass of tropical forest soils. Soil Biol. Biochem., 8: 519-526.
2. Parthasarathi, K., 2006. Aging of pressmund vermicats of *Limpito mauritii* (Kinberg) and *Eudrilus eugeniae* (Kinberg) - Reduction in microbiol population and activity. J. Environ. Biol., 27: 221-223.
3. Alexander, M., 1961. Introduction to Soil Microbiology. John Wiley and Sons, Inc, New York.
4. Speir, T.W. and D.J. Ross, 1978. Soil phosphatase and sulphatase. In: R.G. Burns (ed.), Soil Enzymes. Academic Press, New York, NY. pp: 197-250.
5. Doran, J.W., 1980. Soil microbial and biochemical changes associated with reduced tillage. Soil Sci. Soc. Am., 44: 765-771.
6. Hendrix, P.F., R.W. Parmelee, Jr. D.A. Crossley, D.C. Coleman, E.P. Odum and P.M. Groffman, 1986. Detritus food webs in conventional and no tillage agro systems. Bioscience, 36: 374-379.
7. Miller, R.H., 1973. Recycling municipal sludge and effluents of land. Nat. Assoc. St. Univ. and Land Grand coll. Washington DC, pp: 79-89.
8. Kulkarni, C.Y. and B. Pushpa, 1993. Inhibition of microbial inoculants by Luffa tuberosa under *in vitro* and *in vivo*. Ind. J. Microbiol., 33: 215-217.
9. Tillak, K.V.B.R., 1986. Interaction of vesicular arbiscular mycorrhizae and nitrogen fixer. Proc. Soil Biol. Symp. Hissar, pp: 219-226.
10. Mukharjee, D., S.K. Ghosh and A.C. Das, 1990. A study on chemical and microbiological changes during the decomposition of straw in soil. J. Indian Agriculturist, 34: 1-10.
11. Harden, T., R.G., Joerjenson, B. Meyer and V. Wolters, 1993. Mineralisation of straw and formation of soil microbial biomass in a soil treated with simazine and dinoterb. Soil Biol. Biochem., 25: 1273-1276.
12. Saha, N., A.C. Das and D. Mukharjee, 1995. Influence of organic wastes incorporation on soil microflora. J. Indian Soc. Soil. Sci., 43: 210.
13. Parkinson, D. and D.C. Coleman, 1991. Microbial communities, activity and biomass. Agric. Ecosyst. Environ., 34: 3-33.
14. Wardle, D.A., 1992. A comparative assessment of factors which influence microbial biomass carbin and nitrogen levels in soil. Biol. Rev., 67: 321-358.
15. Hopkins, D.W. and R.S. Shiel, 1996. Size and activity of soil microbial communities in long-term experimental grassland plots treated with manure and inorganic fertilizers. Biol. Fert. Soils, 22: 66-70.
16. Atlas, R.M., G. Sayler, R.S. Burlage and A.K. Bej, 1992. Molecular approaches for environmental monitoring of microorganism. Biotechniques, 12: 706.
17. Head, I.M., J.R. Saunders and R.W. Pickup, 1998. Microbial evolution, diversity and ecology- A decade of ribosomal RNA analysis of uncultivated microorganisms. Microb. Ecol., 35: 1-21.
18. Akkermans, A., J.D. Van Elsas and F.J. Brujin (Ed.), 1995. Molecular microbial ecology manual. Kluwer Academic Publ., Nowell, MA.

19. Ueda, T., Y. Suga and T. Matsuguchi, 1995. Molecular phylogenetic analysis of a soil microbial community in a soybean field. Eur. J. soil Sci., 46: 415-421.
20. Borneman, J. and E.W. Triplett, 1997. Molecular microbial diversity in soils from eastern Amazonia- evidence for unusual microorganisms and microbial population shifts associated with deforestation. Appl. Environ. Microbiol., 63: 2647-2653.
21. Jurgens, G., K. Lindstrom and A. Saano, 1997. Novel group within the kingdom Crenarchaeota from boreal forest soil. Appl. Environ. Microbial., 63: 803-805.
22. Thornton, H.G., 1922. On the development of standardized agar medium for counting soil bacteria with special regard to the repression of spreading colonies. Ann. Applied Biol., 2: 241-274.
23. Thom, C. and K.B. Raper, 1945. Manual of the Aspergili. Williams and Wilkins Co., Baltimore, USA.
24. Marchler–Bauer, A., A.R. Panchenko, B.A. Shoemaker, P.A. Thiessen, L.Y. Geer and S.H. Bryant, 2000. CDD: A database of conserved domain alignments with links to domain three dimensional structure. Nucleic Acids Res., 30: 281-283.
25. Pruitt, K.D., T. Tatusova and D.R. Maglott, 2005. NCBI reference sequence: a cultured non–redundant sequence database of genomes, transcripts, and proteins. Nucleic Acids Res., 33: D501-D504.
26. Tamura, K., J. Dudley, M. Nei and S. Kumar, 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Molecular Biology and Evolution, 24: 1596-1599.
27. Felsenstein, J., 1985. Confidence limits on phylogenies: An approach using the bootstrap. Evolution., 39: 783-791.
28. Batish, D.R., H.P. Singh, J.K. Pandher, V. Arora and R.K. Kohli, 2002. Phytotoxic effect of *Parthenium* residues on the selected soil properties and growth of chickpea and radish. Weed Biology and Management, 2: 73-78.
29. Facelli, J.M. and S.T.A. Pickett, 1991. Plant litter: its dynamics and effects on plant community structure. Botanical Reviews, 57: 1-32.
30. Appel, H.M., 1993. Phenolics in ecological interactions: the importance of oxidation. J. Chemical Ecol., 19: 1521-1552.
31. Ahmed, S., and M.S. Ahmad, 2006. Effect of Insecticides on the number of soil bacteria under laboratory and field conditions. Pak. Entomol., 28(2): 63-67.
32. Jeyalakshmi, C. and V. Valluvaparidasan, 2010. Distribution of microorganisms in the *Parthenium* weed infested soil of Tamil Nadu. J. Biopesticides, 3(3): 523-525.
33. Hillis, D.M. and J. J. Bull, 1993. An empirical test of bootstrapping as a method for assessing confidence in phylogenetic analysis. Systematic Biol., 42: 182-192.
34. Ivanova, N., A. Sorokin, I. Anderson and N. Kyrpides, 2003.Genome sequence of *Bacillus cereus* and comparative analysis with *Bacillus anthracis*. Nature, 423: 87-91.
35. Xu, D. and J.C. Cote, 2003. Phylogenetic relationships between *Bacillus* species and related genera inferred from comparison of 39 end 16S rDNA and 59 end16S–23S ITS nucleotide sequences. International J. Systematic and Evolutionary Microbiol., 53: 695-704.
36. Logan, N.A. and R.C.W. Berkeley, 1984. Identification of *Bacillus* strains using the API system. J. Gen Microbiol., 130: 1871-1882.
37. Claus, D. and R.C.W. Berkeley, 1986. Genus *Bacillus* Cohn 1872, 174AL. In Bergey's Manual of Systematic Bacteriology, 2: 1105-1139..
38. Ash, C., A.E. Farrow, S. Wallbanks and M.D. Collins, 1991. Phylogenetic heterogeneity of the genus *Bacillus* revealed by comparative analysis of small-subunit-ribosomal RNA sequences. Lett. Appl. Microbiol., 13: 202-206.
39. Helgason, E., O.A. Okstad, D.A. Caugant, H.A. Johansen, A. Fouet, M. Mock, I. Hegna and A. B. Kolsto 2000. *Bacillus anthracis, Bacillus cereus, and Bacillus thuringiensis* – one species on the basis of genetic evidence. Appl. Environ. Microbiol., 66: 2627-2630.
40. Cohn, F., 1872. Untersuchungen über Bakterien. Beitrage zur Biologie der Pflanzen, 1875 1 (Heft 2) 1: 127-224.
41. Rossler, D., W. Ludwig, K.H. Schleifer, C. Lin, T.J. McGill, J.D. Wisotzkey, P. Jurtshuk and G.E. Fox, 1991. Phylogenetic diversity in the genus *Bacillus* as seen by 16S rRNA sequencing studies. Syst. Appl. Microbiol., 14: 266-269.
42. Shida, O., H. Takagi, K. Kadowaki, L.K. Nakamura and K. Komagata, 1997. Transfer of *Bacillus alginolyticus*, *Bacillus chondroitinus*, *Bacillus curdlanolyticus*, *Bacillus glucanolyticus*, *Bacillus kobensis*, and *Bacillus thiaminolyticus* to the genus *Paenibacillus*and emended description of the genus Paenibacillus. Int. J. Syst. Bacteriol., 47: 289-298.

43. Jeon, C.O., J.M. Lim, J.M. Lee, L.H. Xu, C.L. Jiang, and C.J. Kim, 2005. Reclassification of *Bacillus haloalkaliphilus* Fritze 1996 as *Alkalibacillus haloalkaliphilus* gen. nov., comb. nov. and the description of *Alkalibacillus salilacus* sp. nov., a novel halophilic bacterium isolated from a salt lake in China. Int. J. Syst. Evol. Microbiol., 55: 1891-1896.
44. Heyrman, J., M. Rodríguez-Díaz, J. Devos, A. Felske, N.A. Logan and P. De Vos, 2005a. *Bacillus arenosi* sp. nov., *Bacillus arvi* sp. nov. and *Bacillus humi* sp. nov., isolated from soil. Int. J. Syst. Evol. Microbiol., 55: 111-117.
45. Shivaji, S., P. Chaturvedi, K. Suresh, G.S. Reddy, C.B. Dutt, M. Wainwright, J.V. Narlikar and P.M. Bhargava, 2006. *Bacillus aerius* sp. nov., *Bacillus aerophilus* sp. nov.,*Bacillus stratosphericus* sp. nov. and *Bacillus altitudinis* sp. nov., isolated from cryogenic tubes used for collecting air samples from high altitudes. Int. J. Syst. Evol. Microbiol., 56: 1465-1473.
46. Ko, K.S., W.S. Oh, M.Y. Lee, J.H. Lee, H. Lee, K.R. Peck, N.Y. Lee and J.H. Song, 2006. *Bacillus infantis* sp. nov. and *Bacillus idriensis* sp. nov., isolated from a patient with neonatal sepsis. Int. J. Syst. Evol. Microbiol., 56: 2541-2544.