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ISOLATION AND CHARACTERIZATION OF PHOSPHORUS SOLUBILIZING MICRO ORGANISMS FROM CARDAMOM RHIZOSPHERE

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Abstract: Cardamom which grow in acidic soils, their rhizosphere are potential habitats where diversity of elite bacteria with phosphorus solubilization remains less explored. Hence, the present investigation was focused to identify and characterize the phosphorus solubilizing bacteria associated with cardamom rhizosphere. The highest solubilization efficiency of 156 ± 4.50 per cent was observed in the isolate Pd 10 which was identified as *Bacillus subtilis.* The presence of gluconic acid responsible for mineral solubilization was also determined by HPLC.
Keywords: Cardamom, phosphorus solubilzation, gluconic acid.

Introduction

Cardamom, *Elettaria cardamomum* (*L.*) Maton, popularly known as 'Queen of spices' is the tall perennial herbaceous plant, belonging to the family Zingiberaceae. India is second compared to Guatemala in world production of cardamom, which is grown in approximately 72,000 ha with an annual production of more than 8,000 tonnes. In 2014-15, as per provisional estimates, of Spices Board of India's production of cardamom is around 18,000 million tonnes with highest percentage of production in Keral (89 per cent).

The bacterial species, mostly associated with the plant rhizosphere, exerts beneficial effect upon plant growth. Therefore, their use as biofertilizers or control agents for improvement in agriculture has been a focus of numerous researchers over the years. Phosphorus (P) is major essential macronutrients for biological growth and development of plants and is applied to soil in the form of phosphatic fertilizers. Most agricultural soils contain large reserves of phosphorus, most of which are accumulated due to regular applications of P fertilizers [9]. A large proportion of the soluble inorganic phosphate fertilizers added to the soil are converted to insoluble form and become unavailable to plants. The cardamom growing soil is acidic and rich in iron and alumina that reacts with the labile phosphorus and renders them unavailable. Despite a large organic matter content in soil and a cover of plant debris that undergo

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continuous decomposition and mineralization, nutrients become limiting to the crop. Integrated nutrient management strategies involving organic farming technologies have been strengthened in cardamom for achieving sustainable production. Nutrient solubilization represents a very interesting feature in plant-associated bacteria. The microbial inoculation that sustain in soil and persist in root region of plants can largely benefit the crop by solubilisation of nutrients like phosphorus, zinc and silicon which contribute significantly to the crop productivity. Once solubilized, such compounds may also become available for absorption by plants [6]. The production of inorganic acids such as gluconic acid, sulphuric acid, nitric acid and carbonic acid seems to be the most frequent agent of mineral solubilization [10]. In the present study an attempt was made to isolate bacteria associated with cardamom rhizosphere and to screen the isolates for phosphorus solubilization. Attempt was also made to identify the best isolate by molecular studies and the type of organic acid secreted by the phosphorus solubilizing organisms.

Materials and methods

Enumeration and isolation of soil microflora in rhizosphere soil samples of cardamom

The rhizosphere soil and roots of cardamom were collected from six different cardamom growing areas like Cumbum mettu, Thadiyankudisai of Tamil Nadu and Kumily, Pampadumpara (Idukki), Mayiladumpara (Idukki) and Wayanad of Kerala state. The population of bacteria were enumerated using serial dilution plate technique on nutrient agar medium [8].

Testing the bacterial isolates for P-solubilization

Plate assay

The bacterial cultures were inoculated in to hydroxy apetite medium [14]. The test organisms were inoculated on these media and incubated at 30°C for 48 h. The diameter of the clearing zones around the colonies were measured. The solubilizing efficiency was calculated as indicated below [15].

$$\text{Solubilization efficiency} = \frac{\text{Diameter of solubilization zone} - \text{colony diameter}}{\text{Colony diameter}} \times 100$$

Broth assay

One ml culture of the test organism containing 2×10^9 cell ml^{-1} was inoculated into the flasks of pikovaskaya's broth containing 100 mg of tricalcium phosphate. An uninoculated control was maintained. At 5, 7, and 9 days intervals the contents were centrifuged at 7000 rpm for 10 min and clear supernatant was used for soluble P estimation following method described

by [7] .One ml of the culture filtrate was pipette into a 25 ml volumetric flask and diluted to 20 ml with water. Four ml of reagent (1.056 g of ascorbic acid in 200 ml of reagent A) was added and the volume was made up to 25 ml with distilled water. The intensity of blue colour was read in colorimeter (Spectronic 20, USA) at 660 nm. The standard curve was prepared with orthophosphate (KH_2PO_4) and amount of P solubilized was calculated by referring to standard graph. The phosphorus content was expressed in terms of mg of phosphorus litre^{-1}.

HPLC chromatographic quantification of gluconic acid produced by mineral solubilizing organisms

The amount of gluconic acid produced by phosphorus solubilizing microorganisms in the presence of various minerals was determined by using High performance liquid chromatography (HPLC). The bacteria were tested for gluconic acid producing ability by growing them in 100 ml Erlenmeyer flasks containing 50 ml of basal liquid medium supplemented with0.5 per cent tricalcium phosphate. To this, 1 ml of 36 h old broth culture of various isolates containing 1×10^9 cells ml^{-1} was inoculated. After incubation of these cultures for 5 days, the culture were centrifuged at 10,000 rpm for 10 min. and the supernatant was passed through 0.2 µ membrane filter so as to obtain the culture filtrate containing only the soluble forms of metal [5]. These filtrates were analysed for the presence of gluconic acid. 20 µl culture filtrate was injected in HPLC (Shimadzu, LC-8 A, Japan) using a Separon SGX C18 column as described by [12]. Elution was performed with an isocratic solvent consisting of acetonitrile:water (3:7 v/v) with a flow rate of 0.8 ml min^{-1}. Gluconic acid present in the culture filtrate was determined by comparing the retention times and peak areas of the sample with the standard of gluconic acid (Sigma).

Authentication of P solubilizing microbes from rhizosphere of cardamom by 16S rRNA sequencing

16S rRNA gene amplification

Full-length 16S rRNA gene (1500 bp) was amplified from the isolates by PCR using the universal forward primer fd1 (5' AGA GTT TGA TCG TGG CTC AG 3') and the universal reverse primer rp2 (5' ACG GCT ACC TTG TTA CCA CTT 3') [18]. The 45 µl PCR reaction mixture consisted of DNA template 50 ng, 1X Taq buffer, 0.2 mM of each of dNTP mixture, 1 µM of each primers, 2.5 mM $MgCl_2$ and 2 U of Taq DNA polymerase (Bangalore Genei, India). PCR amplification was performed in a thermocycler (Eppendorf Master cycler, Germany) with following conditions: initial denaturation at 95°C for 1 min, 35 cycles consisting of 94°C for 1 min (denaturation), 60°C for 1 min (annealing), 72°C for 1 min

(primer extension) and final extension at 72°C for 5 min. The amplified products were analysed by electrophoresis in 1.5 per cent agarose gels. After separation of the PCR products in agarose gel, viewed and photographed using Alpha imager TM1200 gel documentation and analysis system. Sequencing reactions were performed using ABI prism terminator cycle sequencing ready reaction kit and electrophoresis of the products were carried out on an Applied Biosystems (Model 3100) automated sequencer.

Phylogenetic analysis

The identity of 16S rRNA gene sequences was performed by similarity search using BLAST tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The closest species, strain and per cent similarity to the isolates were obtained from the BLAST result.

Results and discussion

The total bacterial population of cardamom rhizospheric soil from different locations were enumerated and presented in Table 1. From the results it was found that the rhizosphere soil samples from Pampadumpara harboured more number of bacteria. A total of forty bacterial isolates were purified and used for further studies.

Assessment of phosphorus solubilizing potential of the isolates

In the present study, the capability of the 40 bacterial isolates from cardamom rhizosphere were tested to solubilize insoluble forms of phosphorus (tri calcium phosphate). It was observed that 26 isolates could solubilize phosphorus with solubilization efficiency ranging from 22 ± 0.64 to 156 ± 4.50 percent (Table 2, Fig 1). The variation in solubilization efficiency may be due to differences in the location from which they were isolated. The highest solubilization efficiency of 156 ± 4.50 per cent was observed in the isolate Pd 10 (identified as *Bacillus subtilis* by molecular studies Table 4). Similar studies were conducted by [4] and confirmed the capacity of *Bacillus* sp. to solubilize insoluble tricalcium phosphate. Among the 26 isolates tested, Pd 10 (*Bacillus subtilis*) solubilized significantly higher amount of P than the other isolates (30.44 ± 0.88 ppm) after nine days of inoculation. The amount of P solubilized increased with incubation time (Table 3). The increase in the level of phosphorus solubilizing activity in *Bacillus* isolate by increase in incubation was already reported by [16]. Reported that the phosphorus solubilized by different *Bacillis* sp in Pikovskaya's broth ranged between 20 to 129 ppm as stated by [17].

Gluconic acid production by mineral solubilizing organisms

For analysis of gluconic acid, five efficient isolates were selected from phosphorus solubilizing organisms. The retention time for gluconic acid was found to be 1.8 min. by using standard gluconic acid under standard conditions. Based on the retention time obtained by injecting culture filtrate preparation, presence of gluconic acid was detected in all the isolates tested (Fig 2). The *Bacillus* strains produced oxalicacid, 2-ketogluconic acid, and succinic acid capable of solubilizing insoluble P as reported by [1, 4]. (1982). According to [2] both gluconic acid and 2-ketogluconic acid produced by *Pseudomonas cepacia* were responsible for the solubilization of insoluble kaolinite and montmorillonite in suspension.

According to [11] bacteria belonging to these groups are widely distributed in the soil. Previous reports showed that *Bacillus* is very common in forest soils due to its spore forming ability as well as low nitrogen requirements [3,13].

Conclusion

From this study, it is clear that several naturally occurring Phosphorus solubilizing isolates are present in cardamom rhizospher soils which could be utilized for increased phosphorus nutrition in soils. Further studies are needed to test these isolates as a bioinoculum in cardamom for the better productivity and as an alternative to phosphatic chemical fertilizers.

Fig 1 Phosphorus solubilization by bacterial isolates from cardamom rhizosphere

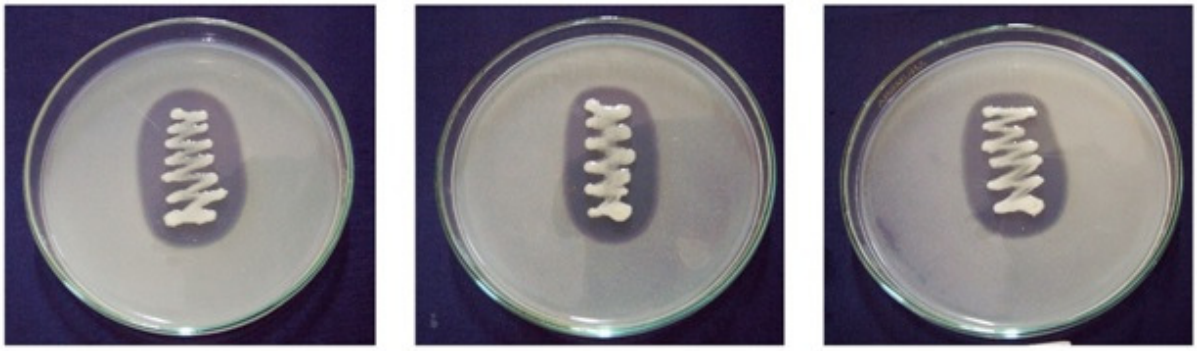

Fig 2 Detection of gluconic acid in phosphorus solubilizing microorganisms

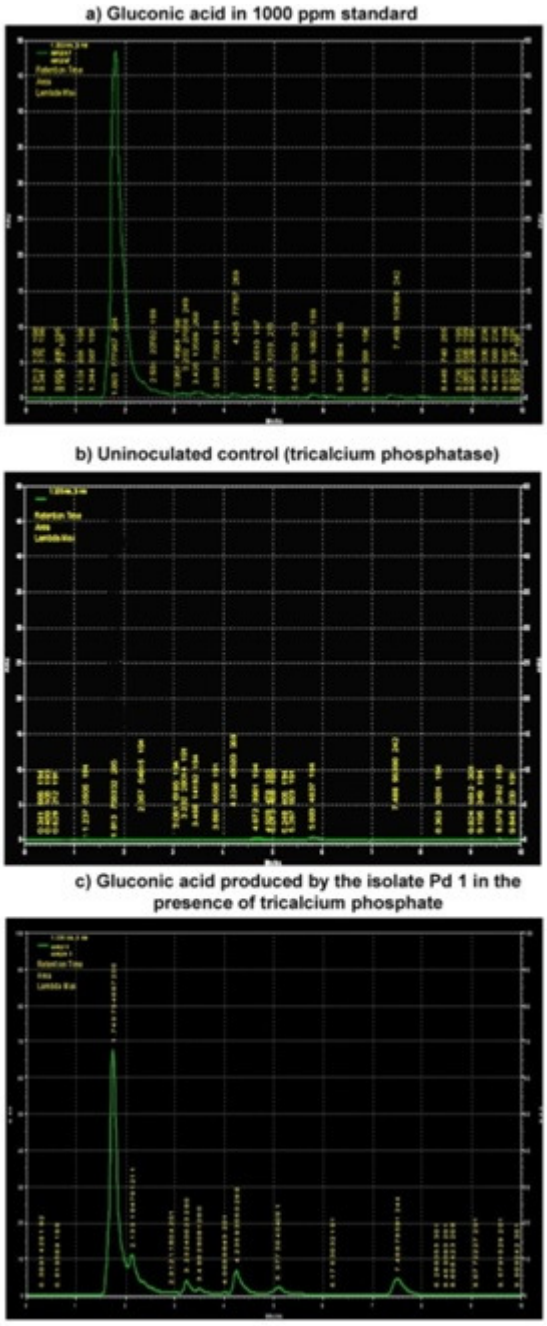

Table 1: Population of total aerobic bacteria in the rhizosphere soil samples of cardamom

Sl. No.	Location	cfu × g $^{-1}$ of soil on dry weight basis
		Total aerobic bacteria population (cfu×10^6)
1	Cumbum mettu	56.5 ± 0.65

2	Thadiyankudisai	60.4 ± 0.70
3	Idukki (Pampadumpara)	78.1 ± 0.90
4	Idukki (Mayiladumpara)	70.2 ± 0.81
5	Wayanad	67.7 ± 0.78
6	Idukki (Kumily)	60.3 ± 0.70

Values are mean ± SE of three replicates

Table 2 *In vitro* phosphorus solubilizing potential of bacterial isolates from cardamom rhizosphere (plate assay)

Sl. No.	Isolates	Colony diameter (mm)	Diameter ofzone of solubilization (mm)	Solubilization efficiency (%)
1	Cd 1	5.5 ± 0.16	9.5 ± 0.27	72 ± 2.08
2	Cd 2	4.3 ± 0.12	6.3 ± 0.18	46 ± 1.33
3	Cd 5	5.2 ± 0.15	7.1 ± 0.20	36 ± 1.04
4	Cd 7	4.0± 0.12	-	-
5	Cd 12	3.7± 0.11	-	-
6	Cd 13	-	-	-
7	Cd 14	-	-	-
8	Cd 15	5.1 ± 0.15	8.0 ± 0.23	56 ± 1.62
9	Td 1	4.0 ± 0.12	8.5 ± 0.25	112 ± 3.23
10	Td 4	4.4 ± 0.13	10.5 ± 0.30	138 ± 3.98
11	Td 5	4.0 ± 0.12	10.0 ± 0.29	150 ± 4.33
12	Td 7	3.8 ± 0.11	-	-
13	Td 9	4.3 ± 0.12	6.9 ± 0.20	60 ± 1.73
14	Td 10	4.5 ± 0.13	6.1 ± 0.18	35 ± 1.01
15	Pd 1	4.2 ± 0.12	9.5 ± 0.27	126 ± 3.64
16	Pd 3	4.1 ± 0.12	7.2 ± 0.21	75 ± 2.17
17	Pd 5	5.0 ± 0.14	7.1 ± 0.20	42 ± 1.21
18	Pd 6	3.8 ± 0.11	4.6 ± 0.13	21 ± 0.61
19	Pd 9	3.7 ± 0.11	4.9 ± 0.14	32 ± 0.92
20	Pd 10	4.1 ± 0.12	11.0 ± 0.32	168 ± 4.85
21	Pd 15	-	-	-
22	Pd 16	5.2 ± 0.15	10.8 ± 0.31	107 ± 3.09
23	Pd 17	-	-	-

24	Md 2	3.9 ± 0.11	6.0 ± 0.17	53 ± 1.53
25	Md 4	5.2 ± 0.15	8.9 ± 0.26	71 ± 2.05
26	Md 5	5.1 ± 0.15	12.1 ± 0.35	135 ± 3.90
27	Md 9	4.3 ± 0.12	8.2 ± 0.24	90 ± 2.60
28	Md 10	3.0 ± 0.09	-	-
29	Md 12	3.3 ± 0.10	-	-
30	Md 14	3.5 ± 0.10	-	-
31	Wd 1	4.0 ± 0.12	5.8 ± 0.17	45 ± 1.30
32	Wd 4	5.3 ± 0.15	6.5 ± 0.19	22 ± 0.64
33	Wd 6	5.1 ± 0.15	7.3 ± 0.21	43 ± 1.24
34	Wd 9	-	-	-
35	Wd 11	5.3 ± 0.15	-	-
36	Wd 13	4.1 ± 0.12	10.5 ± 0.30	156 ± 4.50
37	Kd 8	3.8 ± 0.11	-	-
38	Kd 10	5.3 ± 0.15	-	-
39	Kd 12	3.8 ± 0.11	9.0 ± 0.26	136 ± 3.93
40	Kd 13	4.2 ± 0.12	6.3 ± 0.18	50 ± 1.44

Values are mean ± SE of three replicates
Minus sign (-) indicates no growth

Table 3: Phosphorus solubilizing potential of bacterial isolates from cardamom rhizosphere (broth assay)

	Amount of available phosphorus in the culture filtrate (ppm)		
Isolates	**5 DAI**	**7 DAI**	**9 DAI**
Cd 1	9.7 ± 0.28	10.2 ± 0.29	14.3 ± 0.41
Cd 2	2.5 ± 0.07	3.2 ± 0.09	5.4 ± 0.16
Cd 5	3.6 ± 0.10	5.4 ± 0.16	8.3 ± 0.24
Cd 15	2.1 ± 0.06	3.2 ± 0.09	5.6 ± 0.16
Td 1	7.5 ± 0.22	12.5 ± 0.36	17.8 ± 0.51
Td 4	11.2 ± 0.32	17.4 ± 0.50	20.5 ± 0.59
Td 5	10.7 ± 0.31	17.5 ± 0.51	24.0 ± 0.69
Td 9	5.2 ± 0.15	7.2 ± 0.21	8.7 ± 0.25
Td 10	3.3 ± 0.10	4.3 ± 0.12	5.6 ± 0.16
Pd1	12.0 ± 0.35	16.2 ± 0.47	18.3 ± 0.53

Pd 3	4.2 ± 0.12	7.3 ± 0.21	9.8 ± 0.28
Pd 5	5.3 ± 0.15	6.3 ± 0.18	8.7 ± 0.25
Pd 6	2.5 ± 0.07	3.7 ± 0.11	4.6 ± 0.13
Pd 9	4.2 ± 0.12	5.3 ± 0.15	7.6 ± 0.22
Pd 10	20.1± 0.58	25.4 ± 0.73	30.44 ± 0.88
Pd 16	7.4 ± 0.21	9.5 ± 0.27	12.6 ± 0.36
Md 2	7.8 ± 0.23	8.5 ± 0.25	10.9 ± 0.31
Md 4	5.3 ± 0.15	6.4 ± 0.18	9.7 ± 0.28
Md 5	10.5 ± 0.30	15.6 ± 0.45	22.5 ± 0.65
Md 9	4.2 ± 0.12	5.4 ± 0.16	6.5 ± 0.19
Wd 1	2.1 ± 0.06	3.4 ± 0.10	4.7 ± 0.14
Wd 4	3.4 ± 0.10	4.7 ± 0.14	7.1 ± 0.20
Wd 6	3.5 ± 0.10	5.1 ± 0.15	7.5 ± 0.22
Wd 13	14.51± 0.42	19.6 ± 0.57	27.11± 0.78
Kd 12	13.2 ± 0.38	18.4 ± 0.53	20.2 ± 0.58
Kd 13	4.6 ± 0.13	5.5 ± 0.16	8.3 ± 0.24
Control	0	0	0

Values are mean ± SE of three replicates

Table 4: Identification of bacterial isolates from rhizospheric soils of cardamom by 16S rRNA gene sequence homology

Isolate	Sequence homology			Phylum
	Closest species[a]	Accession No.	Per cent[b] homology	
TAUC2	*Bacillus subtilis*	GU191904	99	Firmicutes

[a] Species identified based on 16S rRNA gene sequence similarity.
[b] Per cent similarity of the sequence in BLAST analysis.

References

[1] Banik, S. and B.K. Dey. 1982. Available phosphate content of an alluvial soil as influenced by inoculation of some isolated phosphate-solubilizing microorganisms. *Plant Soil*, 69: 353-364.

[2] Bar-Yoself, B., R.D. Rogers, J.H. Wolfam and E. Richman. 1999. *Pseudomonas cepacia*-mediated rock phosphate solubilization in kaolinite and montmorillonite suspensions. *Soil Sci. Soc. Am. J.,* 63:1703-1708.

[3] Buchanan, R.E. and N.F. Gibbons. 1974. Bergey's Manual of Determinative Bacteriology, 8th edition. Williams and Wilkins, Baltimore, pp. 1268

[4] Dave, A. and H.H. Patel. 1999. Inorganic phosphate solubilizing soil pseudomonads. *Indian J. Microbiol.,* 39: 161-164.

[5] Francis, A.J., C. Dodge, K. Chendrayan and H. Quinby. 1988. Anaerobic microbial dissolution of lead oxide and production of organic acids. US Patent No.4758345.

[6] Kuklinsky-Sobral, J., W.L. Araujo, R. Mendes, I.O. Geraldi, A.A. Pizzirani- Kleiner and J.L. Azevedo. 2004. Isolation and characterization of soybean-associated bacteria and their potential for plant growth promotion. *Environ.Microbiol*.,12:1244-1251.

[7] Olsen, S.R., C.V. Cole, S. Watanabobe and L.A. Dean. 1954. Estimation of available P in soils by extraction with sodium bicarbonate. U.S. Dept.Agr., Circular, 939: pp. 19.

[8] Parkinson, D., T. R.G. Gray and J. Williams. 1971. Methods for studying the ecology of soil micro-organisms. Blackwell, Oxford, pp: 64-66.

[9] Richardson AE. Soil microorganisms and phosphorus availability. In: Pankhurst CE, Doube BM, Grupta VVSR, Grace PR, editors. Soil Biota, Management in Sustainable Farming Systems. Melbourne, Australia: CSIRO, 1994, pp. 50–62

[10] Rodriguez, H. and R. Fraga. 1999. Phosphate solubilizing bacteria and their role in plant growth promotion. Biotechnol Adv., 285: 260-263.

[11] Rosch, C., A. Mergel and H. Bothe. 2002. Biodiversity of denitrifying and dinitrogen-fixing bacteria in an acid forest soil. *Appl. Environ. Microbiol.,* 68: 3818-3829.

[12] Roukas, T. 2000. Citric and gluconic acid production from fig by *Aspergillus niger* using solid-state fermentation. *J Ind Microbiol Biotechnol* **25**, 298–304.

[13] Schlegel, H.G. 1996. Biobeneficiation of bauxite using *Bacillus* polymyxa. *General Microbiology*, 4: 51-60.

[14] Sperber, J.E. 1958. Solubilization of apatite by soil microorganisms producing organic acids. *Aust. J. Agric. Res.,* 9: 782 -787.

[15] Srivastav, S., K.S. Yadav and B.S. Kundu. 2004. Prospects of using phosphate solubilizing *Pseudomonas* as biofungicide. *Indian J. Microbiol.,* 44: 91-94.

[16] Tank, N. and M. Saraf. 2003. Phosphorous solubilization, exopolysaccharides production and indole acetic acids secretion by rhizobacteria isolated from *Trigonella foenum-raecum*. *Indian. J. Microbiol.,* 43(1): 37-40.

[17] Thakuria, D., N.C. Talukdar, C. Goswami, S. Hazarika, R.C. Boro and M.R. Khan. 2004. Characterization and screening of bacteria from the rhizosphere of rice grown in acidic soils of *Assam. Curr. Sci.,* 86: 978-985.

[18] Weisburg, W.G., S.M. Barns, D.A. Pelletier and D.J. Lane. 1995. 16S ribosomal DNA amplification for phylogenetic study. *J. Bacteriol.,* 173: 697-703.