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Isolation of Plant growth beneficial endophytic bacterial strains from medicinal plants *Gymnema sylvestre* and *Tabernamontana divaricate*

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Abstract
Endophytes are microorganisms that colonize within the living plant cell and mostly have a symbiotic association with the host plant. There is a growing interest to recognize the involvement of the endophytes in the production of the growth promoting bioactive compounds and secondary metabolites that are produced by the plants. Medicinal plants are known to produce several phytochemicals with potential biological activities. In the current study the endophytic bacterial diversity of two valuable medicinal plants *Gymnema sylvestre*, and *Tabernamontana divaricate* are studied. The study aimed at understanding the involvement of the endophytic bacterial community in growth beneficial trait of the host plant viz. IAA(Indole Acetic Acid) production, Phosphate solubilisation and their capacity to grow on Nitrogen free medium so as to assert their role in Nitrogen fixation. A total of 14 bacterial strains were isolated from both the taxa. 11 isolates from *Gymnema sylvestre* and 03 from *Tabernamontana divaricate.* The morphological and biochemical characterization of the isolates were performed. It was found that 13 out of 14 isolates were capable of producing IAA, 04 isolates were able to grow on Nitrogen free media and only one was capable of Phosphate solubilisation.
The study showed that the isolated strains of endophytes have immense plant growth promoting potential which can be used for improvement of growth of crops.

Keywords: endophytes, *Gymnema sylvestre*, *Tabernamontana divaricate*, iaa production, phosphate solubilisation, nitrogen free medium

1. Introduction
At the most basic level, endophytes simply means the location of an organism, with “endo” means “inside” and “phyte” means “plants”. Therefore, endophyte refers to organisms that live within plants. Endophytes are bacterial or fungal microorganisms that colonize healthy plant tissue intercellularly and/or intracellularly without causing any apparent symptoms of disease [1] Some endophytic bacteria exert several beneficial effects on host plants, such as stimulation of plant growth [2], nitrogen fixation [2, 3] and induction of resistance to plant pathogens [4]. Endophytes exist in a range of tissue types within a broad range of plants, colonizing the plant systemically with bacterial colonies and biofilms, residing latently in intercellular spaces, inside the vascular tissue or within cells [5].
Of note, of the nearly 300,000 plant species forming the vegetal biodiversity of earth, each individual plant is host to one or more endophytes and can consequently constitutes an opportunity to find new and interesting endophytic microorganisms [6]. In fact, until now, only few of the existing plants have been completely studied in relation to their endophytic content [7] Endophytic bacteria are now gaining importance for their plant-beneficial traits are potentially excellent plant growth promoters and/or biological control agents for sustainable crop production [8,9] The endophytic bacterial population aid in plant growth promotion in a variety of ways be through its ability to produce growth hormone and N_2 retardation from the air and may other such activities which make them suitable to be used as biofertilizers to increase crop production and significantly reduces the chemical input to the environment [10,11,12].
To deal with endophytes, selection of a proper and promising plant species is necessary. Plants with ethnobotanical history are good candidates for endophytes study since the medical uses for which the plant may have been selected relate more to its population of endophytes than to the plant biochemistry itself [6,13]
In the present investigation, endophytic diversity of two valuable medicinal plants, *Gymnema sylvestre*, and *Tabernamontana divaricata* was studied. *Gymnema sylvestre*a member of Asclepiadaceae family, is considered as one of the major botanicals to treat diabetes in the Ayurvedic system of medicine and is also included in Indian Pharmacopoeia as an anti-diabetic plant [14]. *Tabernamontana divaricate* commonly known as Crape Jasmine belonging to the family Apocynaceae is used to treat fever diarrhoea and is also used as a tonic for brain, liver, and

spleen in folklore medicine [15]. The aim of the study was to isolate and characterize endophytes from these two selected medicinal plants and to assess their plant growth promoting traits with respect to IAA synthesis, their capacity to grow on nitrogen free medium and phosphate solubilisation potential.

2. Materials and methods

2.1 Sample collection

The healthy explants of *Gymnema sylvestre* and *Tabernamontana divaricata* used for the study were collected in sterile bags at the flowering stage from garden, managed and maintained by Bhagwan Mahavir College of Science and Technology, Surat, Gujarat. The plant materials were brought to the laboratory and were washed carefully under tap water to remove any adhering dirt and debris.

2.2 Isolation of Endophytes

Explants (stems, leaves and flowers) were cut into sections 2-3 cm long. The sections were put in beaker, soaked in distilled water and drained. It was rinsed in 70% ethanol for 30 seconds and then sterilized with 0.1% $HgCl_2$ for 3 minute.[16] Surface-disinfected sections were aseptically macerated with homogenizers. Macerated tissue was diluted into 10^{-1} dilution by adding 9 volumes of sterile distilled water. Serial dilution was made up to 10^{-6} and O.1 ml from appropriate dilutions were spread plated on two different media, viz. PDA (Potato Dextrose Agar) and NA (Nutrient Agar) [17]. Plates were sealed using paraflim to minimize contamination and in order to recover maximum possible colonies of endophytes and incubated at 28 ±2°C and observation was made for 48 hrs.

2.3 Morphological and physiological characterization

For Motility test each isolate was spot-inoculated on the centre of semi-solid nutrient agar plates (0.2% agar) and incubatoed at 30^0C. The diffusion of colony was observed and recorded at 24 hours.[18] Gram staining, spore staining and capsule staining were carried out followed standard staining protocols [19].

2.4 Study of metabolic activities of bacteria

To determine metabolic activity of bacteria some of the routine biochemical tests were carried out using standard procedure[20] and the name of biochemical tests mentioned as follows:

Utilizations of carbohydrates and organic acids test was carried out using Methyl-Red (M-R) test, Voges-proskauer (V-P)test, Citrate utilization test. Utilization of nitrogenous compounds test was carried out using Indole production test, Phenylalanine deamination test, Urea hydrolysis test, Nitrate reduction test. Gelatin hydrolysis test was use to indentify Decomposition of large molecules. To indentify Miscellaneous tests, Catalase test was performed. Triple sugar iron agar test was carried out to identify Combined test using composite test media.

2.5 Study of Plant Growth promoting traits

2.5.1 Growth on Ntrogen free media

Semi solid Rennie media[21] was used to screen the Nitrogen fixing capacity of the isolated entophytic strains. Media consisted of (per liter): 0.8 g of K2HPO4, 0.2 g of KH2PO4, 0.1 g of NaCl, 28 mg of Na2FeEDTA, 25 mg of Na2MoO4 · 2H2O, 0.2 g of MgSO4 · 7H2O, 0.06 g of CaCl2 · 2H2O, 100 mg of yeast extract, 3.0 g of mannitol, 5.0 g of sucrose, 0.5 ml of 60%(vol / vol) sodium lactate, 2.0 g of sodium malate, 2.0 g agar, pH 7.0. After autoclaving, filter-sterilized biotin and *para* aminobenzoic acid were added to final concentrations of 5 and 10 µg per liter. [22, 23] Plates were incubated at 37°C for 48hrs and observed for growth.

2.5.2 Phosphate solubilization

To determine phosphate solubilization, Agar medium containing pikovasakya medium [24, 25] was prepared. Loop full of endophytes was dropped at the centre of plate while One plate was kept uninoculated as a control. Plates were incubated in incubator at 37°C for 3-4days till the zone forms upto 5mm in diameter. Observe the zone by consuming the blue color of bromo phenol blue dye to yellow color zone, Phosphate solubilization was detected by color change.

2.5.3 Estimation of IAA

For rapid quantitative estimation of IAA in broth culture, the colorimetric method of Gordon and Weber [26] was used. The cultures were grown in the dark for 7 days, sampled every day, centrifuged at 13000 rpm. for 10 min, and the production of IAA was assayed in duplicated supernatant samples. The presence of IAA in each supernatant was measured colorimetrically by adding two parts of 0.01 M $FeCl_3$ in 35% $HClO_4$ to one part of supernatant followed by reading the optical density at 530 nm after 25 min.The recorded absorbances were read off and a standard curve prepared from pure IAA (Hi-Media). The experiment was done in triplicate.

3. Results

3.1 Isolation of Endophytic Bacteria

A total of 14 isolates were obtained from *Gymnema sylvestre* and *Tabernaemontana divaricate*. Of which 07 isolates designated as S_1, S_2, S_3, S_4, S_5, S_6, and S7 were obtained from stem extract of *Gymnema sylvestre*on NA plates and 03 isolates (L1,L2 and L_3) were obtained from the leaf extracts of the same taxa on PDA media. 01 isolate (CL_1) was obtained from leaf extract of *Tabernaemontana divaricata*on NA plate and02 isolates (CL_2 and CL_3) were obtained from leaf extract of the same plant on PDA.01isolate (CS1) was obtained from stem extract *Tabernaemontana divaricate* on NA plate.

3.2 Morphological and Biochemical s characterization.

The results of Gram staining, spore staining, capsule staining and motility test are presented in Table 1.

Table 1: Morphological traits of isolated endophytes.

Isolate	Gram stain	Cell shape	Spore Stain	Capsule Stain	Motility Test
S_1	-	Rod	-	-	-
S_2	+	Coccus	-	-	-
S_3	-	Coccus	-	-	-
S_4	+	Coccus	-	-	-

S_5	+	Rod	-	-	-
S_6	+	Coccus	-	-	-
S_7	+	Coccus	-	-	-
L_1	-	Rod	-	-	+
L_2	+	Coccus	-	-	-
L_3	+	Coccus	-	-	-
CL_1	+	Coccus	-	-	-
CL_2	+	Rod	-	-	-
CL_3	-	Rod	-	-	-
CS_1	+	Rod	-	-	-

3.3 Study of metabolic activities of bacteria

The results of Biochemical tests are presented in Table 2.

Table 2: Biochemical traits of isolated endophytes.

Test	**Results**													
	S_1	**S_2**	**S_3**	**S_4**	**S_5**	**S_6**	**S_7**	**L_1**	**L_2**	**L_3**	**CL_1**	**CL_2**	**CL_3**	**CS_1**
Methyl production	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Vogous-proskur	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Indole production	+	-	-	-	-	-	-	+	-	-	+	-	-	-
H_2S production	-	-	-	-	+	-	-	-	-	-	-	-	-	+
Urea test	+	+	+	+	-	-	+	-	-	+	-	+	+	+
Nitrate reduction	-	+	+	-	-	-	-	+	-	-	+	-	-	-
Gelati liquification	+	+	+	-	-	+	+	+	-	-	+	+	+	+
Catalase test	+	+	+	+	+	+	+	+	+	+	+	+	+	+
TSI agar test	+	-	+	+	+	-	-	+	-	+	-	-	+	+
Sugar fermentation														
Glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Sucrose	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Fructose	+	+	+	+	-	+	-	+	+	+	-	+	+	-
Maltose	+	+	+	+	-	-	-	+	+	+	-	+	+	-
Lactose	+	+	+	+	+	-	+	+	+	+	+	+	+	+

3.4 Plant growth promoting traits of the isolated strains

Among 14 isolates only 4 isolates showed growth on nitrogen free media. Of these 4 isolates, 03 were gram positive (S_2, S_7 and CL_1) and one was gram negative (S_3).

Only 01 isolate (S_1) was able to solubilize phosphate. 13 isolates did not show any phosphate solubilizing activity.

After 7 Days of optimum growth under dark condition, IAA production was observed in 13 of the total 14 isolates in varying concentration of minimum of 07µg/ml to a maximum of 23 µg/ml. However no IAA production was observed in the isolate designates as CL_2.

The isolate designated as L1 was found to produce maximum of 23 µg/ml.

Table 3: Plant growth promoting properties of isolates

Isolate	**Nitrogen fixation**	**Phosphate solubilization**	**IAA production**
S_1	-	+	+
S_2	+	-	+
S_3	+	-	+
S_4	-	-	+
S_5	-	-	+
S_6	-	-	+
S_7	+	-	+
L_1	-	-	+
L_2	-	-	+
L_3	-	-	+
CL_1	+	-	+
CL_2	-	-	-
CL_3	-	-	+
CS_1	-	-	+

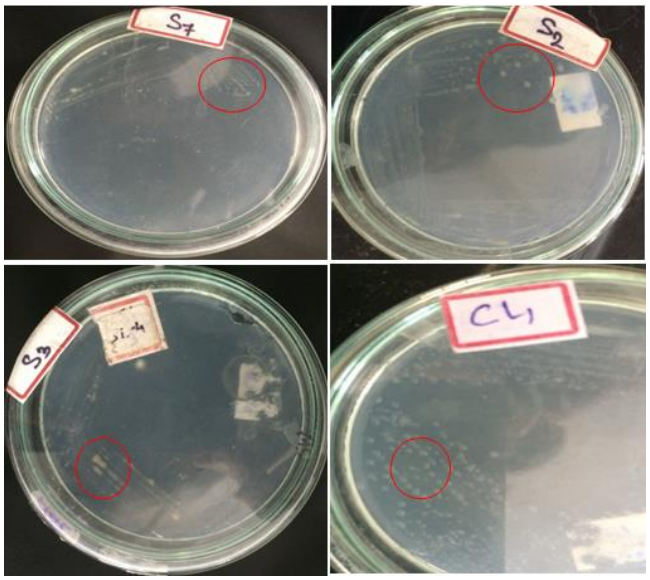

Fig 1: Isolated strains showing growth on Nitrogen free media

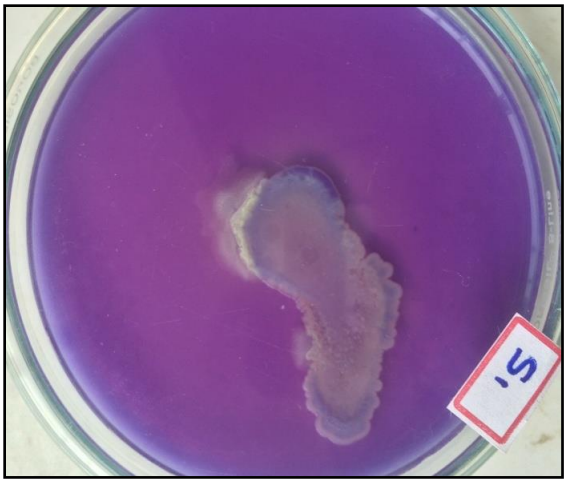

Fig 2: Phosphate solubilizing isolate

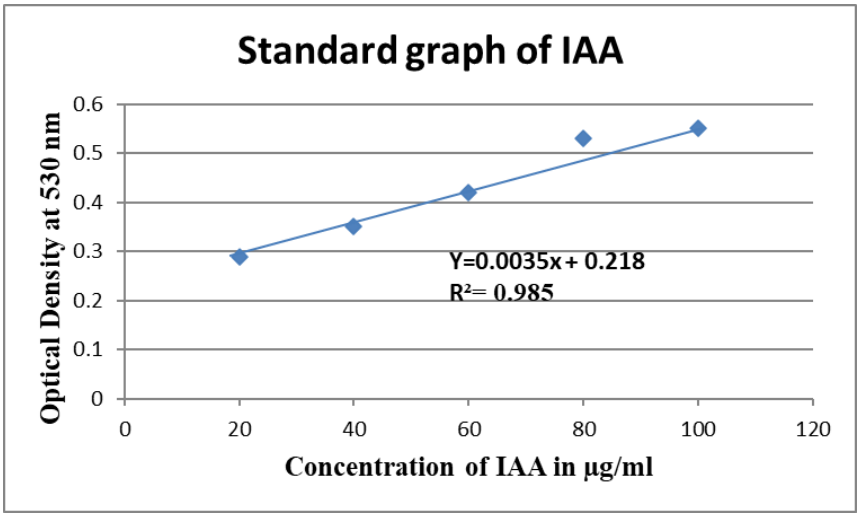

Fig 3: Standard graph of IAA

Table 4: IAA production by the isolated endophytic strains on day 7th

Isolate	IAA production(µg/ml)
S_1	19.00± 0.42
S_2	12.33± 0.65
S_3	11.66± 1.44
S_4	11.33± 0.56
S_5	9.33± 0.18
S_6	8.00±3.22
S_7	9.16 ± 0.87
L_1	23.66± 0.34
L_2	12.00± 1.61
L_3	11.85 ± 0.81
CL_1	13.20 ±1.28
CL_2	0
CL_3	11.66± 0.93
CS_1	7.33 ± 2.19

Each value represents the mean of three replicates ±Standard Deviation.

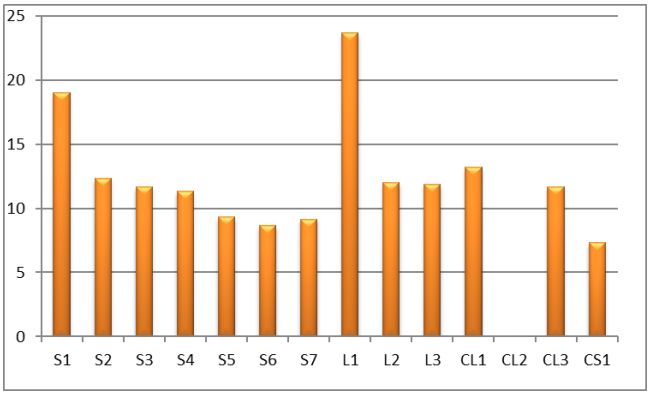

Fig 4: IAA production by the isolated endophytic strains on the 7th day

4. Discussion

Endophytes isolated from the two medicinal plant species exhibited phenotypic diversity and all of them showed plant growth promoting traits.

Four of the isolated endophytic bacterial strains were found to be capable of growing on Nitrogen free media. Nitrogen is the most limiting nutritional factor for the growth of plants. Since plants cannot reduce atmospheric N_2, they require exogenously fixed nitrogen for growth and development. Nitrogen-fixing endophytic bacteria can make available the fixed nitrogen directly to plants. [27]

The, rod shaped Gram negative bacterial strain designated as S1,showed many promising biochemical properties and was found to be capable of phosphate solubilisation. Phosphorus is one of the essential nutrients required for plant growth. Although it is moderately available in nature, it is a deficient nutrient in most soils.[28] Microorganisms is integral in the natural phosphorus cycle. Phosphate solubilising microorganisms (PSMs) play very important role in replenishing the soil with the much needed Phosphorous required for the healthy growth of the plant.[29]

13out of 14 endophytic bacterial strains isolated in this study were found to be capable of IAA production with a yield ranging from approximately 8.00µg/mlto 23.66µg/ml**.**

IAA is the most active plant growth regulators responsible for a number of growth promoting activities in plants. The ability of endophytic microorganisms to produce plant hormones such as IAAcan help plants to grow better.[10] It was observed in a previous work that Auxin produced by endophytic bacteria *Burkholderiakururiensis* in peanut plants cause plant growth to be better with the number of roots, and it makes lateral roots of the plant increases. Plant growth is rapid, and it gives high yielding products.[30]

5. Conclusion

Currently lot of researches is carried out on the mechanism involved in plant-endophyte interaction in various plant beneficial trait which in turn will be deterministic in use of suitable formulations of endophytic bacteria to be used as biofertilizer for sustainable agriculture.[27, 29]

The strains isolated in this investigation showed promising plant growth promoting activities which can be utilized at commercial level after their molecular identification.

6. Reference

1. Wilson D. Endophyte - the evolution of a term, and clarification of its use and definition. Oikos. 1995; 73(2): 274-276.
2. Sturz AV, Christie BR, Matheson BG, Nowak J. Biodiversity of endophytic bacteria which colonize red clover nodules, roots, stems and foliage and their influence on host growth. Biol. Fertil.Soils. 1997; 25:13-19.
3. Kirchhorf, G, Reis VM, Baldani JI, Eckert B, Döbereiner J and Hartmann A. Occurrence, physiological and molecular analysis of endophytic diazotrophic bacteria in gramineous energy plants. Plant Soil. 1997; 194:45-55.
4. Chen C, Bauske EM, Mussan G, R Rodriguez-Kabana, Kloepper JW. Biological control of Fusarium wilt on cotton by use of endophytic bacteria. Biol. Control. 1995; 5:83-91.
5. Ulrich K, Ulrich A, Ewald D. Diversity of endophytic bacterial communities in poplar grown under field conditions. FEMS Microbiol Ecol. 2008; 63:169–180.
6. Strobel G, Daisy B. Bioprospecting for Microbial Endophytes and Their Natural Products. Microbiol. Molecul. Biol. Rev. 2003; 491-502.
7. Strobel G. The Emergence of Endophytic Microbes and Their Biological Promise. J. Fungi. 2018; 4:57.

8. Di Fiore S, Del Gallo M. Endophytic bacteria: their possible role in the host plant // *Azospirillum*VI and Related Microorganisms / Ed. I. Fendrik. – Berlin Heidelberg: Springer. 1995; G37:169-187.
9. Strobel G. *Muscodoralbus*and its biological promise. J. Indust. Microbiol.Biotech. 2006; 33:514-522.
10. Herlina L, KedatiPukan K, Mustikaningtyas D. The endophytic bacteria producing IAA (Indole Acetic Acid) in *Arachishypogaea* CELL Biology & Development. 2017; 1: 31-35.
11. Luo SL, Xu TY, Chen L, Chen JL, Rao C, Xiao X, *et.al.* Endophyte-assisted promotion of biomass production and metal-uptake of energy crop sweet sorghum by plant-growth-promoting endophyte *Bacillus* sp. SLS18. Appl Microbiol Biotechnol. 2012; 93:1745-1753.
12. Ahemad M, Kibret M. Mechanisms and applications of plant growth promoting rhizobacteria: current perspective. J King Saud Univ Sci. 2014; 26:11-20.
13. Yu H, Zhang L, Li L, Zheng C, Guo L, LiW, Sun P, Qin L. Recent developments and future prospects of antimicrobial metabolites produced by endophytes. Microbiol. Res. 2010; 165:437–449.
14. Singh VK, Umar S, Ansari SA, Iqbal M. *Gymnemasylvestre* for diabetics. Journal of Herbs. J. Herbs Spices Med. Plants. 2008; 14:88–106.
15. Purushothaman M, Preetam Raj Jp, Khusro A, Panicker Sg. Antibacterial Activity of *Tabernaemontana divaricate (Apocynaceae)* Secondary Metabolites Capped Silver And Gold Nanoparticles. Asian J Pharm Clin Res. 2016; 9(1):63-65.
16. Gagne S, Richard C, Roussean H, Antoun H. Xylem-residing bacteria in alfalfa roots. Can. J. Microbiol. 1987; 33:996-1000.
17. Pham QH, Annapurna K. Isolation and characterization of endophytic Bacteria in soyabean (Glycine sp.) Omonrice 2004; 2:92-101.
18. Elbeltagy A, Nishioka K, Suzuki H, Sato T, Sato YI, Morisaki H, *et al.* Isolation and characterization of endophytic bacteria from wild and traditionally cultivated rice varieties. Soil Sci. Plant Nutr. 2000; 46:617-629.
19. Rakesh P, kiran P. Experimental Microbiology, Aditya Publication, Ahemdabad, India. 2015; 1(8)54-65.
20. Rakesh P, kiran P. Experimental Microbiology, Aditya Publication, Ahemdabad, India. 2015; 1(8)110-126.
21. Rennie RJ. A single medium for the isolation of acetylene-reducing (dinitrogen – fixing) bacteria from soils.Can J Microbiol. 1981; 27:8–14.
22. Elbeltagy A, Nishioka K, Sato T. *et al*. Endophytic Colonization and In Planta Nitrogen Fixation by a *Herbaspirillum*sp. Isolated from Wild Rice Species.Appl Environ Microbiol. 2001; 67:5285–93.
23. Inga M and Odeta B IAA production and other plant growth promoting traits of endophytic bacteria from apple tree. Biologija. 2011; 57(2): 98–102.
24. Pikovskaya RI. Mobilization of phosphorous in soil in connection with vital activity of some microbial species. Microbiologiya. 1948; 17:362-70.
25. SundaraRao WVB, Sinha MK. Phosphate dissolving organisms in the soil and rhizosphere. J Indian J AgricSci 1963; 33:272-8.
26. Gordon SA, Weber RP. Colorimetric estimation of indoleacetic acid. Plant Physiol 1950; 30(1):86–8.
27. Gupta G, Panwar J, Akhtar MS, Jha PN. Endophytic Nitrogen-Fixing Bacteria as Biofertilizer. In: Lichtfouse E. (eds) Sustainable Agriculture Reviews. Sustainable Agriculture Reviews, 2012, 11.
28. Hellal F, El-Sayed S, Zewainy R, Amer A. Importance of phosphate pock application for sustaining agricultural production in Egypt. Bulletin of the National Research Centre. 2019; 43:11.
29. Kalayu G. Phosphate Solubilizing Microorganisms: Promising Approach as Biofertilizers, International Journal of Agronomy. 2019; 1-7.
30. Mattos KA, Padua VLM, Romerio A, Hallack LF, Neves BC, UlissesTMU, *et. al.* Endophytic colonization of rice (*Oryzasativa*L) by the diazaotrophicbacteriu *Burkholderiakururiensis* and its ability. Ann Acad Bras Cienc. 2008; 80(3):477-493.