View metadata, citation and similar papers at core.ac.uk
brought to you by CORE
provided by E-Journal Center for Plant Conservation Botanic Gardens-LIPI (Indonesian Institute of...

LIPI
SATREPS
Science and Technology Research Partnership for Sustainable Development Program
Proceedia 2017
JICA
JST
The 1st SATREPS Conference, Bogor Nov 14th, 2016
"The Project for Producing Biomass Energy and Material through Revegetation of Alang-alang (*Imperata cylindrica*) Fields"

Isolation and Characterization of Nitrogen Fixing Endophytic Bacteria Associated with Sweet Sorghum (*Sorghum bicolor*)

Tri Ratna Sulistiyani[a1], Siti Meliah[a]

[a] *Research Center for Biology, Indonesian Institute of Sciences*
Jl. Raya Jakarta Bogor Km. 46, Cibinong 16911, Indonesia.

Abstract

Sweet sorghum (*Sorghum bicolor*) has high adaptability to the dryland and resistant to drought. Many endophytic bacteria live in association with their host and play crucial role in health and development of plant. The aims of this work were to isolate and characterize nitrogen-fixing endophytic bacteria associated with sorghum. Sorghum samples were collected from Cibinong Science Center, Cibinong, Bogor, Indonesia. Sorghum werecultivated in four different treatment (compost and inoculants (CI), inoculants (TI), compost without addition inoculants (CN), without compost and inoculants (TN). The samples were sterilized by surface sterilization method and isolation of endophytic bacteria was carried out using spread plate method on TSA medium 1/10. Characterization of endophytic bacteria to fix nitrogen were done using Jensen's media and tested by specific primers to amplify *nif*H gene. A total of 89 isolates were successfully isolated from root and stem. Sixteen isolates were able to grow on Jensen's media. Based on PCR amplification of *nif*H gene was observed the expected size of the *nif*H region.

Keywords: endophytic bacteria, nitrogen fixing, *nif*H gene, *Sorghum bicolor*

1. Introduction

Sweet sorghum (*Sorghum bicolor*) is considered as one of the most important cereal crops in some parts of the world, particularly in arid or semi-arid region. This is due to their adaptability to the dryland and their resistivity to drought condition. Sorghum is critical component of food security for more than 300 million people in Africa [1]. Their stalks contain high amount of sugar and can be utilized as livestock feed, syrup component, wine, or material to produce biofuel. Therefore, developing sorghum will play an important role in promoting the development of agricultural production, livestock husbandry, energy, and refining sugar.

In this research, these beneficial roles of sorghum plant were used to control trouble some weedy species, such as alang-alang (*Imperata cylindrica*) through revegetation strategy. Alang-alang responsible for the decrease of agronomic crops production in Africa and the loss of thousands of hectares of native habitat in southeastern US [2]. It also less in commercial uses, therefore, planting sorghum plants in area covered with persistence alang-alang can add value to this relatively unproductive land. To ensure a successful re-vegetation strategy, the health and productivity of sorghum plants must be taken into account.

Almost all vascular plant species, including sorghum, are found to be associated with endophytic bacteria, which may produce various bioactive compounds related to the host [3]. *Bacillus*, *Corynebacterium*, and *Microbacterium* are the most prevalent

[1] Corresponding author. Tel.: +62 21 8765066; fax: +62 21 8765062
E-mail address: *lishadari@yahoo.co.id*

LIPI
SATREPS
Science and Technology Research Partnership for Sustainable Development Program
Proceedia 2017
The 1st SATREPS Conference, Bogor Nov 14th, 2016
"The Project for Producing Biomass Energy and Material through Revegetation of Alang-alang (*Imperata cylindrica*) Fields"

endophytic bacteria genera isolated from Sorghum [4]. These endophytic bacteria play crucial role in health and development of their host plant. They are considered as agents to stimulate plant growth by solubilizing phosphate, producing phytohormone (such as indole acetic acid), enhancing uptake minerals, and fixing nitrogen (N_2) [5].

Endophytic bacteria that can fix N_2 live in the intracellular spaces in the plants vascular system and take dissolved N_2 gas from the sap flow and convert it into amines and ammonium nitrogen for plant use. These bacteria had been well studied in non-legume plants, particularly rice plant. Inoculation of N_2-fixing bacteria to rice plants resulted in the increase of growth and grain yield [6]. For this reason, the use of N_2-fixing bacteria to crop plants can reduce chemical N-fertilizer application. The ability to fix free N_2 in endophytic bacteria is encoded by N_2-fixing genes. Nitrogenase is the enzyme responsible for fixation. This enzyme consist of two components, they are component I encoded by the *nifD* and *nifK* genes and component II encoded by *nifH* [7]. Therefore, the aim of this works were to isolate endophytic bacteria associated with sorghum and characterize their N_2-fixing ability based on their growth on nitrogen free medium and the present of *nifH* gene.

2. Methods

2.1. Source of samples

Sorghum bicolor samples were collected from Cibinong Science Center, Cibinong, Bogor, West Java, Indonesia. Sorghum were cultivated in four different treatments (compost and inoculants (CI), inoculants (TI), compost without addition inoculants (CN), without compost and inoculants (TN)). From each plant materials, root and stem were collected for endophytic bacteria isolation.

2.2. Isolation of endophytic bacteria

Endophytic bacteria were isolated from root and stem of sorghum plants. The plant organs were sterilized using surface-sterilization modified protocol by Maropola [8]. The roots and stem of sweet sorghum were washed using tap water to remove soil from their surface. Briefly, 1 g of roots and 5 g of stem were washed with 70% ethanol for 10 min with vigorous shaking, followed by 2.5% sodium hypochlorite solution for 20 min, and finally rinsed four times with sterilized distilled water for 2 min. The samples were dried using sterilized towel tissue in laminar airflow. The roots and stem were sterilized separately. To confirm sterility, 100 µL of the last distilled water was spread on to TSA plate (1/10 strength of TSA) and incubated at room temperature for three days. Sterilization was considered successful when no colonies were observed on the plate. If any present bacterial growth on the plate, the isolation procedure was discarded.

For the isolation of endophytic bacteria, 1 g of the sterilized sample were aseptically ground using autoclaved mortar. Serial dilution were made using 9 mL of 0.85% NaCl solution, and 100 µL of the 10^{-3} to 10^{-5}dilution were spread on to medium were supplemented with cycloheximide 50 µg/mL to avoid the growth of fungi. Plates were incubated at 28^{o}C for 2-15 days. Bacterial colonies which appeared on the media were picked up based on several phenotypic characteristics and then purified to obtain a single colony. Based on their different morphological characteristics, the endophytic bacteria were selected for further studies.

2.3. Screening of nitrogen fixing activty of endophytic bacteria

Endophytic bacteria associated with sorghum plant were screened for nitrogen fixing activity by Ahmad, Ahmad, and Khan [9]. Screening of nitrogen fixing activity was done using Jensen's medium andBromothymol Blue (BTB) as a color indicator.

LIPI
SATREPS
Science and Technology Research Partnership for Sustainable Development Program
Proceedia 2017
The 1st SATREPS Conference, Bogor Nov 14th, 2016
"The Project for Producing Biomass Energy and Material through Revegetation of Alang-alang (*Imperata cylindrica*) Fields"

After 3-8 days, the color of medium will change from greenish blue to dark blue. Nitrogen fixing activity of endophytic bacteria was estimated by colour alteration of media todark blue. Here is the composition of Jensen's medium: 20 g sucrose, 1 g K_2HPO_4, 0.500 g $MgSO_4$, 0.500 g NaCl, 0.100 g Fe_2S0_4, 0.005 g $Na_2MoO_4{\cdot}2H_2O$, 2.000 g $CaCO_3$, and 15.000 g Bacto agar.

2.4. Nucleic acid extraction

DNA extraction from bacteria was conducted using a modified protocol from Franco-Correa *et al.* [10]. The bacteria were grown for 24-48 hours in Nutrient Agar (NA). The bacteria was harvested and cleaned with TE buffer and centrifuged at 13,500 rpm for 10min. The pellet was used for DNA extraction. Pellet of cell bacteria was dispersed in 500 µL lyses buffer and incubated at 37^{o}C for 60 min. Ten µL of of a proteinase K solution was modified to one µL (20 mg ml−1) was added and the suspension was re-incubated at 65^{o}C for 30 min. The lysate was extracted with an equal volume of cool phenol, mixed gently and centrifuged at 13,500 rpm for 10 min. The aqueous layer was re-extracted with equal volume of phenol:chloroform (1:1) and centrifuged at 13,500 rpm for 10 min. The supernatant was collected andfollowed by the addition of cool NaCl and 2 volumes of cool 95% (v/v) ethanol. The solution was centrifuged at 13,500 rpm for 10 min. The precipitated DNA was cleaned with 50 µl of 70% (v/v) ethanol and centrifuged at 13,500 rpm for 10min. The pellet DNA was dried and resuspended in 50 µl of nuclease free water. Finally DNA was stored at −20^{o}C.

2.3. nifH gene amplification

Amplification of *nif*H gene was performed using Gradient PCR machines (Eppendorf Mastercycler Gradient PCR System 5331). PCR was conduccted using primer pair of *nif*HF (5'-GGCAAGGGCGGTATCGGCAAGTC-3') and *nif*HR (5'-CCATCGTGATCGGGTCGGGATG-3'). The *nif*H gene amplification was carried out in a total volume of 25 µL containing Ultrapure water, 5x Taq buffer, 50 µM of each primer, 25 mM $MgCl_2$, 100 µM dNTPs, 5U/µL Taq polymerase, and DNA template. The PCR conditions was set as follows: initial denaturation at 95°C for 90 sec, followed by 30 cycles of denaturation at 94°C, for 60 sec; annealing at 57^{o}C, for 60 sec; elongation at 72^{o}C, for 60 sec and final extension at 72^{o}C for 5 min, finally at 4^{o}C for 20 min. PCR products were analyzed using 2% agarose gel. Gel was soaked in ethidium bromide solution (5 µgmL^1) for 30 min, rinsed with 1X TAE buffer, and the results were detected using a UV transilluminator.

3. Result and Discussion

The plant material was taken from experiment garden in Cibinong Science Center, which previously covered by Alang-alang (*I. cylindrica*). Invasions of non-native species (*Imperata cylindrica*) can suppress biodiversity and alter ecosystem functions. It showed that the soilwas used for sorghum cultivation haslow microbial diversity and organic matter. Conversion of *Imperata* grassland to agricultural land is considered as a way to improve the soil ecosystem.

Endophytic bacteria were successfully isolated from sorghum plant using spread plate method. Population of endophytic bacteria associated with sorghum ranging from 3 to 4 log (cfu/g). Population of endophytic bacteria recovered from sorghum plant were not differed between treatments. There was no discernible pattern in the population among treatment and concentration. However, endophytic bacteria population differed between part of plants. Population of endophytic bacteria obtained from root were higher compared to stem (Fig 1). This may be caused by the content of root compounds. The plant uses the root to store starch, protein, fat and other nutrients which are useful

LIPI
SATREPS
Science and Technology Research Partnership for Sustainable Development Program
Proceedia 2017
JICA
JST
The 1st SATREPS Conference, Bogor Nov 14th, 2016
"The Project for Producing Biomass Energy and Material through Revegetation of Alang-alang (*Imperata cylindrica*) Fields"

for the plant and its endosymbionts. Population of endophytic microbes in root were the highest compared other part of plants, due to root is the earliest place for microbes entering the plant [11].

Endophytic bacteria appear to originate from seeds, vegetative planting material, rhizosphere soil and the phylloplane [12]. Bacteria entry into plant tissue could be via stomata, lenticels, wounds (including broken trichomes), areas of emergence of lateral roots, and germinating radicles [12]. However, the main entry for endophytic bacteria appears to be through wounds that naturally occur as a result of plant growth. The highest population of endophytic bacteria could be found from sorghum treatmented with compost and inoculant. It could be possible reason that bacterial from compost and inoculant entry into plant and colonize parts of plant, stay for all or part of their life cycle without causing damage or illness to host.

Population of endophytic bacteria influenced by species, age, tillage, and environment conditions. Age of sorghum were 2 months and this periode sorghum plants are in the reproductive stage. Microbial population will reach a maximum in the reproductive stage compared to other growth stage. As a mature plants, all of the nutrients needed by bacteria are abundant, therefore stable population of endophytic bacteria will be obtained [11]. Vendan *et al.* reported that age of plants influence the variation of endophytic community in the gingseng plants [13]. The diversity of microbial endophyte is a function of the different maturation stages,it may influence type and amount of root exudates [14].

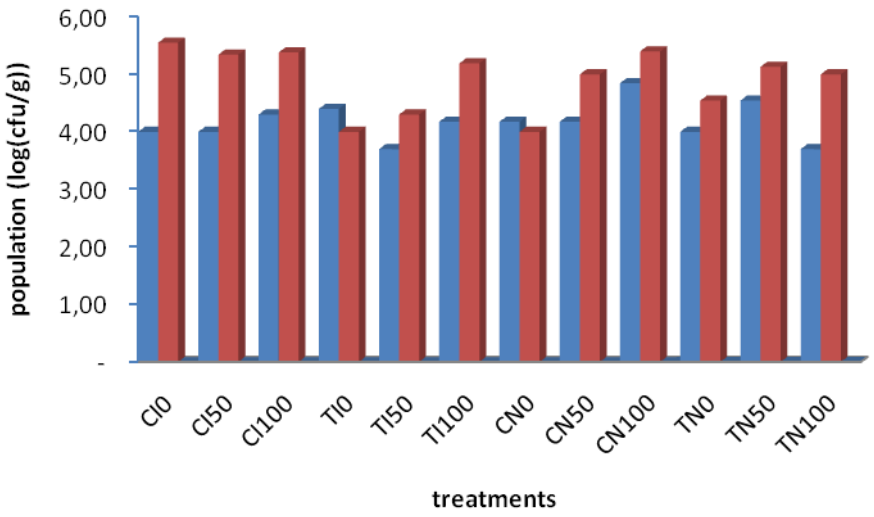

Fig 1. Population of endophytic bacteria associated with sorghum plant (stem root)
Note: CI: compost and inoculants; TI: inoculants; CN: compost without addition inoculants; TN without compost and inoculant

A total of 220 isolates of endophytic bacteria were obtained by surface sterilization method. Based on the morphological characteristics, 89 were selected for further studies (Fig 2). Among the 89 selected isolates, 51 isolates from stem (57%) and 38 from root (43%) (Table 1). Based on the data presented in Table 1, bacteria from stem more diverse compared to root. Here inafter the selected isolates were used for futher studies.

LIPI
SATREPS
Science and Technology Research Partnership for Sustainable Development Program
Proceedia 2017
JICA
JST
The 1st SATREPS Conference, Bogor Nov 14th, 2016
"The Project for Producing Biomass Energy and Material through Revegetation of Alang-alang (*Imperata cylindrica*) Fields"

Table 1. The number of selected endophytic bacterial isolates obtained from stem and root of sorghum plant

Treatment	Endophytic bacteria population	
	Stem	Root
CI	11	4
TI	12	15
CN	15	12
TN	13	7
Total	51	38

Note: CI: compost and inoculants; TI: inoculant, CN: compost without addition inoculants; TN: without compost and inoculants.

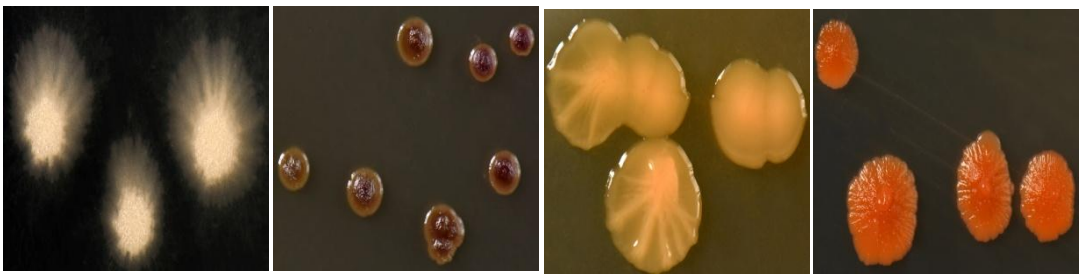

Fig 2. Endophytic bacteria associated with sorghum plant

A collection of 188 putative endophytes from surface-sterilized stems and roots were constructed and characterized. Bacterial isolates were shown belongs to different genera including *Pantoea*, *Enterobacter*, *Pseudomonas*, *Acinetobacter*, *Stenotrophomonas*, *Ralstonia*, *Herbaspirillum*, *Achromobacter*, *Rhizobium*, *Chryseobacterium*, *Kocuria*, *Brevibacillus*, *Paenibacillus*, *Bacillus* and *Staphylococcus* [15]. A total of 301 isolates of endophytic bacteria were collected from Maize plant cultivated on Acrisols of the Eastern of South Vietnam was reported by Thanh and Diep [16]. Hameed *et al.* also reported that as much as 52 endophytic bacterial encompassed 5 classes and 20 genera obtained from various tissues of four different rice cultivars [17]. Therefore, it seems that endophytic bacteria in sorghum plant were collected from revegetation of Alang-alang fields were diverse. It could be possible reason that the addition of inoculant could improve the microbial diversity in soil and plants were planted in those area.

Observation on Jensen's medium streaked by endophytic bacteria revealed that 16 out of 89 isolates were able to grow on Jensen's medium. Most of the colonies growing on Jensen's medium appeared to be translucent and mucilaginous (Fig 3). Jensen's medium is nitrogen free medium and is designed for detection and cultivateon of N_2-fixing bacteria. Bacterial growth on Jensen's medium indicate that these bacteria are able to fix nitrogen. Colour alteration of Jensen's media supplemented by bromothymol blue from greenish blue to dark blue suggested that the ammonium has been produced. Ammonium is alkaline and can increase the pH of the medium. Increasing of pH in the media after inoculated with endophytic bacteria resulted in the changes of their colour. This simple method to detect N_2-fixing bacteria using Jensen's medium was also performed by Kayasth*et al.* on free-living N_2-fixing isolated from Haryana soil samples [18]. Several of nitrogen fixing endophytic bacteria as acid producing. It could be seen from the colour canges from greenish blue to yellow.

LIPI
SATREPS
Science and Technology Research Partnership for Sustainable Development Program
Proceedia
2017
The 1st SATREPS Conference, Bogor Nov 14th, 2016
"The Project for Producing Biomass Energy and Material through Revegetation of Alang-alang (*Imperata cylindrica*) Fields"

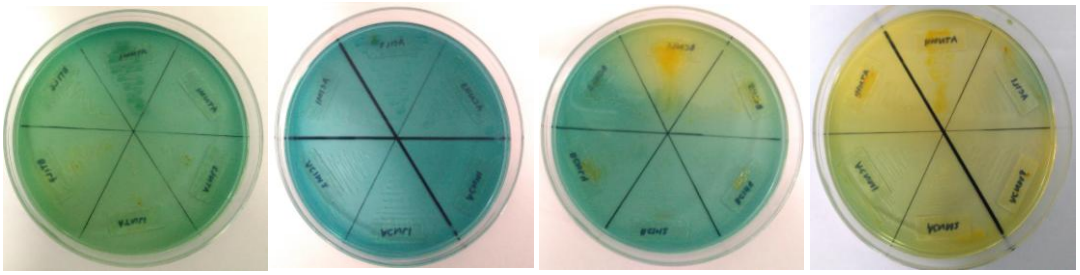

Fig 3. Endophytic bacteria grown on Jensen's medium agar plate (greenish blue no nitrogen fixing activity; dark blue: nitrogen fixing activity yellow: acid producing

Table 2.Endophytic bacteria associated with sorghum plants with nitrogen fixing activity

No.	Isolate	Nitrogen fixing activity
1	BCIM1	+
2	BCNL1	+
3	BCNL2	+
4	ACIL2	+
5	ATIM2	+
6	ATIM5	+
7	ATIM7	+
8	ATIH1	+
10	ATIH3	+
11	ACNM2	+
12	ACNM4	+
13	ACNM5	+
14	ACNM6	+
15	ATNM2	+
16	ATNM4	+

Based on *nif*H gene PCR amplification of 3 isolates, 2 isolates (ATIM2 and ACNM5) showed a proprietary of *nif*H gene. This was indicated by the presence of expected size of DNA in the gel electrophoresis as about 270 bp (Fig 4). Among 13 isolates of 16 isolates still on going study. In this study *Escherichia coli*was used as a negative control was not found the DNA band as that produced by *Rhizobium* isolate was used as a positive control. Other genera notoriously for their ability to fix nitrogen, such as *Bradyrhizobium*, *Klebsiella*, *Burkholderia*share the same trait by having *nif*H gene in their genomes.

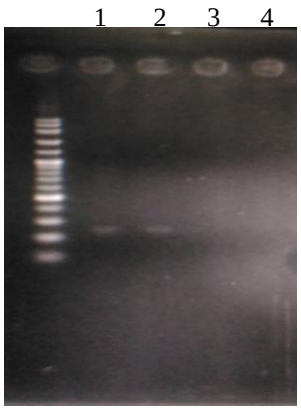

Fig 4. Gel electrophoresis of *nifH* gene amplified by PCR. Well: (1) Marker, (2) ATIM2, (3) ACNM5, (4) BCNL2

LIPI
SATREPS
Science and Technology Research Partnership for Sustainable Development Program
Proceedia 2017
The 1st SATREPS Conference, Bogor Nov 14th, 2016
"The Project for Producing Biomass Energy and Material through Revegetation of Alang-alang (*Imperata cylindrica*) Fields"

The *nifH* gene encoded nitrogen as ereductase subunit is the most sequenced gene and has become the marker gene for studying phylogeny, diversity, and abundance of N_2-fixing microorganisms [19]. It was also reported that many features of *nifH*-based phylogenetic tree are entirely consistent with 16S rRNA-based phylogeny of N_2-fixing bacteria. For this reason, *nifH* gene can be used to study bacterial community capable of fixing nitrogen [20].

Nitrogen fixing endophytic bacteria have been isolated from sugarcane [21] and other plants, e.g., rice, kallar grass and maize, and these bacteria supply fixed nitrogen (N) to their hosts [12]. Other nitrogen fixing bacteria, including *Azospirillum* spp., *Herbaspirillum* spp., *Burkholderia* spp., *Enterobacter cloacae*, *Klebsiellaoxytoca*, *Klebsiellapneumoniae* and *Pantoea* sp., were reported to have been isolated from the roots, stems and leaves of sugarcane [22]. Jia*et al*. also reported that 576 endophytic bacteria were succesfully isolated from the leaves, stems, and roots of 10 rice cultivars and based on *nif* gene amplification, 12 isolates were able to fixing nitrogen [23].In conclusion, that there are differences in the population and number of endophytic bacteria isolates recovered from part of sorgum plant were cultivated in revegetation of Alang-alang fields and several endophytic bacteria from sorgum has activity to fixing nitrogen.

4. Acknowledgements

This research was funded by SATREPS-JST-JICA program entitled "Producing biomass energy and material through revegetation of Alang-alang (*Imperatacy lindrica*) fields". We also would like to thank Rinatu Siswi and Riesca Martdiyanti for their help during the research period.

5. References

1. Amelework B, Shimelis H, Tongoona P, Laing M. Physiological mechanisms of drought tolerance in sorghum, genetic basis, and breeding methods: A review. *Afr J Agr Res*. 2015. 10(31): 3029-3040. DOI: 10.5897/AJAR2015.9595.
2. MacDonald GE.Cogongrass (*Imperatacylindrica*)—biology, ecology, and management. *Critical Reviews in Plant Sciences*. 2004. 23: 367-380. http://dx.doi.org/10.1080/07352680490505114.
3. Sari WE, Solihin SS, Lestari Y. Identification of endophyticactinomycetes from Indonesia rice plant based on 16S rRNA and *nif*H genes analyses. *AdvEnviron Biol*. 2014. 8(7): 2357-2365.
4. Zinniel DK, Lambrecht P, Harris NB, Feng Z, Kuczmarski D, Higley P, Ishimaru CA, Arunakumari A, Barletta RG, Vidaver AK. Isolation and characterization of endophytic colonizing bacteria from agronomic crops and prairie plants. *Appl Environ Microbiol*. 2002. 68(5): 2198-2208. doi: 10.1128/AEM.68.5.2198-2208.
5. Ryan RP, Germaine K, Franks A, Ryan DJ, Dowling DN. Bacterial endophytes: recent developments and applications. *FEMS MicrobiolLett*. 2008. 278: 1-9. DOI:10.1111/j.1574-6968.2007.00918.x
6. Hongrittipun P,Youpensuk S, Rerkasem B. Screening of nitrogen fixing endophytic bacteria in *Oryza sativa* L. JAgrSci. 2014. 6(6): 66-74. doi:10.5539/jas.v6n6p66.
7. Minerdi D, Fani R, Gallo R, Boarino A, Bonfante P. Nitrogen fixation genes in an endosymbiotic*Burkholderia* strain. *Appl Environ Microbiol*. 2001. 67 (2): 725-732. DOI: 10.1128/AEM.67.2.725–732.2001.
8. Maropola MK, Ramond JB, Trindade M.Impact of metagenomic DNA extraction procedures on the identifiable endophytic bacterial diversity in Sorghum bicolor (L. Moench). *J Microbiol M*eth. 2015.112:104-17. doi: 10.1016/j.mimet.2015.03.012.

9. Ahmad F, Ahmad I, Khan MS. Screening of free-living rhizospheric bacteria for their multiple plant growth promoting activities. *Microbiol Res.* 2008. 163: 173–181.
10. Franco-Correa M, Quintana A, Duque C, Suarez C, Rodríguez MX, Barea J. Evaluation of actinomycete strains for key traits related with plant growth promotion and mycorrhiza helping activities. *Appl Soil Ecol*.45. 2010. 209–217.
11. Dalal J, Kulkarni N. Population dynamics and diversity of endophytic bacteria associated with soybean (*Glycine max* (L) Merril). *Brit Microbiol Res* J.2013. 3:96−105.
12. Hallmann J,Quadt-Hallmann A, Mahaffee WF, Kloepper JW.Bacterial endophytes in agricultural crops: Reviews. 1997. *Can J Microbial*. 43: 895 -914.
13. Vendan RT, Yu YJ, Lee SH, Rhee YH. Diversity of endophytic bacteria in ginseng and their potential for plant growth promotion. *J Microbiol*. 2010. 48:559−565.
14. Ferreira A, Quecine MC, Lacava PT, OdaS, Azevedo JL, Araújo WL. Diversity of endophytic bacteria from *Eucalyptus* speciesseeds and colonization of seedlings by*Pantoeaagglomerans*. *FEMS MicrobiolLett*.2008. 287:8−14.
15. Mareque C, Taule C, Beracochea M, Battistoni F. Isolation, characterization and plant growth promotion effect of putative bacterial endophytes associated with sweet sorghum (*Sorghum bicolor* (L) Moench). *Ann Microbiol*. 2015. 65: 1057-1067. doi:10.1007/s13213-014-0951-7.
16. Dang Thi Ngoc Thanh, Cao Ngoc Diep. Isolation, Characterization and Identification of Endophytic Bacter.
17. ia in Maize (*Zea Mays* L.) Cultivated on Acrisols of the Southeast of Vietnam. Am *J Life Sci*. 2014. 2(4): 224-233. doi: 10.11648/j.ajls.20140204.16 .
18. Hameed A, Meng-Wei Y, Yu-Ting H, Wei-Ching C, Chaur-Tsuen L, Li-Sen Y. Diversity and functional characterization of bacterialendophytes dwelling in various rice (*Oryza sativa* L.) tissues,and their seed-borne dissemination into rhizosphere under gnotobiotic P-stress. *Plant Soil*. 2015. 394:177–197. DOI 10.1007/s11104-015-2506-5.
19. Kayasth M, Gera R, Dudeja SS, Sharma PK, Kumar V. Studies on salinization in Haryana soils on free-living nitrogen-fixing bacterial populations and their activity. *J Basic Microbiol*. 2014. 54(3):170-179. doi: 10.1002/jobm.201200158.
20. Gaby JC, Buckley DH. A Comprehensive Evaluation of PCR Primers to Amplify the *nif*H Gene of Nitrogenase. *PLoS ONE*. 2012. 7(7): e42149.doi:10.1371/journal. pone.0042149.
21. Poly F, Monrozier LJ, Bally R. Improvement in the RLFP procedur for studying the diversity of *nif*H genes in communities of nitrogen fixers in soil. 2001. *Res Microbiol* 152: 95-103. S0923-2508(00)01172-4/FLA.
22. Loiret FG, Ortega E, Kleiner D, Ortega-Rode´s P, Rode´s R and Dong Z.A putative new endophytic nitrogen-fixingbacterium *Pantoea* sp. from sugarcane. *J Appl Microbiol*. 2004, 97, 504–511 doi:10.1111/j.1365-2672.2004.02329.x
23. Mendes R,Pizzirani-Kleiner AA,Araujo WL,Raaijmakers JM. Diversity of Cultivated Endophytic Bacteria from Sugarcane: Genetic and Biochemical Characterization of *Burkholderiacepacia* Complex Isolates.*Appl Environ Microbiol*. 2007 Nov; 73(22): 7259–7267.doi: 10.1128/AEM.01222-07.
24. Jia SH, Gururanib MA, Chuna S. Isolation and characterization of plant growth promoting endophyticdiazotrophic bacteria from Korean rice cultivars. *Microbiol Res*. 2014.169:83– 98.