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Plant growth promotion characterization of endophytic bacteria from *Sesbania sesban* (L.) Merr. collected in Tan Hung district, Long An province, Vietnam

Nguyen Thi Phuong Vy [1], Dang Thi Ngoc Thanh [2], Pham Van Ngot [1] and Hoang Minh Tam [2,*]

[1] *Faculty of Biology, HCMC University of Education, 280 An Duong Vuong Street, Ward 4, District 5, Ho Chi Minh City, Vietnam*
[2] *Faculty of Natural Science Pedagogy, Sai Gon University, 273 An Duong Vuong Street, Ward 3, District 5, Ho Chi Minh City, Vietnam.*

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Abstract

The study aimed to isolate and characterize endophytic bacteria associated with *Sesbania sesban* (L.) Merr. collected in Tan Hung district, Long An province, Vietnam. Bacterial endophytes were isolated and screened capacity of plant growth promotion by using LGI, Burks' nitrogen free, NBRIP medium. Quantifying bacterial strain's probability of nitrogen fixation, phosphate solubilization and IAA production was based on colorimetric methods. Isolates with the most potential of nitrogen fixation, phosphate solubilization were identified by using MALDI-TOF Mass Spectrometry. The results were that eighteen bacterial endophytes were isolated from *Sesbania sesban* (L.) Merr. roots. They had characteristics of promoting plant growth by nitrogen fixation, phosphate solubilization and IAA biosynthesis. DD2 and DD18 with the best capacity of nitrogen fixation and phosphate solubilization were identified as *Klesiella pneumonia* and *Priestia megaterium*, respectively. Both were previously reported as plant growth promotion bacteria.

Keywords: Bacterial endophyte; IAA production; Nitrogen fixation; Plant growth promotion; Plant growth promoting bacteria; Phosphate solubilization

1. Introduction

The genus *Sesbania* with about 70 species were widely distributed in tropical and subtropical regions. In various countries, *Sesbania* were commonly used to improve soil fertility. *Sesbania cannabina* was used as a green manure on oceanic Islands. In Fiji and India, *Sesbania cannabina* was used as green manure for coconut, rice, sugarcane [1]. *Sesbania grandifora* was grown as a green manure crop providing green manure along rice paddies in Southeast Asia. *Sesbania speciosa* has been grown as a green manure in South India. The reasons for applying *Sesbania* as green manure were *Sesbania* species contained high nitrogen content and micronutrients. A study of application of *Sesbania bispinosa, Sesbania rostrata*, and *Sesbania speciosa* as "biofertilizers" for rice was found that *Sesbania rostrata* formed root and stem nodules and consisted of the highest nitrogen content being twice as much as *Sesbania bispinosa* and *Sesbania speciosa*. Among three species, *Sesbania rostrata* accumulated the highest amount of manganese, zinc, and copper and *Sesbania bispinosa* consisted of the highest iron content.

High nitrogen content in *Sesbania* was from symbiosis with diazotrophic bacteria. About 40 *Sesbania* species formed nodules after infection with diazotrophic bacteria - symbiotic nitrogen-fixing bacteria [2]. Diazotrophic bacteria symbiosing with *Sesbania* ssp. belonged to genus *Rhizobium, Mesorhizobium, Sinorhizobium, Azorhizobium*, *Neorhizobium, Bradyrhizobium, Ensifer* and Agrobacterium [2, 3]. *Neorhizobium huautlense* interacted symbiotically with *Sesbania herbacea* [4]; *Azorhizobium caulinodans* and *Bradyrhizobium sp*. associated symbiotically with *S. rostrata*

* Corresponding author: Hoang Minh Tam.

[5, 6, 7]; *A. doebereinerae* formed nodules with *S. virgate* [8 16]; *Ensifer teranga* and *E. saheli* associated symbiotically with *S. rostrate* and *S. cannabina* [5]; *Mesorhizobium plurifarium* isolated from *S. punicea, S. sericea* and S. *herbacea*; *N. huautlense* isolated from *S. sericea* and *S. exasperate* [9, 10]; *Rhizobium gallicum* associated symbiotically with *S. sericea* and *S. sesban*. *Agrobacterium salinitolerans* sp. nov., a saline-alkaline-tolerant bacterium isolated from root nodule of *Sesbania cannabina* [11]. *S. sesban* had the broader spectrum of nodule-inducing rhizobial species from *Rhizobium, Mesorhizobium, Ensifer* and *Allorhizobium* [12, 13].

Eight *Sesbania* species were found in Vietnam [14] and applied for soil improvement in agriculture. *Sesbania rostrata* L. had the ability to fix biological nitrogen and formed nodules on both stems and roots and often grown as a green manure crop in rice fields [15]. Burying *Sesbania sesban* plants effectively increased the content of digestible nitrogen (NH_4^+ and NO_3^-) and nitrogen total in the soil [16]. Growing *Sesbania* improved nutrients of saline rice soil and planting *sesbania* on non-saline land and 3‰ salinity soil increased rice productivity [17].

However, up to now, when conveyed interaction of plant growth-stimulating bacteria with *Sesbania* ssp., majority of researches seemed to focused on nitrgen-fixing bacteria related to *Sesbania* ssp. nodules. In Vietnam, researches on plant growth promotion bacteria associating with the genus *Sesbania* have been still very limited. Therefore, to better understand the relationship between plant growth-promoting bacteria and *Sesbania* ssp., this study was conducted. The aims of this study were to (1) isolate endophytic bacteria from *Sesbania sesban* (L.) Merr. roots without nodules collected in Tan Hung district, Long An province, Vietnam and screen their capacity of nitrogen fixation and phosphate solubilization, (2) quantify capacity of nitrogen fixation, phosphate solubilization and IAA production of isolated bacterial strains and (3) identify bacteria isolates with high potential of nitrogen fixation and phosphate solubilization.

2. Material and methods

2.1. Sample collection and preparation

Roots without nodules of *Sesbania sesban* (L.) Merr growing in Tan Hung district, Long An province, Vietnam were collected, cut into segments with 2 - 3 cm in length, washed with tap water to remove attached soil, let be dried at room condition and stored at 4 - 5 ℃ for later uses. The root samples were rewashed with distilled water for 3 - 4 times, immersed and shook in 70% ethanol in one minute, washed with distilled water 3 - 4 times. The roots then shook with fresh sodium hypochlorite solution 5% for 15 - 30 minutes, rinsed with sterile distilled water for 3 - 4 times. The aliquots of the last washed water were streaked on TYGA medium petri dishes. The plates were incubated at 28 ± 2 ℃ for 24 hours. If there were no growing microorganisms, the root surface were sterilized and used for further experiments [18].

2.2. Isolation of endophytic bacteria

Sterilized root samples were macerated with a sterile mortar and pestle and mixed well with 1.0 mL of distilled water. Tissue extracts were then made a serial dilution of up to 10^{-6} dilution by adding 1 mL of well-shaken suspension and into 9.0 mL water blank tubes. 500 µl-aliquot samples placed on test tubes containing 3.0 mL semi-solid LGI medium. The tubes were incubated at 28 ± 2 ℃ for 2 - 4 days. Bacteria, growing a white or yellow pellicle at a depth of 1 to 4 mm was streaked on LGI agar plates and then subculture for purification purpose to obtain distinct colonies [19].

2.3. Morphological characterization of bacterial isolates

Morphology of colonies such as form, elevation, margin, surface and size were recorded after 48 hours of cultures on solid LB medium. Size, shape and motion of bacteria cells were observed by light microscopy. Gram of isolates was determined by the method as described by Nguyen et al., (2003) [20].

2.4. Preparation of standard bacterial suspensions

All endophytic bacteria were subcultured on Burk's nitrogen free medium [21] for detecting nitrogen fixation bacteria and on NBRIP medium for obtaining phosphate solubilization bacteria. Isolates grown on Burk's nitrogen free medium were of capacity of nitrogen fixation and developed on NBRIP medium with clear zones had ability to solubilize insoluble phosphates. All endophytic bacteria were assessed capacity of IAA synthesis.

For preparation of standard bacterial suspensions, bacteria strains were subcultured in flasks containing Burk's nitrogen free liquid medium and liquid NBRIP medium for quantifying ability of nitrogen fixation, IAA production; and phosphate solubilization, respectively [22]. Flasks were incubated on a shaker at 30 ℃, 120 rpm. After incubating for one - two days, the suspension of each strain was determined the cell density with a spectrophotometer at light

wavelength of 600 nm and adjusted to the McFarland standard 0.5 with concentration of 1.5 x 10^8 $CFUmL^{-1}$ (called standard bacterial suspensions) [23].

2.5. Quantification of nitrogen fixation, phosphate solubilization and IAA synthesis

Colorimetric methods were used to determining concentration of NH_4^+, P_2O_5 and IAA produced by targeted bacterial isolates. Five hundred µL of each standard bacterial suspension was grown in a falcon containing 20 mL of corresponding liquid media (Burk's nitrogen free medium for quantitating NH_4^+, IAA and NBRIP medium for quantitating P_2O_5). The falcons were placed on a shaker at 100 rpm and 30 °C. Culture fluid were collected at 2, 4, 6, and 8 days for quantitating NH_4^+, IAA and at 5, 10, 15 and 20 days for quantitating P_2O_5 and centrifuged at 12,000 rpm for 5 minutes to collect supernatants. Each supernatant then mixed with phenol nitroprusside, or ammonium molypdate or Salkowki reagent in volumetric ratio of 5:1, 5:1 and 2:1, respectively. The mixtures were incubated for 30 minutes to develop color. Optical density (OD) of colored solutions containing NH_4^+, P_2O_5 and IAA was recorded at light wavelength of 640 nm, 880 nm and 530 mm, respectively. Concentration (mgL^{-1}) of NH_4^+, P_2O_5 and IAA produced in the cell free supernatants were determined by comparing the recorded OD values against corresponding standard curves of NH_4^+, IAA and P_2O_5 solutions [24].

2.6. Identification of selected bacteria isolates

Isolates with the best capacity of nitrogen fixation and phosphate solubilization were chosen for identification. The isolates were cultured on LB for 24 hours and identified by MALDI-TOF Mass Spectrometry as descripted by Dang et al., 2018 [25].

2.7. Experiment design and data analysis

Experiments were set up in a completely randomized design and repeated three times. One-factor analysis of variance and Duncan's test with the value $\alpha = 0.01$ were carried out by using IBM SPSS Statistics 20.0.

3. Results and discussion

3.1. Morphological characterization of endophyte bacteria

Eighteen endophytic bacteria isolated from roots of *Sesbania sesban* (L) Merr. collected in Tan Hung district, Long An province, Vietnam and named as DD1 to DD18. Bacterial morphology of colonies was descripted as in Table 1 and Figure 1. Most of colonies were ivory white (44.44%) and opaque (33.33%). Light yellow and transparent colonies occupied of 16.67% and 5.56%, respectively. 83.33% colonies were with circular shape while 16.67% of colonies had irregular one. Regard to colony margin, colonies with entire margin accounted about 88.89% and with serrated margin were of 11.11%. About 77,78% and 22,22% colonies had raised and flat elevation. Colony diameters were arranged from 1.0 mm to 7.0 mm.

Table 1 Colony morphology of bacterial isolates on solid LB medium

Colony Morphology		**Number**	**Percentage (%)**
Colour	Transparent	1	5.56
	Opaque	6	33.33
	Ivory white	8	44.44
	Light yellow	3	16.67
Shape	Circular	15	83.33
	Irregular	3	16.67
Margin	Entire	16	88.89
	Serrated	2	11.11
Elevation	Raised	14	77.78
	Flat	4	22.22

Figure 1 Morphology of some bacterial colonies

(a): DD10; (b): DD16; (c): DD18

Regard to cell characteristics, among isolated endophytic bacterial strains, most of bacterial cells were short rod-shaped, occupied 72.22%. Bacterial cells with spherical shape occupied about 22.22% while with long rod- shaped cells just accounted for 5,56%. Percentage of Gram-negative bacteria was 55.56% and Gram-positive bacteria was 44.44% (Figure 2). Most strains of bacteria were capable of movement.

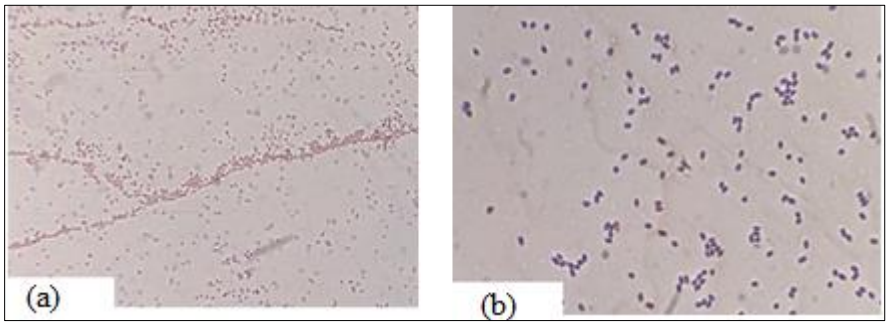

Figure 2 Gram of some bacteria

(a): DD3-Gram (-), (b): DD18-Gram (+)

Endophytes were microorganisms found inside the tissues and maintained their ability to infect plants [26]; in a strict sense, without causing symptoms to the plant [27]. In legume plants, large numbers of bacterial endophytes isolated from different tissues: roots, nodules, leaves, flowers and sprouts have been reported [27]. However, for *Sesbania ssp*., majority of studies of isolating endophytic bacteria focused on nodules. Presently, isolating bacterial endophyte from other tissues of these plants was noted. The results showed that besides colonizing in nodules, bacteria with diverse morphology resided and were found in other tissues of *Sesbania*. In research of diversity of culture dependent endophytic bacteria isolated from leguminous agroforestry trees in western Kenya, seven pure endophytic bacteria strains were isolated from *S. Sesban* leaves, roots and stems [28], in which, endophytic bacteria isolated from roots accounted 27.4%. Bacterial colonies had white, yellow and cream colour; most colony elevations were raised; and most colony margins were entire. In another study of bioactivity of endophytes from *Calliandra calothyrsus*, *Leucaena diversifolia* and *Sesbania sesban* against *Cercospora zeae*-maydis, twelve endophytic bacteria were isolated from leaves, roots and stem of *Sesbania sesban*. About 25% bacterial strains were isolated from roots [29]. Thus, investigation on bacterial endophytes from various tissues of *Sesbania sesban* had important contributions to study of bacteria endophyte diversity in *Sesbania sesban* in particular and legume plants in general.

3.2. Screening plant growth promotion characteristics

3.2.1. Ability of nitrogen fixation

Eighteen isolated endophytic strains all grew on Burks' nitrogen free medium. Capacity of isolates' nitrogen fixation was evaluated and presented in Table 2. Concentration of NH_4^+ produced by isolates were arranged from 0.183 to 0.357 mgL^{-1}. Three strains with the best capability of fixing nitrogen were DD12, DD2 and DD1with [NH_4^+] of 0.357 mgL^{-1}, 0.354 $mgL^{-1}$and 0.351 mgL^{-1}, respectively. However, these values were not statistically different. Four strains with ability to fix nitrogen ranking second among eighteen endophytic strains were DD3, DD4, DD5 and DD7 producing [NH_4^+] of 0.276 mgL^{-1}, 0.282 mgL^{-1}, 0.273 mgL^{-1} and 0.303 mgL^{-1} but not be statistically different.

Table 2 Quantification of nitrogen fixation ability of endophytic isolates

Isolate	$[NH_4^+]$ (mgL^{-1})	Isolate	$[NH_4^+]$ (mgL^{-1})
Negative control	0.00^{e}	Negative control	0.00^{e}
DD1	0.351 ± 0.019^{a}	DD10	0.321 ± 0.02^{ab}
DD2	0.354 ± 0.02^{a}	DD11	0.204 ± 0.019^{bcd}
DD3	0.276 ± 0.019^{abcd}	DD12	0.357 ± 0.033^{a}
DD4	0.282 ± 0.015^{abcd}	DD13	0.285 ± 0.014^{abcd}
DD5	0.273 ± 0.036^{abcd}	DD14	0.228 ± 0.016^{abcd}
DD6	0.201 ± 0.028^{bcd}	DD15	0.294 ± 0.009^{abcd}
DD7	0.303 ± 0.039^{abcd}	DD16	0.183 ± 0.012^{d}
DD8	0.222 ± 0.009^{bcd}	DD17	0.186 ± 0.035^{cd}
DD9	0.318 ± 0.024^{abc}	DD18	0.189 ± 0.014^{bcd}

Values in the same vertical column followed by one or more of the same letters are not significantly different at the 0.01 significance level according to Duncan's test.

3.2.2. Ability of phosphate-solubilization

All eighteen endophytic bacteria had phosphate solubilizing characteristics. Their phosphate solubilizing ability was determined through concentration (mgL^{-1}) of P_2O_5 produced in cell free supernatant and described in Table 3. $[P_2O_5]$ produced by isolates were arranged from 56.052 mgL^{-1} to 69.66 mgL^{-1}. Two isolates showing the highest phosphate solubilizing ability were DD18 and DD16 with $[P_2O_5]$ of 69.664 mgL^{-1} and 68.244 mgL^{-1}, correspondently. The next six isolates with ability of solubilizing phosphate ranking second were DD2, DD3, DD13, DD14, DD15 and DD17 with quantified $[P_2O_5]$ arranged from 60.62 mgL^{-1} to 64.95 mgL^{-1}.

Table 3 Quantification of phosphate solubilizing ability of endophytic isolates

Isolate	$[P_2O_5]$ (mgL^{-1})	Isolate	$[P_2O_5]$ (mgL^{-1})
Negative control	0.00^{g}	Negative ontrol	0.00^{e}
DD1	56.868 ± 0.029^{f}	DD10	57.104 ± 0.28^{f}
DD2	60.624 ± 1.522^{cdef}	DD11	57.596 ± 1.347^{ef}
DD3	61.356 ± 0.381^{cdef}	DD12	59.524 ± 0.457^{cdef}
DD4	57.876 ± 0.265^{ef}	DD13	60.712 ± 0.47^{cdef}
DD5	57.644 ± 0.751^{ef}	DD14	64.74 ± 1.289^{abcd}
DD6	56.052 ± 0.534^{f}	DD15	63.772 ± 0.485^{bcde}
DD7	58.716 ± 1.133^{def}	DD16	68.244 ± 0.638^{ab}
DD8	57.848 ± 0.273^{ef}	DD17	64.952 ± 0.999^{abc}
DD9	58.776 ± 1.045^{def}	DD18	69.664 ± 0.218^{a}

Values in the same vertical column followed by one or more of the same letters are not significantly different at the 0.01 significance level according to Duncan's test.

3.2.3. Ability of IAA synthesis

Similar to phosphate solubilization, all isolated endophytic bacteria had capacity of biosynthesis of IAA. Their capacity of IAA production was evaluated through concentration (mgL^{-1}) of IAA produced in cell free supernatant and presented in Table 4. [IAA] produced by isolates were arranged from 0.796 mgL^{-1} to 1.543 mgL^{-1}. DD16 produced the highest [IAA] of 1.543 mgL^{-1} and followed by DD9 with [IAA] of 1.39 mgL^{-1}.

Table 4 Quantification of IAA biosynthesis ability of endophytic isolates

Isolate	[IAA] (mgL^{-1})	Isolate	[IAA] (mgL^{-1})
Negative control	0.00^{e}	Negative control	0.00^{e}
DD1	0.951 ± 0.129^{cd}	DD10	1.128 ± 0.131^{bcd}
DD2	1.129 ± 0.17^{bcd}	DD11	1.173 ± 0.169^{bc}
DD3	1.275 ± 0.093^{abc}	DD12	1.257 ± 0.157^{abc}
DD4	1.111 ± 0.099^{bcd}	DD13	1.122 ± 0.06^{bcd}
DD5	0.796 ± 0.099^{d}	DD14	1.095 ± 0.073^{bcd}
DD6	0.982 ± 0.143^{bcd}	DD15	1.117 ± 0.065^{bcd}
DD7	1.058 ± 0.122^{bcd}	DD16	1.543 ± 0.357^{a}
DD8	1.299 ± 0.402^{abc}	DD17	1.111 ± 0.05^{bcd}
DD9	1.319 ± 0.297^{ab}	DD18	1.137 ± 0.163^{bcd}

Values in the same vertical column followed by one or more of the same letters are not significantly different at the 0.01 significance level according to Duncan's test.

Bacterial endophytes could accelerate and enhance plant growth through mechanism such as nitrogen fixation, phosphate solubilization, phytohormones… As discussed above, most bacterial endophytes of legume plants including *Sesbania* ssp were isolated from nodules and were described as rhizobia [2-13]. The results of investigation on plant growth promoting activities of bacterial isolates from *Sesbania bispinosa* showed that 20 isolates collected from the root nodules of *Sesbania bispinosa* could grew on nitrogen free medium and gave positive effects on ammonia production test [30]. Various number of endophytic bacteria showed ability to solubilize insoluble phosphate and synthesize IAA. Twenty rhizobia were isolated from *Sesbania grandiflora* root nodules and 80% of those rhizobial isolates were found to be phosphate solubilizers. Phosphate solubilisation index (P-SI) of these isolates ranged from 1.96 to 4.85. Five isolates were of excellent phosphate solubilization and suggested as biofertilizers [31]. Rhizobia strains, *Rhizobium* sp. U9709-SC isolated from *S. cannabina* was evaluated as a phosphate solubilizer. It produced a clear zone on the agar medium supplemented with insoluble phosphate [32]. 20 isolates were collected from the root nodules of *Sesbania bispinosa* had positive effects and created halo zones on NBRIP medium and exhibited high production of IAA [30]. In research on bioproduction of indole acetic acid by Rhizobium strains isolated from *Sesbania sesban* (L.) Merr, twenty-six Rhizobium strains were isolated from *Sesbania sesban* (L.) Merr. root nodules collected from regions of Andhra Pradesh. All of them were able to synthesize IAA. Five out of twenty-six strains produced maximum IAA when supplied with 2.5 $mgml^{-1}$ L-tryptophan [33]. In summary, for *Sesbania* ssp., besides plant growth promotion bacteria isolated from nodules, in this study, bacterial endophytes with plant growth promoting characteristics were also isolated from root tissues (instead of in nodules).

3.3. Identification of selected bacterial endophytes

Two bacterial endophytes with the highest potential of nitrogen fixation and phosphate solubilization, DD2 producing [NH_4^+] of 0.354 mgL^{-1} and DD18 producing [P_2O_5] of 69.664 mgL^{-1}, respectively were selected for identification. Using MALDI-TOF Mass Spectrometry, DD2 and DD18 were identified as *Klesiella pneumonia* and *Priestia megaterium*, respectively. *Klebsiella pneumoniae* belonging to Enterobacteriaceae was described as a gram-negative, encapsulate, non-motile, and rod-shaped bacteria bacterium [34]. Isolating source of *Klebsiella pneumonia* were various. In plants, *Klebsiella pneumonia* was isolated from maize, legume plants, rice…[35, 36]. *Klebsiella pneumonia* was a plant growth promoting bacterium that promoted plant growth by fixing nitrogen [37], solubilizing phosphate [38] and synthesizing indole-3-acetic acid [39]. *Priestia megaterium* (previously known as *Bacillus megaterium)* was a Gram-positive, rod shaped bacterium and found in *Phaseolus vulgaris, Trifolium pretense [*36, 40*]. P. megaterium* was described as a plant growth promoting bacterium. Its mechanism of plant growth promotion included transformation of phosphorus in minerals and organic sources to their bioavailable forms [41, 42]. *P. megaterium* was reported to produce IAA resulting in a plant growth promoting effect on different plants [43, 44].

4. Conclusion

Eighteen bacterial endophytes were isolated from *Sesbania sesban* (L.) Merr. roots. All strains had characteristics of promoting plant growth by nitrogen fixation, phosphate solubilization and IAA biosynthesis. Two strains with the best capacity of nitrogen fixation and phosphate solubilization were DD2 and DD18 and identified as *Klesiella pneumonia* and *Priestia megaterium*, respectively. Both were previously reported as plant growth promotion bacteria.

Compliance with ethical standards

Acknowledgments

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Disclosure of conflict of interest

There is no conflict of interest.

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