EVALUATION OF THE IAA-PRODUCING ABILITY OF SOME ENDOPHYTIC BACTERIA ISOLATED FROM *Panax pseudoginseng* ROOTS

Nguyen Van Giang[1], Nguyen Thanh Quan[2], Nguyen Thanh Nhung[2], Ta Thị Dieu Linh[2], To Hoang Anh Minh[2], Do Thanh Trung[1], Tran Thi Dieu Thuy[1], Nguyen Phan Bao Tran[3], Khuat Huu Trung[2], Tran Dang Khanh[2*]

ABSTRACT

The objective of this research was to evaluate the Indole-3-acetic acid (IAA) producing ability of isolated endophytic bacteria from *Panax pseudoginseng* roots through *in vitro* culture conditions. As a result attained, 16 endophytic bacterial strains, including both gram-positive and gram-negative bacteria were isolated from the collected roots of *P. pseudoginseng* and all of these isolates could produce IAA with different concentrations, which ranged from 4.61 µg/mL to 85.11 µg/mL, respectively. Among the strains, NN1 and NN6 resulted in the most productive IAA by 85.11 µg/mL and 69.29 µg/mL, respectively. Underlay of NA liquid medium combined with L-tryptophan, 6-day dark incubation with pH 6-8 showed a better synthesis of IAA. While, KNO_3 and $(NH_4)_2 HPO_4$ were considered the optimal nitrogen sources for IAA production of NN1 (56.36 µg/mL and 55.52 µg/mL) and NN6 (54.17 µg/mL and 42.93 µg/mL, respectively) strains, dextrin and saccharose were the best suitable carbon sources for bacterial isolates NN1 (59.17 µg/mL) and NN6 (67.24 µg/mL). The attained results suggest that the diversity of endophytic bacterial species in *P. pseudoginseng* roots and their IAA-producing potential are largely based on cultural techniques. Besides, the findings of this study can serve as a foundation for developing natural bioactivity products to improve the productivity of ginsengs.

Keywords: *Endophytic bacteria, Indole-3-acetic acid (IAA), carbon and nitrogen sources, Panax pseudoginseng, plant growth promotion.*

Received: 2 May 2022; revised: 12 July 2022; accepted: 20 August 2022

1. INTRODUCTION

Endophytic bacteria reside within living plant tissues without any harmfulness or nutrient competition with host plants [1]. These endophytic microorganisms can even restrain disease expression caused by some plant pathogens and promote plant growth [1, 2]. According to previous studies, the community of endophytic bacteria mostly exists in the rhizosphere or root surface, but they can break the endodermal barrier and move from the root cortex into the vascular system, and then inhabit tubers, stems, leaves, and other parts of the plants [3, 4]. Endophytic bacterial species are involved in promoting plant growth by diverse mechanisms, such as solubilizing mineral phosphates, fixing nitrogen, supplying micronutrients, producing siderophore, activating photosynthetic process, stimulating endogenous hormone activity, and promoting beneficial plant-microbe symbiosis [3, 5, 6, 7].

[1] Department of Biotechnology, Vietnam National University of Agriculture
[2] Institute of Genetics Agricultural, Vietnam Academy of Agricultural Sciences
[3] Grier School, 2522 Grier School Rd, Tyrone, PA 16686, USA
* Corresponding author: tdkhanh@vaas.vn

Furthermore, bacterial endophytes also play an important role in producing antibiotics and secondary metabolites to enhance the plant defence systems against soil pathogens and various environmental stresses [7, 8]. Several phytohormones including IAA, gibberellins, and cytokinins synthesized by the stem and root endophyte bacteria strains enable to promoting plant physiological processes and protect the host plant from biotic and abiotic stresses [9, 10]. IAA is an important endogenous auxin hormone for cell division and enlargement contributing to the formation and elongation of plant roots, root hairs, and branch roots [9-11].

P. pseudoginseng has been known as one of the most precious oriental herbs. In Vietnam, *P. pseudoginseng* is called "Tam That" which implies "three-seven roots" and it is usually used as a health tonic in traditional medicine. According to popular remedies, *P. pseudoginseng* can help to treat some human diseases, such as insomnia, nervous depression, tumors, cancer, cardiovascular neurasthenia, lumbar pain, haemorrhage, and nosebleeds [12, 13]. The studies have reported that this plant also possesses a high content of ginseng saponins, the most important phytoconstituent in *Panax* species, such as ginsenosides-Rb_3, -Rd, -Re, -Rg_1, -Rt_1, -F_{11} and -F_8 [14, 15], respectively. In recent years, due to the high increase of using the demand for medicinal plants, *P. pseudoginseng* is facing over-exploitation. Besides, present sources of *P. pseudoginseng* mostly depend on field cultivation, which requires time and farming techniques. To reduce the time of cultivation as well as shorten the growth cycle for harvesting, farmers usually use chemical reagents and fertilizers to enhance plant growth parameters. However, this farming method causes harmful impacts on the soil environment, it also affects the plant-beneficial microbes interaction, decreasing the accumulation of ginsenosides and the quality of *P. pseudoginseng* as well. To date, although numerous endophytic microbes from various crop plants have been isolated and assessed for their biocontrol and plant growth stimulation abilities, however, little available information on the characterization of bacterial endophytes from ginseng is reported. Therefore, this study aimed to examine the diversity of endophytic bacteria strains in *P. pseudoginseng* roots and evaluate their levels of IAA biosynthesis under the *in vitro* culture condition. The utility of some of these isolated bacterial endophytes as biocontrol reagents promoting plant growth will be estimated and further applied to develop natural bioactive products for improving the quality and productivity of *P. pseudoginseng*.

2. MATERIALS AND METHODS

2.1. Materials

The sample of *P. pseudoginseng* was collected in Lai Chau province and kept at Agricultural Genetics Institute (AGI), km 2, Pham Van Dong road, Co Nhue, Bac Tu Liem, Ha Noi, Vietnam.

The bacterial cultural mediums used in the study include: NA medium (g/L): pepton-5; NaCl-5; meat extract-2; yeast extract-3; agar-18; 1000 mL of H_2O. NBRIP culture (g/L): glucose-10; $Ca_3(PO_4)_2$-10; $MgCl_2.6H_2O$-5; $MgSO_4.7H_2O$, 0.25; KCl-0.2; $(NH_4)_2SO_4$, 0.1; 1000 mL of H_2O; pH 7.0. Basal mineral medium (g/L): K_2HPO_4-1.73; KH_2PO_4-0.68; $MgSO_4.7H_2O$-0.25; NaCl-4; $FeSO_4.7H_2O$-0.03; NH_4NO_3-1, $CaCl_2.2H_2O$-0.02; glucose-5.

2.2. Methods

The root samples of *P. pseudoginseng* were collected from healthy *P. pseudoginseng* plants and temporarily stored at 4°C until conducting isolation. Endophytic bacteria isolation was performed following a method described by Chen *et al.* (2014) [16]. Briefly, to remove the soil, *P. pseudoginseng*'s roots were washed under water tap, then they were cut into small pieces and

soaked in 75% ethanol for 2.5 minutes. Root sterilization was continuously treated with 3% sodium hypochlorite for 2 minutes and 75% ethanol for 30 seconds. After each time of sterilizing treatment, root samples were washed again with distilled water. To make sure the root surface is entirely sterilized, washing samples of distilled water would be tested on the NA-contained Petri dishes without any living microorganisms after 2 days of 30°C incubation. Next, sterilized root samples were inoculated into NA medium using Petri dishes and these dishes were put in an incubator at 30°C. The development of endophytic microorganisms was observed after several days of culture. As soon as appearing the development of individual single colonies, they were picked up and transferred to a new cultural medium and kept at 4°C for further experiments.

Indole acetic acid (IAA) production: Endophytic bacterial samples were inoculated into 20 mL of NA liquid medium containing L-tryptophan. Afterward, the solution was shaken at 200 rpm, incubated at 30°C for 48 hours, and then centrifuged at 3000 rpm for 15 minutes. Finally, to determine the IAA concentrations of isolated bacterial strains, 1 mL of supernatant from centrifugation was mixed with 2 mL of Salkowski reagent and incubated for 30 minutes. The appearance of red color in test mixtures showed a synthesis of IAA from bacterial isolates and the concentration was determined according to the Vietnamese National Standard TCVN 10784: 2015 [17].

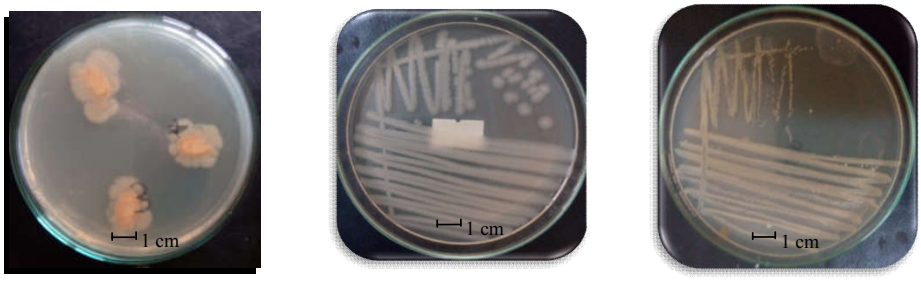
Effect of incubation time and pH on the production of IAA: In dark conditions, isolated bacterial strains were cultured in NA liquid medium supplemented with L-tryptophan (100 mg/L). The IAA content of each bacterial strain was separately measured every 24 hours of culture and in different pH values (ranging from 3.0 to 10.0). The measurement of IAA concentration was conducted following the Salkowski colorimetric method with the wavelength of ultraviolet absorption of the detector at 530 nm [17].

Effect of carbon and nitrogen sources on the production of IAA: The carbon sources were supplemented in basal medium (Trp^+NA liquid medium) including starch, lactose, dextrin, sucrose, D-sorbitol, and mannitol. The added sources of nitrogen were $(NH_4)_2SO_4$, NH_4Cl, NH_4NO_3, NH_4Cl, $(NH_4)_2HPO_4$, KNO_3, and peptone. Whereas, the controls only used basic mediums. The concentration of PO_4^{3-} is determined according to the method described above.

Statistical analyses: All the experiments were replicated three times. The data were analyzed by using Microsoft Excel ver 2016 and presented as the mean values.

3. RESULTS AND DISCUSSION

3.1. Isolating endophytic bacteria from the root of *P. pseudoginseng*



Isolation results NN1 Sample NN6 Sample

Figure 1. Endophytic bacteria isolated from the root of *P. pseudoginseng*

The sterilized samples of *P. pseudoginseng* roots were cultured in NA medium using Petri dishes and incubated at 30°C (Figure 1). In this experiment, a total of sixteen different endophytic bacteria were isolated.

Endophytic bacteria appeared from two root segments placed on the agar plate. Microbial samples were transplanted into LB medium for purification. Colonies of isolated bacteria samples expressed the diversity of shape, color, and size. Most of the colonies had milky white and yellow colors, with smooth surfaces. Among sixteen endophytic bacteria, there were 13 gram-positive bacteria and 3 gram-negative bacteria. Thus, these new strains of isolated endophytic bacteria may belong to different species or genera.

3.2. Evaluation of IAA synthesizing capacity of isolated bacterial strains under laboratory conditions

The results of IAA biosynthesis showed that all sixteen bacteria isolates were able to produce IAA (Figure 2). The highest contents of IAA were 85.11 µg/mL and 69.29 µg/mL produced by strains NN1 and NN6, respectively. Other bacteria samples synthesized lower IAA contents with different concentrations. According to the report of Tuyet and Giang [18], isolated twenty-one endophytic bacteria species from the turmeric's roots and almost all strains could synthesize IAA with various concentrations, ranging from 3.33 µg/mL to 65.38 µg/mL. Similarly, in the experiment of Hassan *et al.* (2017) [5], IAA contents synthesized by fungal and bacterial endophytes in the medicinal plant of *Teucrium polium* L. were 19.9 - 63.5 µg/mL and 4.1 - 23.4 µg/mL, respectively. From the roots of *Aloe vera*, Nguyen Van Giang *et al.* (2016) [6] found out 14 isolates of endophytic bacteria could synthesize IAA with contents from 16.49 - 36.32 µg/mL. In addition, Chandra *et al.* (2018) [19] also isolated two endophytic bacteria samples from *Stevia rebaudiana* rhizosphere CA1001 and CA2004 could produce very high contents of IAA, 91.7 µg/mL and 81.7 µg/mL, respectively. Our findings revealed that IAA biosynthesis from endophytic bacteria in *P. pseudoginseng*'s roots was quite popular and necessary for plant growth promotion. Among them, two isolates NN1 and NN6 showed the highest contents of IAA production, and they were selected for further analysis.

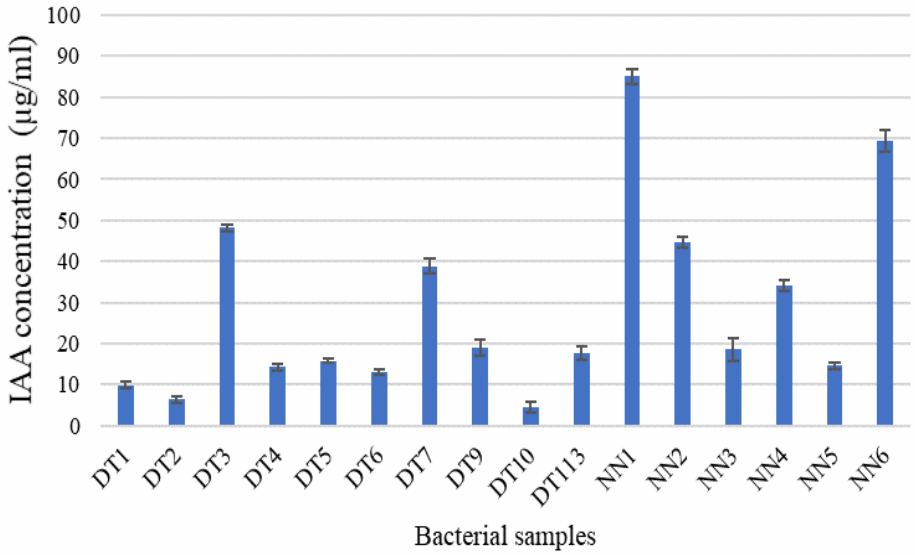


Figure 2. The amount of IAA production synthesized by endophytic bacteria

3.3. Effect of *in vitro* culture conditions on the ability of IAA production of NN1 and NN6 strains

3.3.1. Effect of incubation time

Generally, the content of IAA generated by NN1 and NN6 strains trends gradually increased in the first 6 days of culture (Figure 3). On the 6th day (after 144 hours), the concentration of IAA caused by NN1 and NN6 were the highest, comparing other days, 85.11 µg/mL and 69.29 µg/mL, respectively. However, on the 7th day, IAA content was reduced in both NN1 and NN6 strains (82.08 µg/mL and 64.22 µg/mL, respectively), suggesting that the capacity of IAA synthesis of NN1 and NN6 gets maximum value when nutrient sources are adequately supplied in a certain time of culture. On the contrary, in case of nutrient sources are insufficient or exhausted, the ability of IAA production of endophytic bacteria could be remarkably decreased. On the other hand, IAA-producing property also depends on the different characteristics of each bacterial species. Maximization of IAA content was determined the incubative time of *P. pseudoginseng* rhizobacteria in this study which is similar to several previous reports, but with diverse endophytic isolates from various plants. For instance, Kumari *et al.* (2018) [20] optimized IAA production (137.81 µg/mL) in rhizospheric bacteria of roadside weed, *Eragrostis cynosuroides* after 4-day incubation (96 hours) in JNFb$^-$ Trp$^+$ broth medium. Apine *et al.* (2011) [21] also reported that IAA synthesis caused by a bacterium *Pantoea agglomerans* strains from *Nicotiana tobacum* (leaf) explants were optimized under conditions as a medium containing meat extract and tryptophan, pH 7, and 2-day incubation in 30°C.

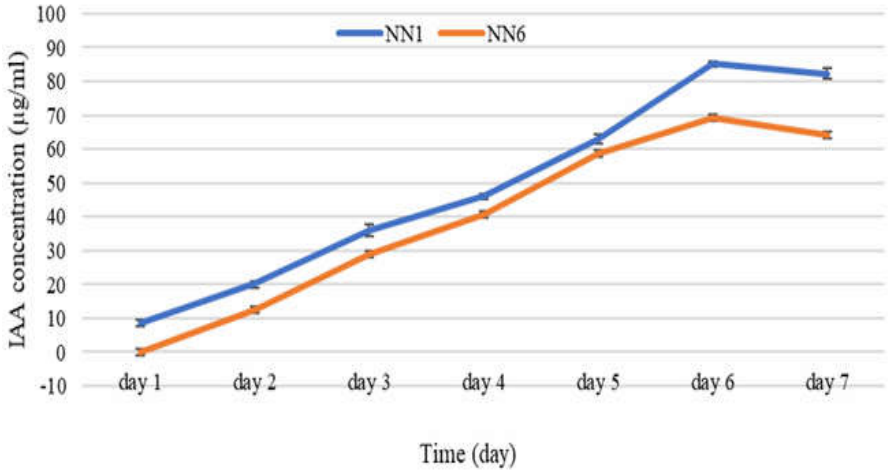


Figure 3. Effect of culture time on IAA synthesis ability

3.3.2. Effect of pH on IAA production of NN1 and NN6

The result exhibited all endophytic bacterial strains could produce IAA in the range of pH from 3.0 to 10.0. However, rhizobacterial strains cultured in Trp$^+$ NA liquid medium with the adjustment of pH 6.0-8.0 were assessed as the most appropriate for optimal producing IAA (Figure 4). Nita *et al.* (2011) [22] proved that the highest IAA content caused by *A. dazotrophicus* L1 strain was cultured in media with pH 6.0. In the experiment of Chandra *et al.* (2018) [19], bacterial isolates from *Stevia rebaudiana* rhizosphere including CA1001, CA2003, and CA2004 expressed the most synthesis ability of IAA contents ranging pH adjustment from 5.0 to 9.0, respectively. In other investigations, pH 6.0-7.0 was estimated as the most suitable and common adjustment for

rhizobacteria isolated from turmeric roots [18] and *Aloe vera* roots [6] to produce optimal IAA hormone.

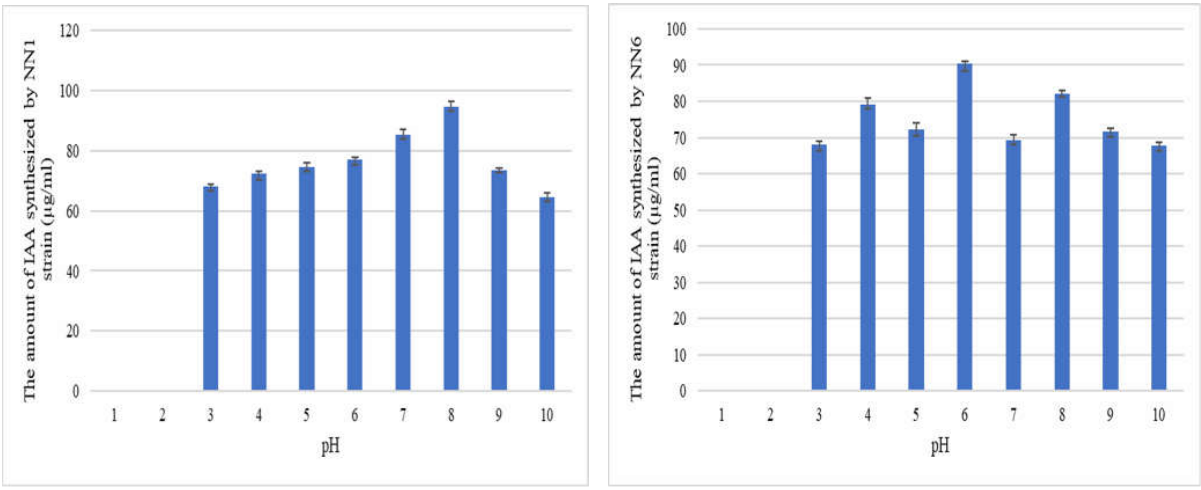


Figure 4. Effect of pH on the ability of IAA synthesis of NN1 (left) and NN6 (right)

3.3.3. Effect of carbon source on IAA production of NN1 and NN6

Carbon is one of the most important nutrient sources for endophytic microorganisms to synthesize the necessary components, essential enzymes or plant growth hormones. In this research, NN1 and NN6 strains cultured in Trp^+ NA medium were separately supplied with different carbon sources, including dextrin, lactose, sorbitol, saccharose, fructose, and xylose. Glucose was subjected to a carbon source used in the control sample.

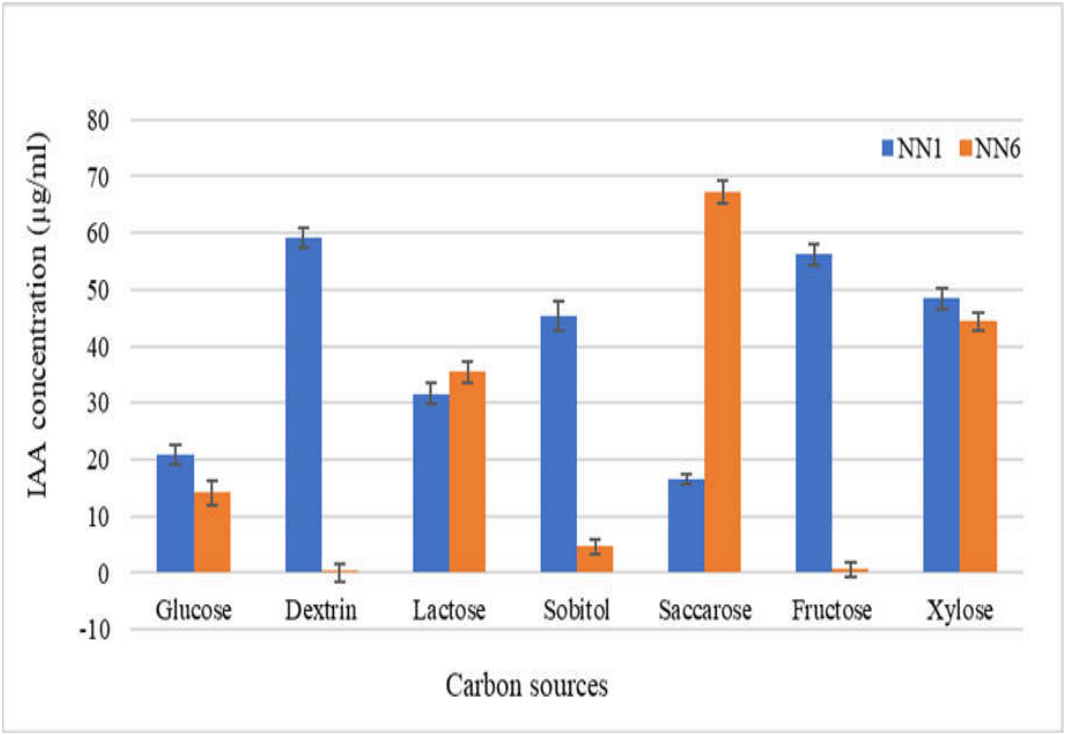


Figure 5. Effect of carbon source on IAA production of NN1 and NN6

The capacity of carbon absorption to produce IAA hormone in NN1 and NN6 strains are shown in figure 5. As a result, dextrin was revealed to be a suitable carbon source for NN1 when generating a maximum content of IAA (59.27 µg/mL). However, this carbon source virtually did not affect the IAA synthesis of NN6 (0.05 µg/mL). Nevertheless, saccharose was the appropriate carbon for NN6 strain to create the highest amount of IAA (67.24 µg/mL) and saccharose induced the inefficiency of IAA production in NN1 strain (16.63 µg/mL). Our results were in agreement with the report of Nita *et al.* (2011) [22] found that saccharose is a potential carbon source for the productive synthesis of IAA in *A. diazotrophicus* L1 strains from sugarcane.

3.3.4. Effect of nitrogen sources on the IAA production of NN1 and NN6

The influence of nitrogen sources including $(NH_4)SO_4$, NH_4Cl, NH_4NO_3, NH_4Cl, $(NH_4)HPO_4$, KNO_3, and peptone on the IAA-producing levels of NN1 and NN6 bacteria are shown in figure 6.

Generally, IAA content was synthesized by NN1 and NN6 strains which had significant differences between nitrogen sources. Supplement of the nitrogen sources KNO_3 and $(NH_4)_2HPO_4$ resulted in the highest contents of IAA production in both NN1 (56.36 µg/mL and 55.52 µg/mL, respectively) and NN6 (54.17 µg/mL and 42.93 µg/mL, respectively). This result is inconsistent with the results of Kumari *et al.* (2018) [20] who reported that in an environment containing KNO_3, the concentration of IAA was not high. However, Mohite *et al.* (2013) [23] concluded that a suitable source of nitrogen for synthesizing IAA of samples is KNO_3 and peptone.

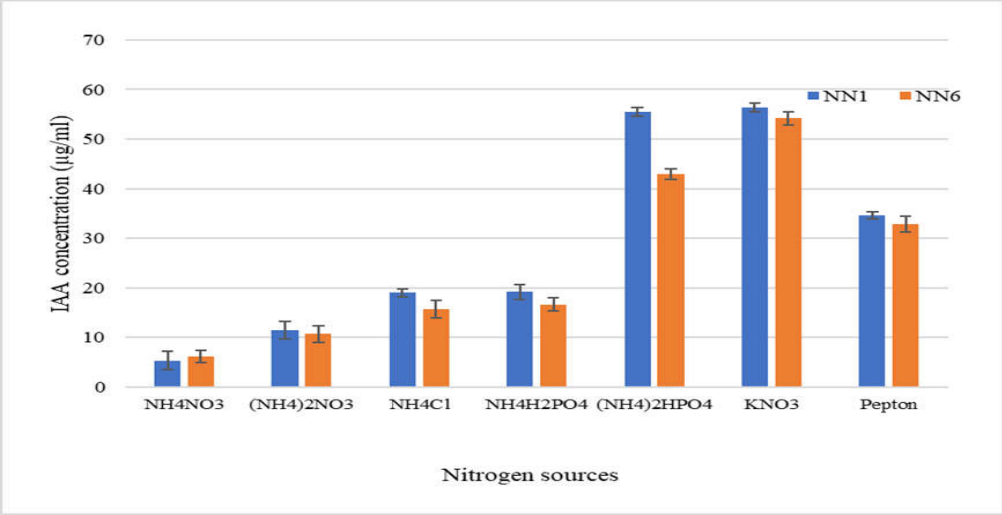


Figure 6. The effect of nitrogen on the IAA production of NN1 and NN6

3.4. Biological characteristics of endophytic bacteria

Table 1. Some biological characteristics of NN1 and NN6

Reaction	NN1	NN6
Gram staining	+	-
Catalase	+	+
Citrate	+	+
Mobility	+	+
MR reaction	-	-
VP reaction	+	+

Some biochemical characteristics of NN1 and NN6 strains were investigated and the main biological characteristics are presented in table 1 and figure 7.

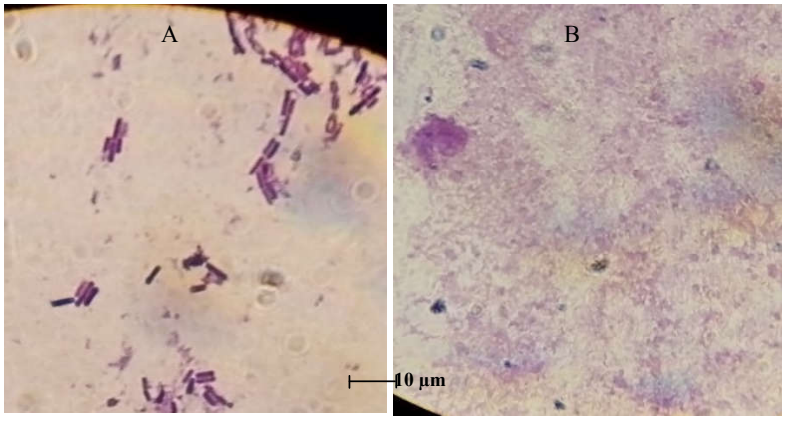


Figure 7. Gram stain results of NN1 (A) and NN6 (B)

4. CONCLUSIONS

From the roots of *P. pseudoginseng,* 16 samples of endophytic bacteria were isolated. All 16 bacteria samples were able to synthesize IAA, of which the samples NN1 and NN6 synthesized the most amount of IAA, with the concentrations at 85.11 µg/mL and 69.29 µg/mL, respectively. NN1 and NN6 bacteria strains produced the highest IAA concentration at 6 days in the nitrogen-contained medium of KNO_3 and (NH_4) HPO_4, pH 6-8. Lastly, dextrin and saccharose are suitable carbon sources for NN1 and NN6 bacteria to synthesize IAA with maximum levels. Cells of endophytic NN1 bacteria samples are bacilli, gram-positive, capable of mobility, cells of endophytic bacteria samples NN6 are bacilli, gram-negative, motile. Both NN1 and NN6 had positive reactions to catalase, VP, citrate reduction, and MR negative reaction. NN1 and NN6 bacteria strains are the potential candidates for the utmost production of IAA in laboratory conditions and these bacteria can be utilized as biofertilizers for promoting plant growth of *P. pseudoginseng*.

REFERENCES

1. Hallmann, J., Quadt-Hallmann, A., Mahaffee, W. F., & Kloepper, J. W. (1997). Bacterial endophytes in agricultural crops. *Canadian journal of microbiology, 43(10),* 895-914.

2. Chen, C., Bauske, E. M., Musson, G., Rodriguezkabana, R., & Kloepper, J. W. (1995). Biological control of Fusarium wilt on cotton by use of endophytic bacteria. Biological control, 5(1), 83-91.

3. Compant, S., Duffy, B., Nowak, J., Clément, C., & Barka, E. A. (2005). Use of plant growth-promoting bacteria for biocontrol of plant diseases: principles, mechanisms of action, and future prospects. Applied and environmental microbiology, 71(9), 4951-4959.

4. Liu, X., Dou, G., & Ma, Y. (2016). Potential of endophytes from medicinal plants for biocontrol and plant growth promotion. *Journal of general plant pathology,* 82(3), 165-173.

5. Hassan Saad El-Din (2017). Plant growth-promoting activities for bacterial and fungal endophytes isolated from medicinal plant of *Teucrium polium L. Journal of Advanced Research,* 8(6). 687-695.

6. Nguyen Van Giang, Tran Thi Dao, and Trinh Thi Thuy An (2016). Isolation and characteristics of some bacterial endophytes from root of *Aloe vera. Vietnam Journal of Agricultural Science,* 14(5), 772-778.

7. Perez-Rosales, E., Alcaraz-Meléndez, L., Puente, M. E., Vázquez-Juárez, R., Quiroz-Guzmán, E., Zenteno-Savín, T., & Morales-Bojórquez, E. (2017). Isolation and characterization of endophytic bacteria associated

with roots of jojoba (*Simmondsia chinensis* (Link) Schneid). Current Science, 396-401.

8. Ardanov, P., Sessitsch, A., Häggman, H., Kozyrovska, N., & Pirttilä, A. M. (2012). Methylobacterium-induced endophyte community changes correspond with protection of plants against pathogen attack. PLoS One, 7(10), 1-7.

9. Syamsia, S., Idhan, A., Firmansyah, A. P., Noerfitryani, N., Rahim, I., Kesaulya, H., & Armus, R. (2021). Combination on endophytic fungal as the Plant Growth-Promoting Fungi (PGPF) on cucumber (*Cucumis sativus*). *Biodiversitas Journal of Biological Diversity*, 22(3), 1194-1202.

10. Widowati, T., Nuriyanah, N., & Sukiman, H. (2013, December). Potency of endophyte bacterium isolated from *Shorea selanica* on producing IAA hormone and supporting the growth of soybean. *In Annales Bogorienses*, 17(2), 35-41.

11. Herlina L., Pukan K. K., and Mustikaningtyas D. (2017). The endophytic bacteria producing IAA (Indole Acetic Acid) in *Arachis hypogaea*. Cell Biology & Development. 1(1), 31-35.

12. Hanh, T. T. H., Cham, P. T., Anh, D. H., Cuong, N. T., Trung, N. Q., Quang, T. H., Cuong, N. X., Nam, N. H. and Minh, C. V. (2021). Dammarane-type triterpenoid saponins from the flower buds of *Panax pseudoginseng* with cytotoxic activity. Natural Product Research, pp.1-9.

13. Shukla, Y. N., Thakur, R. S., & Pachaly, P. (1992). A bidesmosidic oleanolic acid saponin from *Panax pseudoginseng*. Phytochemistry, 31 (3), 1046-1048.

14. Morita, T., Zhou, J., & Tanaka, O. (1986).Saponins from *Panax pseudoginseng* WALL.subsp. pseudo-ginseng HARA Collected at Nielamu, Tibet, China. Chemical and pharmaceutical bulletin, 34(11), 4833-4835.

15. Tanaka, O., & Yahara, S. (1978). Dammarane saponins of leaves of *Panax pseudoginseng* subsp. himalaicus. Phytochemi stry, 17(8), 1353-1358.

16. Chen T., Z. Chen, G. H. Ma, B. H. Du, B. Shen, Y. Q. Ding and K. Xu (2014). Diversity and potentialapplication of endophytic bacteria in ginger. Genetics and Molecular Research 13 (3), 4918-4931.

17. Vietnam Ministry of Science and Technology (2015). TCVN 10784: 2015: Microorganisms - Determination of indole-3-acetic acid (IAA) synthesis capability.

18. Tran Thi Tuyet and Nguyen Van Giang (2017). Isolation and characteristics of some bacterial endophytes from root of turmeric (*Curcuma longa* L.). *Vietnam Journal of Agricultural Science.,* 9(82), 76 - 81.

19. Chandra Shella, Kazim Askari, Madhumita Kumari (2018). Optimization of indole acetic acid production by isolated bacteria from*Stevia rebaudiana* rhizosphere and its effects on plant growth. *Journal of Genetic Engineering and Biotechnology, 16, 581-586.*

20. Kumari S., C. Prabha, A. Singh, S. Kumari, and S. Kiran (2018). Optimization of Indole-3-acetic acid Production by *Diazotrophic B. subtilis* DR2 (KP455653), Isolated from Rhizosphere of *Eragrostis cynosuroides*. *International Journal of Pharma Medicine and Biological Sciences.* 7(2), 20-27.

21. Apine, O. A. and Jadhav, J.P. (2011). Optimization of medium for indole-3-acetic acid production usingPantoeaagglomerans strain PVM. *Journal of Applied Microbiology.* 110, 1235-1244.

22. Nita B. Patil, Milind Gajbhiye, Sangita S. Ahiwale, Aparna B. Gunjal, Balasaheb P. Kapadnis (2011). Optimization of Indole 3-acetic acid (IAA) production by *Acetobacter diazotrophicus* L1 isolated from sugarcane. *International journal of Environmental sciences,* 2(1), 307 - 314.

23. Mohite B., (2013). Isolation and characterization of indole acetic acid (IAA) producing bacteria from rhizospheric soil and its effect on plant growth. *Journal of Soil Science and Plant Nutrition,* 13(3), 638-649.