



ISSN NO. 2320-5407

Journal Homepage: - www.journalijar.com

INTERNATIONAL JOURNAL OF ADVANCED RESEARCH (IJAR)

Article DOI: 10.21474/IJAR01/12179
DOI URL: <http://dx.doi.org/10.21474/IJAR01/12179>



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Journal Homepage: <http://www.journalijar.com>
Journal DOI: 10.21474/IJAR01

RESEARCH ARTICLE

IN VITRO SCREENING OF SIDEROPHORE-PRODUCERS FROM UPLAND RICE ROOT ENDOPHYTIC BACTERIA FOR ANTAGONISTIC ACTIVITIES AGAINST *FUSARIUM OXYSPORUM*, AND *XANTHOMONAS ORYZEA* PV. *ORYZAE*

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Manuscript Info

Manuscript History

Received: 15 October 2020

Final Accepted: 19 November 2020

Published: December 2020

Key words:-

Endophytic bacteria, *Fusarium oxysporum*, Plant Growth Promoting, Siderophore Production, *Xanthomonas oryzae* pv. *oryzae*

Abstract

Rice is a staple crop for a large part of the world population including Vietnam. Although it's export is ranged of second place over the world just after Thailand, rice production in Vietnam is challenging by various issues. The most significant is application of agrochemicals, which are not eco-friendly, leading to side effects on the ecosystem and human health. Endophytic bacteria (EB) possess different beneficial traits such as Plant Growth Promoting (PGP) and antagonistic activities against plant pathogens. In this study, a collection of 150 EB strains have been isolated from upland rice roots grown on the mountain-farm in the Tung village, Nam Co commune, Mu Cang Chai district, Yen Bai province, Vietnam. 119/150 isolates showed siderophore production ability including 12 isolates showed the halo orange zone surrounding colonies of 1 – 10 mm in diameter (D_h), considering them as low level of siderophore production, 80 strains exhibited the moderate activity, D_h of 11 – 30 mm and 27 strains showed strong/high activity, D_h more than 30 mm. All 119 endophytic bacterial isolates were screened for antifungal activity against *Fusarium oxysporum* (Fo), and antibacterial activity against *Xanthomonas oryzae* pv. *oryzae* (Xoo). Of them, thirty isolates showed either antifungal or antibacterial or both activities. Indeed, five isolates showed antifungal activity; fifteen isolates exhibited the antibacterial activity. Especially, ten isolates named NC88, NC89, NC126, NC129, NC130, NC131, NC134, NC152, NC153, and NC156 showed antimicrobial activity against both tested pathogens. These results are the starting-up for further investigation in planta under conditions of net house and field trial in order to reveal the potential strains for the development of bioinoculants toward to control the diseases caused by Fo and Xoo.

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Introduction:-

Rice is one of the most important staple crops, which is feeding almost half of the world population. In modern society, farmers usually use agrochemicals such as chemical fertilizers, herbicide, and pesticide in order to gain higher productivity and prevent pathogens and weed. The use of agrochemicals is known to have an adverse effect on disrupting ecosystem balance and decreasing the number of microbes present in the soil as well as human health due to the amount of residue of toxic chemicals (Fitri et al., 2020). Another effort that could be done to overcome this problem is by applying beneficial endophytic bacteria to complement and mitigate the use of agrochemicals ensuring a healthier environment (Moronta-Barrios et al., 2018; Fitri et al., 2020).

Endophytic bacteria (EB) are those bacteria that colonize in the internal tissue of the plant with no external sign of infection or harmful effect on their host (Holliday, 1989; Schulz and Boyle, 2006). Several studies have focused on their interactions with host plants, especially on a large number of plant crops including rice (Reinhold-Hurek and Hurek, 2011). Hence, these studies contributed promising results to develop sustainable agriculture. Indeed, they have proved some endophytic bacteria isolates that are able to accelerate the growth of rice plants by observation of the root length and canopy height parameters (Fitri et al., 2020). Another study found an endophytic bacteria strain could assist plant nutrient uptake and prevent plant diseases on the basis of its biocontrol activity, siderophore production, and nitrogen fixation, fungal antagonistic (Zhao et al., 2018).

In fact, many of these interactions have been shown to benefit the hosts, namely Plant Growth Promoting (PGP) properties, which increased nutrient availability and has the ability to chemically stimulate the growth and/or tolerance of the host to abiotic stress (Moronta-Barrios et al., 2018). Iron is one of the most essential micronutrients required for the development of plants and microorganisms. Moreover, iron is not easily available in neutral to alkaline soils, which makes plants iron-deficient despite its abundance (Rout and Sahoo, 2015). Siderophores are low-molecular-weight chelating agents (200-2000 Da) with high Fe^{3+} chelating affinity responsible for facilitating the uptake of iron (Singh et al., 2019). Siderophore-producing EB are able to solubilize and chelate the iron from organic and inorganic insoluble forms present in soil to the beneficial forms for the plants and other microorganisms (Singh et al., 2019). Siderophore thus not only enhances plant growth, but also helps in protection of plant from phytopathogens who are inhibited by iron starvation or competitive exclusion in iron deficient conditions. Furthermore, endophytic bacteria have been widely known for their antagonistic activities in competition for substrate or production inhibitory chemicals. The latter property is important in inducing plant systemic resistance against pathogens (Compant et al., 2005), particularly the antifungal activity against *Fusarium oxysporum* (Fo), which is the causal agent of root rot or wilt diseases in many plant species and the antibacterial activity against *Xanthomonas oryzae* pv. *oryzae* (Xoo), which is the causal agent of rice bacterial blight disease.

The aim of this study is to find out potential beneficial rice root EB from the collection of 150 EB strains isolated from upland rice root grown on the mountain in the Tung village, Nam Co commune, Mu Cang Chai district, Yen Bai province, Vietnam. All of 119 siderophore-producers were screened for antifungal activity against *Fusarium oxysporum* (Fo), and antibacterial activity against *Xanthomonas oryzae* pv. *oryzae* (Xoo). These results are the starting-up for further investigation in planta under conditions of net house and field trial in order to reveal the potential strains for development of bioinoculant toward controlling the diseases caused by Fo and Xoo. Furthermore, the study hence could contribute to preservation of the environment and agriculture sustainability in Vietnam and the beneficial endophytic bacteria would become a trend in agriculture by reducing chemical fertilizers and pesticides.

Materials and Methods:-**Sample site and collection samples:**

The rice root samples were collected from the mountain-paddy in the Tung village, Nam Co commune, Mu Cang Chai district, Yen Bai province, Vietnam (21°51'50.01"N; 104°16'25.38"E). Three healthy rice plants were randomly selected at three points along the paddy's diagonal. Plants were harvested at three different growth stages: tillering, booting and milky maturation. Whole rice plants were collected independently to obtain a total of nine and categorized separately in zip bags and transferred to the laboratory for further processing.

Surface sterilization of rice samples:

All plant samples were washed with many volumes of tap water. The root samples were surface sterilized by submerging successively in 75% ethanol for 2 min., in 50% hypochlorite (4% of active Cl) for 2 min. and again in ethanol 75% for 1 min. The samples were rinsed abundantly with sterile water between each of these surface sterilization steps and at the end of the treatment (5 times). The extent of sterilization of the material was verified by both plating 2 ml of the final wash as well as by placing the sterilized roots on Tryptic Soy Agar (TSA) plates for 60 min. before maceration. The plates were then checked for bacterial growth in the next 72 h.

Isolation of endophytic bacteria:

The surface-sterilized root samples were crushed aseptically using mortar and pestle in 10 ml of PSB solution. A serial dilution was plated on 1/6 TSA medium. Plates were incubated at $28 \pm 2^\circ \text{C}$ for 72 h. Independent colonies of putative EBs showing distinct colony morphology were picket and streaked again on 1/6 TSA plates to obtain pure cultures. Pure bacterial cultures were maintained stored at -80°C in 1/6 TSA and 18% glycerol.

Detection of siderophore production on plate culture:

Siderophore production by the EBs was tested by a change in colour of the universal Chrome Azurol S (CAS) medium from blue to orange (Schwyn and Neilands, 1987). Isolates grown on 1/6 TSB medium at $28 \pm 2^\circ \text{C}$ for 48 h was spotted on the CAS agar plates and incubate at $28 \pm 2^\circ \text{C}$ for 7 days. The presence of yellow to light orange halo surrounding the colony indicates the production of siderophore (Ghosh et al. 2015). Depending on the diameter of the halo zone (D_h), the level production was identified as follow: low production (D_h : 1 - 10 mm), moderate production (D_h : 11 - 30 mm) and high production ($D_h > 30$ mm). Positive control was the strain *Pseudomonas fluorescens*.

Antifungal activity against *Fusarium oxysporum*:

The antifungal activity against Fo assay was followed protocol taken from Rahman and colleagues (2007). Fo was cultured with a petri dish containing 1/6 TSA medium and sterile filter papers of 6 mm placed around a petri dish. Six mm diameter pieces of fungal mycelium were taken from a seven-days old cultured dish and kept at the center of the new plates containing 1/6 TSA medium. Each EB was streaked a line by using a sterile toothpick and the distance from this line to fungal is 3 cm. The plates were sealed with parafilm and incubated at $28 \pm 2^\circ \text{C}$ for up to 7 days. If EB had ability against fungal, fungal could not grow through the bacteria's line. Conversely, if these bacteria did not have this ability, fungal can grow through the bacteria's line. The negative control plates were without the addition of the bacteria to the fungal plate.

Besides, the percentage of inhibition was calculated by the following formula (Rahman et al., 2007):

$$I = \frac{C - T}{C} 100 (\%)$$

Where I – The percentage of inhibition (%); C – Radical growth of fungi in the control plate (mm); T – Radical growth of fungi in the test plate (mm)

All isolates were tested in triplicate.

Antibacterial activity against *Xanthomonas oryzae* pv. *oryzae*:

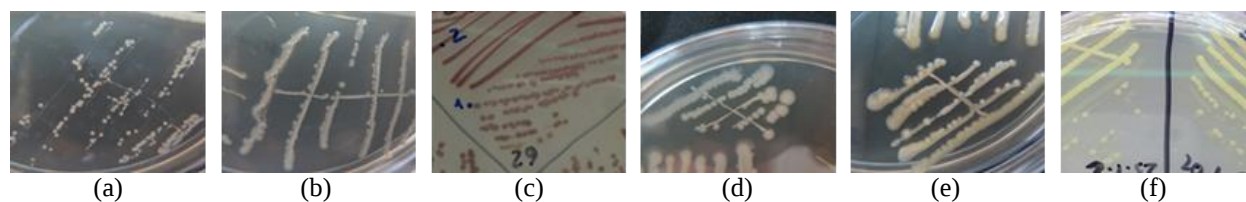
The antibacterial activity of the endophytic bacteria were evaluated by the dual culture test following Khoa and colleagues (2016) with some modifications. Indeed, the Xoo strain X19.2 provided by Agricultural Genetics Institute were recovered on PDA medium for 48 h then transferred to 1/2 TSA medium for 48 h. The bacteria were then suspended in PSB solution, pH 6.8 and adjust to $\text{OD}_{600} = 1$. Two hundred microliters of the Xoo suspension was spreaded out on 1/2 TSA medium by sterilized spreaders until the plate dried completely. Following this, cells from actively growing 24-hour-old cultures of each bacterial endophyte were dotted on the center of tested plate. This was done by touching one of the bacterial colonies with the sterilized tip of an inoculation loop (diameter 2 mm) and subsequently touching the plate. The experiment was performed with ten replicates per isolate. Inhibition zones formed by the isolates against Xoo after 48 h of inoculation at $28 \pm 2^\circ \text{C}$ were recorded for selection of strong antagonists to be further analyzed.

Statistical analysis:

Statistical analysis of the data was made with 5% level of significance using ANOVA in the Microsoft Office Excel 2016 assuming a normal distribution.

Results and Discussion:-**Isolation of bacterial endophytes from upland rice roots and colony characteristics:**

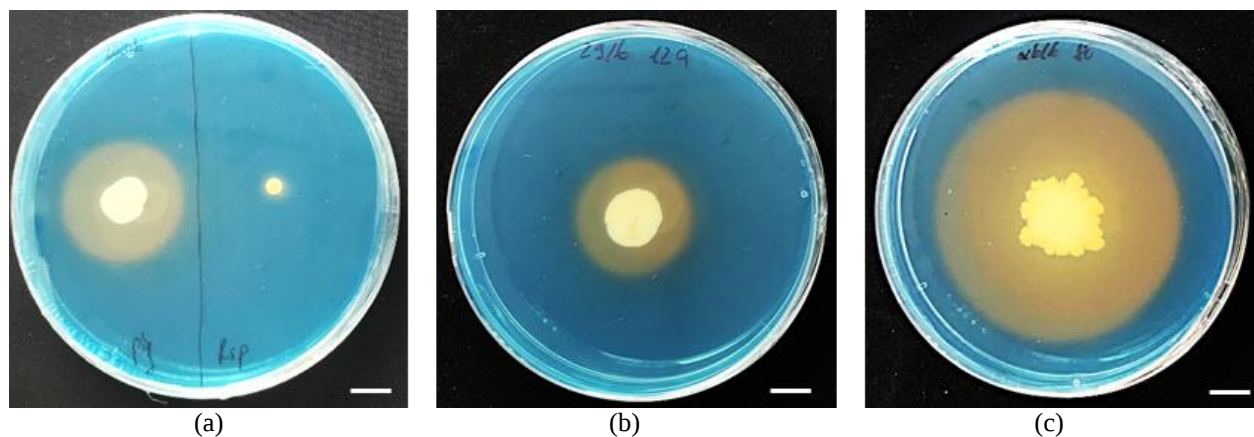
A total 150 culturable bacterial endophytes were isolated from the upland rice roots on the 1/6 TSA medium. All isolates grow very well on this medium from 24 – 48 h at $28 \pm 2^\circ \text{C}$. Their colonies had round-shape, slimy, smooth, colourless, milk-colour, yellow, brown purple and the size of colonies varies from 1 to 5 mm (Fig 1, Table 1).

Fig 1:- Characteristics of 24-hour-old rice roots endophytic bacterial colonies on 1/6 TSA media.**Table 1:-** Distribution of bacterial endophytic isolates by colour and shape of colonies.

Colony characteristics	Types	No of Isolates	Per cent (%)
Colour	Light yellow	57	38,00
	Yellow	36	24,00
	Milk colour	40	26,67
	Colourless	15	10
	Brown purple	2	1,33
Shape	Circular raised	127	84,67
	Irregular undulate	23	15,33

In vitro detection for siderophore production of bacterial endophytes:

In this study, we described the screening for siderophore-producers having the antagonistic traits from the collection of endophytic bacteria isolated from the upland rice roots in Vietnam. Among 150 endophytic bacteria isolated in this study, 119 isolates showed siderophore production ability with different levels based on the diameter of orange zone surrounding the colony after 7 days of incubation on CAS agar plates. Results were visually distinct in terms of halo formation against the blue medium as shown in Fig 2. 119 out of 150 tested isolates (79.3 %) were positive for siderophore production, where 12 isolates exhibited low level (D_h : 1 – 10 mm), 80 isolates showed moderate level (D_h : 11 – 30 mm), and 27 isolates with high level (D_h : > 30 mm), a clear and large yellow halo zone (Fig 2, Table 2). 31 out of 150 tested isolates (20.7%) showed no change in the color of the medium CAS blue dye. In particular, among 27 isolates showed high-level production, 6 isolates NC3, NC4, NC115, NC133, NC143, and NC144 had the highest diameter of the orange halo zone, up to 60 mm (Fig 2c).

Fig 2:- Siderophore production of EB on CAS medium.

(a) – Positive control *P. fluorescens* (left) and *Rhizobium* sp. (right); (b) and (c) – Different levels of siderophore production of EB isolates NC52 and NC3, respectively. Bar – 10 mm.

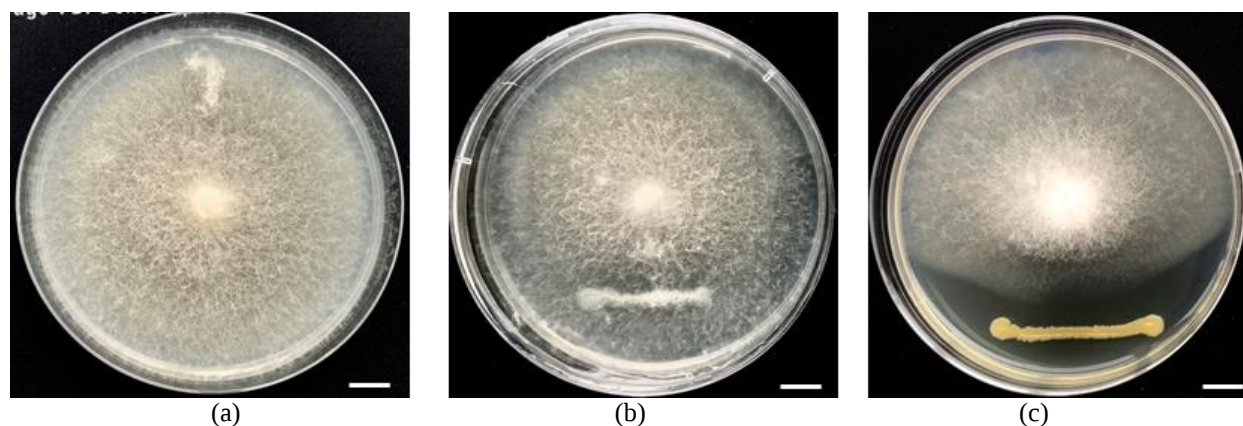
Table 2:- Siderophore production levels of 150 EB isolates.

Siderophore production level				
Diameter of orange halo zone (mm)	Negative	Low D_h : (1– 10mm)	Moderate D_h : (11– 30mm)	Strong/High D_h : (>31mm)
Number of EB isolates	31	12	80	27
Total	31	119		

Antifungal activity against *Fusarium oxysporum*:

All 119 siderophore-producers were screened for antifungal activity against Fo by dual assay on the agar plates. 15 tested isolates (12.6%) were found positive and the remaining 104 isolates (87.4%) did not show antifungal activity against Fo. Overall, 8 isolates named NC3, NC4, NC87, NC88, NC89, NC152, NC153 and NC156 showed antifungal activity against Fo at the highest level, with the percentage of inhibition was more than 50%, especially isolate NC87 showed 73.33% of inhibition (Fig 3, Table 3). The observation of these isolates under the microscope has been shown that the closer mycelia to the bacteria, the fewer number of spores and also the structure of mycelium changed. This observation has already been reported in the other studies (Rahman et al., 2007; Zhao et al., 2018). After several days of culturing with EB, fungal spores were more inhibited and the morphological features of the mycelia became abnormal compared to the control, such as the fungus become fractured, etc. (Zhao et al., 2018). Therefore, further studies are needed to identify deeper bacteria-fungi interaction, then develop a commercial product as commercial products containing Burkholderia cepacia are available in the market for control of many solid-borne pathogens (Fravel et al., 1998).

Fig 3:- Antifungal effect of EB on growth of *Fusarium oxysporum* mycelia.



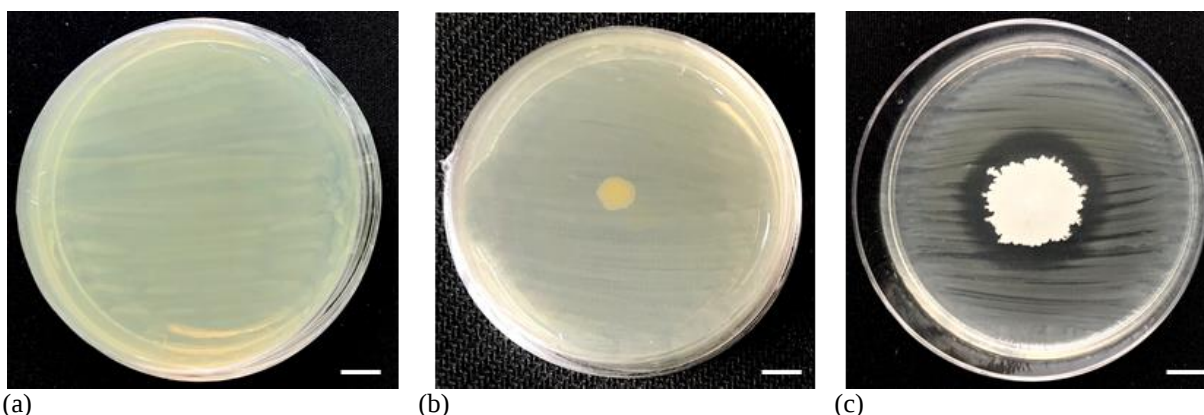
(a) – Control plate (no EB); (b) – isolate NC5 showed no antifungal effect on growth of fungi; (c) - isolate NC89 showed antifungal effect on growth of fungal mycelia. Bar – 10 mm.

Table 3:- The percentage of inhibition of radical growth of *Fusarium oxysporum* by positive EBs.

Strains code	Percentage inhibition (%)	Strains code	Percentage inhibition (%)	Strains code	Percentage inhibition (%)
NC3	62.22± 2.22	NC89	52.29± 3.39	NC131	40.37± 3.57
NC4	57.78± 7.70	NC124	44.07± 5.13	NC134	42.59± 0.64
NC23	48.52± 3.39	NC126	41.85± 1.28	NC152	54.44± 1.92
NC87	73.33± 4.44	NC129	42.07± 0.26	NC153	51.48± 1.70
NC88	50.74± 3.39	NC130	42.96± 3.57	NC156	52.39± 3.39

Antibacterial activity against *Xanthomonas oryzae* pv. *oryzae*:

A total of 119 siderophore-producers were screened to identify the antibacterial activity against Xoo, where 25 isolates (21%) demonstrated positive results. The isolates with positive antibacterial activity were determined by producing a halo zone of inhibition around the bacteria colony on the medium (Fig 4). Therefore, these isolates were considered as potential antagonists. Among them, two endophytic isolates NC152 and NC153 presented the highest means of inhibition diameter of 6 and 10 mm respectively, while the other isolates demonstrated a range of antagonistic activity from 0.2 to 0.55 mm.

Fig 4:- Antibacterial effect on growth of Xoo.

(a) – Control plate (no EB); (b) – EB isolate NC147 showed no antibacterial activity; (c) – EB isolate NC153 showed antibacterial effect on growth of Xoo indicated by the halo zone surrounding EB colony. Bar – 10 mm.

Based on these primary results, further investigation in planta under conditions of net house and field trial will be necessary to implemented. From that, we can conclude exactly the antibacterial activity of each endophytic bacterial. For instance, under greenhouse conditions, El-shakh and colleagues have found out the strain D29 showing the highest level of antibacterial activity (57.86%) against Xoo followed by A15, H8 and A13 respectively (El-shakh et al., 2015). Moreover, many studies demonstrated all *Bacillus* strains tested reduced the leaf blight, lesion length and wilting in rice plants compared to control plants (El-shakh et al., 2015; Thanh Trung et al., 2017; Huỳnh Nhã Uyên et al., 2018). Another study of Niño-Liu had the same conclusion that the highest percentage tested *Bacillus* strains against Xoo (Niño-Liu et al., 2006). Therefore, in the future, we will identify endophytic bacterial strains and compare the results with them.

Taken together, in this study, among 150 isolates, 10 isolates (NC88, NC89, NC126, NC129, NC130, NC131, NC134, NC152, NC153, and NC156) showed remarkable inhibition of bacterial and fungal phytopathogens and also the ability of siderophore production. Furthermore, in this study, the experiments were evaluated in a qualitative assay, so there is a need to test in the quantitative assay and tested *in planta* as well to confirm the results. Indeed, it is required to apply these strains to the seed, leaf and also in field trials at different locations to determine the antagonistic activity against leaf blight disease caused by Xoo and root rot or wilt diseases caused by Fo. In addition, further investigation of other plant growth promoting traits on these strains is necessary such as the capability to produce IAA, gelatinase, HCN, hydrolyze starch, solubilizing phosphate, fix nitrogen, etc. Following this path, we can expect to come up with biocontrol strategies and bioinoculant formulations that are not only more sustainable but also more efficient. For instance, the yield of *Basella alba* L. in testing experiments fertilizer increased by 89.51% as compared with the use of NPK fertilizer (Hai Van and Thi Minh 2017).

Conclusion:-

In this study, we have found several rice root endophytic bacterial isolates that are promising for at least one of the three traits namely the production siderophore and antagonistic activity against *F. oxysporum*, Xoo. Of 119 siderophore-producers, thirty isolates showed either antifungal or antibacterial or both activities. Indeed, five isolates showed antifungal activity; fifteen isolates exhibited the antibacterial activity. Especially, ten isolates named NC88, NC89, NC126, NC129, NC130, NC131, NC134, NC152, NC153, and NC156 showed antimicrobial activity against both tested fungi. Further investigation will be performed to be executed in order to confirm bioinoculants and also the contributions of bacterial properties to plant growth and biocontrol development.

Acknowledgements: This study is granted by Graduated University of Science and Technology (GUST), Vietnam Academy of Science and Technology (VAST) under the Project number: GUST.STS.ĐT2017-SH05. The authors would like to thank the Mixed International Laboratory LMIRICE2 for providing the strains *Magnaporthe oryzae*, *Pseudomonas fluorescens*, *Fusarium oxysporum*, *Xanthomonas oryzae* pv. *oryzae*. The authors also dedicated the gratefulness to bachelor students from Vietnam National University of Agriculture and University of Science and Technology of Hanoi (USTH), VAST.

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