http://dx.doi.org/10.21172/ijiet.134.04

Isolation and Characterization of Actinobacteria in Rhizosphere of Crinum latifolium (L.) Cultivated in Tay Ninh Province, Vietnam

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Abstract- Characteristics of plant growth promotion and biological antagonism of rhizospheric actinobacteria were initially investigated. Rhizospheric soil samples were collected from medicinal plant farms of Trang Bang district, Tay Ninh province. Actinobacteria were isolated by spreading soil suspension on Gauze's medium No.1 plates. The pure colonies were selected and streaked over the agar surfaces of Burk's N free and NBRIP media plates. Sixty eight isolates had grown on both of media, indicated their abilities of nitrogen fixation and phosphate solubilization. Quantitative results showed that the nitrogen fixation ability of 68 isolates ranged from 0.4 – 8.8 mg L-1 NH4+, and the ability of phosphate solubilization of these were equivalent to 17.6 – 59.1 mg L-1 P2O5. There were 40 in the total 68 isolates showed positive reactions to Salkowski reagent. Hence quantification of IAA produced by these isolates had been conducted under the condition of adding 100 mg L-1 tryptophan into the cultural medium. The IAA production of 40 isolates ranged from 0.3 – 11.5 mg L-1. Eleven isolates that showing outstanding ability in nitrogen fixation, phosphate solubilization and IAA production had been tested ability of producing siderophore and antifungal activity. There were 3 isolates including C15A, C1, H6P that showed the production of siderophore in the CAS assay. Especially, C15A also showed antifungal ability. These three isolates were identified by using the MALDI Biotyper System (Germany). The identification result showed that C15A was Streptomyces aureofaciens, C1 was Streptomyces fabae. Only isolate H6P was not identified because no peak had found in the analysis. Results of this research indicate that two isolates C15A and C1 are promising candidates for application as inoculant for medicinal plants such as Crinum latifolium because they possess good characteristics of plant growth promotion and biological control.

Keywords – PGP actinobacteria, MALDI-TOF MS, Crinum latifolium (L.), nitrogen fixation, phosphate solubilization, IAA production, siderophore production, biological antagonism.

I. INTRODUCTION

Crinum is a large genus in the family Amaryllidaceae that includes many herbs are valuable in terms of decoration, economics and medicinal materials. The folk remedies of Asian countries such as India, China and Vietnam show the extracts from Crinum leaves and bulbs is often used to treat vomiting, ear-ache, rheumatism, fistula. Extracts of Crinum species, especially Crinum latifolium, have been reported to have anti-cancer, immune stimulating, analgesic, antiviral, antimicrobial and antifungal effects [1] [2] [3]. Crinum latifolium plants (Pink-striped Trumpet Lily) are grown in many provinces of the South East of Vietnam incuding Tay Ninh (Figure 1) [4]. Planting Crinum latifolium requires special care techniques such as organic fertilizer applying, insect hand-catching to limit using of chemicals. So the growth of the plant depends mainly on the metabolism of nutrients in soil in which microorganism plays an important role.

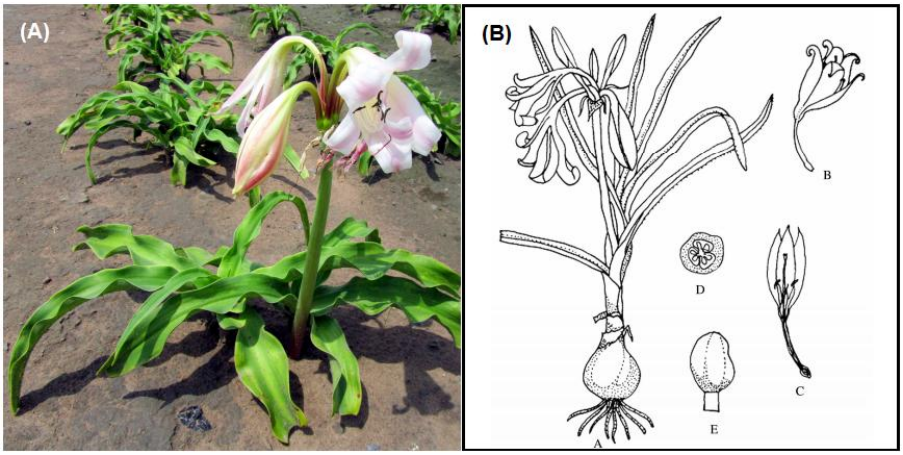

Figure 1. Crinum latifolium L. var. crilae Tram & Khanh, var. n. cultivated in Vietnam (A) [4], and plant morphology of Crinum latifolium (B) [1]

Among soil microorganisms, many bacteria that exist in the rhizosphere including actinobacteria have been shown having a good impact on plant growth. In addition, these bacteria are also capable of producing important secondary compounds, helping to improve the quality of medicinal materials produced by host plants [5]. Although reports on plant growth promoting (PGP) characteristics of bacteria in the phylum Actinobacteria are few [6], many authors have directly used the terms "Plant Growth Promoting Actinobacteria" [6] [7] [8], or "Plant Growth Promoting Actinomycetes" [9] [10] [11] (PGPA for short) to refer this subject. The plant growth promoting mechanisms of actinobacteria are not different from the common PGP bacteria. It is often divided into two groups: direct and indirect. Direct mechanisms include (1) providing plant growth stimulating compounds such as the IAA, cytokinin for plants, and (2) supporting plants to absorb nutrients through out nitrogen fixing, producing iron-chelating compounds as siderophores, solubilizing minerals. Indirect mechanisms include (1) biological control, and (2) reducing the harmful effects of biotic and abiotic stress on plants [6] [12] [13].

Studies on PGPA associated with medicinal plants are common in Asian countries such as China, India and Thailand. The medicinal plants in these research are wild or cultivated herbs such as Camptotheca acuminate, Taxus chinensis, Curcuma phaeocaulis, Ginkgo biloba. The effects of these folk medicinal plants have been demonstrated in the treatments of tumors, obesity, diabetic, infection, asthma, dizziness [14] [15] [16] [17] [18]. The isolated actinobacteria have showed the capacities of production of PGP compounds such as ammonia, IAA, HCN, siderophores, phosphate solubilization, antibacterial and antifungal activities, or inhibiting cancer cells [19] [20] [21] [17]. The popular genera of isolated PGPA are Streptomyces, Nocardia, Micromonospora, ect., but the most abundant is Streptomyces [15] [22] [23] [17]. It is for the above reasons that this study was done to initially learn about the PGPA associated with a famous medicinal herb of Vietnam - Crinum latifolium.

II. MATERIALS AND METHODS

2.1 Samples collection and preparation

The location of Tay Ninh province is at 10o57'08" - 11o46'36" N latitude and 105o48'43" - 106o22'48" E longitude. This province has an area of 4,028 km², and subdivided into 8 districts and one provincial city, in which Trang Bang is one of its three southern districts. Twenty five samples of 3-months-old Crinum latifolium plants were collected in 3 farms of An Tinh ward, Trang Bang district (Figure 2). Aerial parts of each plant were cut off. Whole bulb, roots and soil of each plant was collected, kept in a plastic bag, sealed, labeled and transported to the laboratory. Collection of rhizospheric soil samples was carried out according to the method of [7] (shaking method, ID: 13_Turpault). Soil samples had been dried at approximately 40°C to constant mass (air-dry soil).

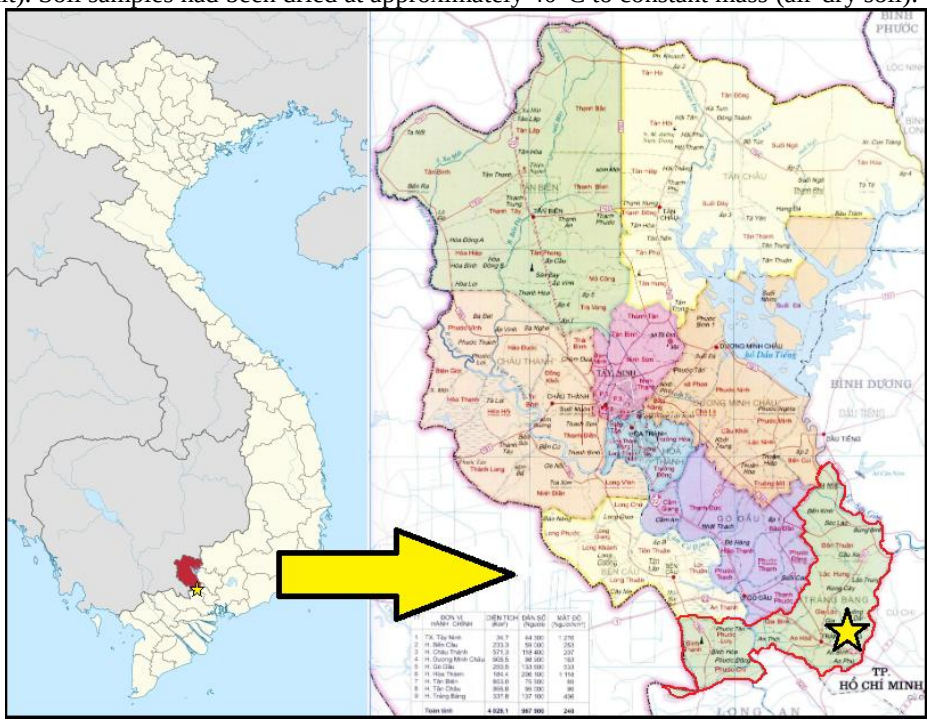

Figure 2. Map of Tay Ninh province with Trang Bang district and place of sampling
(Star shapes used to illustrate An Tinh ward of Trang Bang district)

2.2 Isolation and collection of nitrogen-fixing and phosphate-solubilizing actinobacteria

One gram of air-dry soil was suspended in 99 mL sterile water, shaked at 200 rpm for 30 minutes and deposited for 3 hours. Atfer 3 hours, the supernatant was continuously diluted in sterile water up to 10−3 by ten-fold serial dilution method. Then 100 µL of the 10-3 diltution was collected and spread on each Gauze's medium No.1 agar plate. These plates were incubated at 28oC for 5 – 10 days to observe and select the colony that show powdery, consistency, and firmly sticking to agar surface [24] [25]. Pure colonies of actinobacteria were streaked over the agar surfaces of Burk's N free and NBRIP media plates. Bacterial isolates that had grown on both of media were nitrogen-fixing and phosphate-solubilizing bacteria. These isolates were preserved temporarily in 40% glycerol solution for use in subsequent studies [19].

2.3 Morphological characterization of bacterial isolates

Colony morphology including form, elevation, surface, color of aerial and substrate mycelium, diffusible pigment, and size were recorded for at least 7 days of growth on Gauze's No.1 medium at 28°C. The mycelium was also observed by light microscopy [24].

2.4 PGP funtional characterization of bacterial isolates

2.4.1 Quantification of nitrogen fixation and phosphate solubilization

One pure colony of each isolates (Φ=5 mm) was inoculated into 5 mL of Burk's broth (for testing nitrogen fixation) or NBRIP broth (for testing phosphate-solubilizing), then incubated at 28oC and 120 rpm (rounds per minute) for 5 days. After 5 days, 500 µL of suspension was collected, and transferred to 10 mL of Burk's or NBRIP solution, then incubated continuously within 8 days (for testing nitrogen fixation) or 20 days (for testing phosphate-solubilizing) at 120 rpm, 28oC. Periodically, 10 mL suspension was collected at 2, 4, 6, 8 DAI (days after inoculation) or at 5, 10, 15, 20 DAI, then centrifuged at 12,000 rpm in 5 minutes to obtain the supernatant for the next colorimetric analysis. Colorimetric procedures were based on the description of [26].

2.4.2 Detection and quantification of IAA production

The bacterial isolates were grown in SCB (Starch Casein Broth) [27] with 100 mg L-1 tryptophan adding to the cultural medium. After 8 days of culture, the ability of IAA production was detected by dropping 100 µL Salkowski reagent (formulas described by [28]) into 500 µL of bacterial suspension, then incubated in dark for 15 min. The appearance of pink to red color on solution means positive result [29]. So these isolates were subsequently quantified the production of IAA.

For quantification, the preparation of the cultural suspension was similar to that of the nitrogen quantitative experiment with the replacement of Burk's broth with SCB. Periodic sampling occurred each of 2 days within 8 DAI. Colorimetric method followed the description of [26].

2.4.3 Preliminary survey of biological control ability of selected isolates

This experiment was limited among outstanding isolates (having the best quantitative results of N2-fixation, phosphate-solubilization, and IAA production).

Qualitative experiment of siderophore production was conducted by using CAS (Chrome Azurol Sulphonate) assay. One colony of each bacterial isolate was inoculated into 5 mL of SCB, incubated at 28oC and 120 rpm for 7 days. After 7 days, 1.5 mL of suspension had been collected, conducted the centrifugation process at 12,000 rpm for 5 minutes. After centrifugation, 2 mL of supernatant was transferred to fresh tube, then an equal volume of the CAS solution (formulas according to [26]) was added to each tube, and the mixture incubated in the dark for 30 minutes at 28oC. The color change of the blue reagent into other color such as green, orange or purple indicates the presence of siderophore. Testing of antifungal activities of selected isolates against the indicator Fusarium sp. was based on method of [30].

2.5 Identifying selected isolates by their molecular fingerprinting

This experiment was limited to the selected actinobacteria with the best results after going through all the surveys. These selected isolates were cultured on LB for 24 hours, and identified by using MALDI-TOF MS method. This method was used to determine the unique proteomic fingerprint of an organism. The characteristic spectrum pattern of this proteomic fingerprint is used to reliably and accurately identify a particular microorganism by matching thousands of reference spectra from microorganism strains [31].

2.6 Experimental design and Data analyses

Quantitative experiments were random assignment (Completely Randomized Design) with three replicates. Negative controls were conducted similarly to treatments, did not use bacterial suspension but put on sterilized corresponding medium instead.

Statistics methods were ANOVA (Analysis of Variance) one factor and Duncan test at α=0.05 by using IBM SPSS Statistics 20.0.

III. RESULTS AND DISCUSSION

3.1 Bacterial isolation

Seventy nine actinobateria isolates were collected from spreading and pure culturing on Gauze's medium No.1. After were transferred to Burk's medium and NBRIP medium plates, there were 68 isolates (accounting for 86.1%) growing on both media (Table 1). These were actinobacteria isolates having the ability of nitrogen fixation and phosphate solubilization that were found out.

Table - 1 Origin and ability to grow on the media of the isolates

Origin and number of samples	Number of isolates collected on Gauze's medium	Number of isolates grew on Burk's medium	Number of isolates grew on NBRIP medium	Number of isolates grew on Burk's and NBRIP media
Farm No.1: 5 samples	20	17	18	17
Farm No.2: 10 samples	32	29	30	29
Farm No.3: 10 samples	27	23	25	22
Total: 25	79	69	73	68

This study showed that the ratio of phosphate solubilizing isolates was higher than that of nitrogen fixation isolates: 92.4% (73 isolates) compared to 87.3% (69 isolates) (Table 1). Meanwhile, the research of Damam et al. (2016) [19] gave the opposite result: 29% and 75%, respectively. Insoluble phosphates derived from fertilizers exist in soil with a large amount. Therefore, the presence of phosphate solubilizing bacteria plays an important role in agriculture.

3.2 Morphological characterization

On Gauze's medium, the colors of aerial mycelium were gray colors (33.8%), white colors (29.4%), brown colors (16.2%) and yellow colors (10.3%), meanwhile the ratios of these color groups of the substrate mycelium were 16.2%, 22.1%, 23.5%, 22.1%, respectively (Figure 3). These were also three common color groups of actinobacteria colonies that had been reported [32] [19].

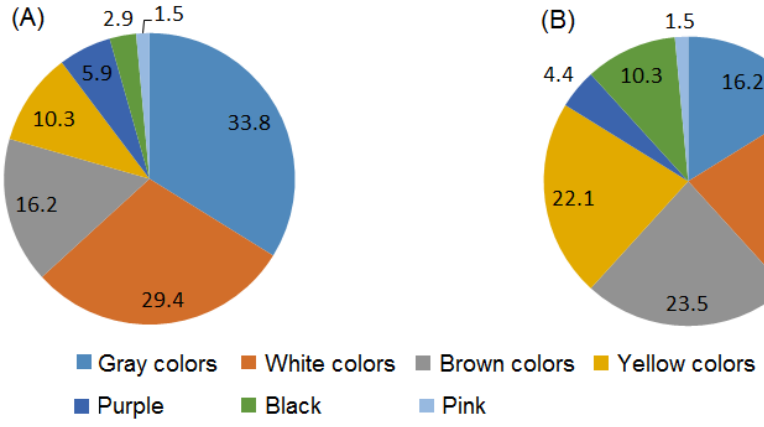

Figure 3. Ratio (%) for color of aerial mycelium (A) and substrate mycelium (B) of actinobacteria isolates

Substrate hyphae often produce pigments. Water-soluble pigments will penetrate into the cultural medium to create corresponding colors. The color of substrate fibers and soluble pigments is an important reference in identifying species [33]. In this study, there were 43 isolates which produced water-soluble pigments make the medium dyed brown, yellow, purple, ect. (Firgure 4).

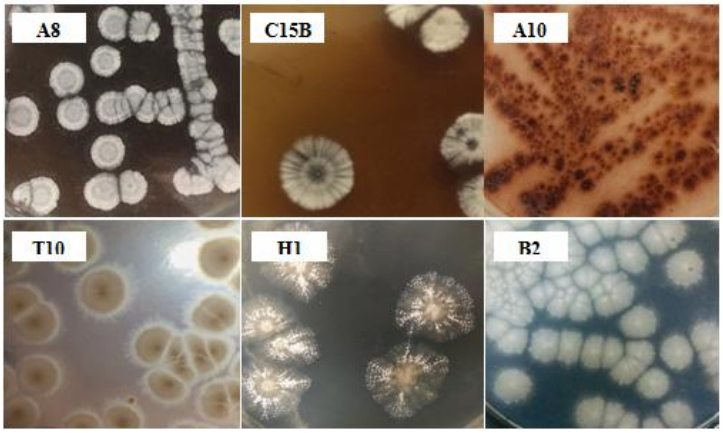

Figure 4. Colony morphology of actinobacteria on Gauze's medium (images above: aerial mycelium; images below: substrate mycelium)

3.3 PGP funtional characterization

3.3.1 The ability of nitrogen fixation and phosphate solubilization

All of 68 isolates were examined the ability of nitrogen fixation and phosphate solubilization by colorimetric method. The average values of 4 times of measurement at 2-day distance were in range from 0.4 – 8.8 mg L-1 NH4+ while the average values of dissolved phosphate contents of 4 times of measurement at 5-day distance were in range from 17.6 – 59.1 mg L-1 P2O5. Particularly, the optimal time-point for nitrogen fixation of the majority of bacterial isolates was "4 DAI" while "10 DAI" seemed to be the suitable time-point for phosphate solubilization as some authors had reported [26][31] [35].

3.3.2 The ability of IAA production

The qualitative experiment showed that 40 isolates out of 68 isolates indicated the ability to produce IAA (accounting for 58.8%) through the appearance of pink in solution after adding reagent. In quantitative experiment, the average values of IAA contents of 4 times of measurement at 2-day distance with the presence of tryptophan in culture medium of 40 isolates ranged from 0.3 – 11.5 mg L-1. The measured IAA content was similar to the results of the research of some authors [19] [36]. Appropriate time for IAA production was 2 DAI, similar to some authors' reports [26] [31] [34]. Phytohormones are often produced by plant associated bacteria. There were 80% of rhizospheric bacteria have the ability of IAA production to interfere host plant physiology [19] [31].

Base on the results of quantitative surveys mentioned above, there were 11 isolates had expressed superiority in capabilities of nitrogen fixation, phophate solubilization and IAA production (Table 2). Therefore, these 11 isolates would be preliminary examined the ability of biocontrol including the siderophore production and antifungal activity.

Table 1- In vitro Plant Growth Promoting functional characterization of ten selected isolates

Isolate	In vitro Plant Growth Promoting Functional Characterization					
	N2-fixation (mg NH4+/L)		P-solubilization (mg P2O5/L)		IAA-production (mg IAA/L)	
	4 DAI	Averagefor 8 days	10 DAI	Averagefor 20 days	2 DAI	Averagefor 8 days
A8P	0.38	4.10	63.98	25.50	2.59	0.87
A10	0.39	0.40	12.42	21.85	6.37	1.82
B5P	8.65	5.88	9.37	17.95	4.43	1.36
C1	0.57	0.52	13.08	33.75	3.68	1.29
C9	1.44	0.64	34.53	22.13	5.79	1.78
C12	0.46	0.49	26.58	23.39	4.41	1.74
C15A	2.37	0.89	24.25	23.22	2.85	0.86
H6P	0.58	2.97	27.45	18.12	16.03	5.62
H3	0.48	0.52	35.79	29.98	3.71	1.05
H9P	0.33	3.53	8.45	17.59	12.91	4.00

T10	1.00	0.52	18.67	19.05	6.21	2.07

3.4 Siderophore production and antifungal activity of selected isolates

The results of qualitative experiments showed that 3 isolates C1, C15A and H6P had the ability to produce siderophores (accounting for 27.3%), but only the isolate C15A showed antifungal ability (Figure 5).

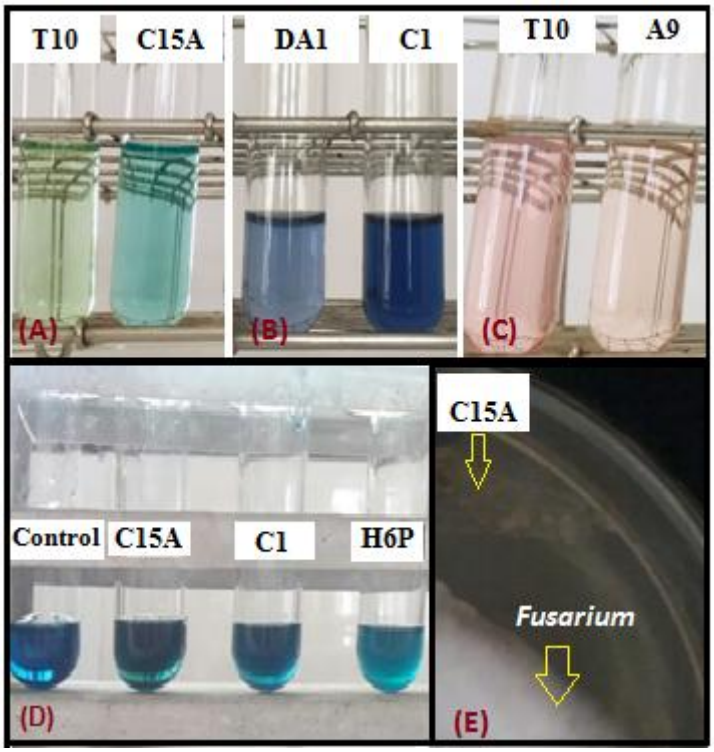

Figure 5. The abilities of nitrogen fixation (A), phosphate solubilization (B). IAA production (C), siderophore production (D), and antifungal activity (E) of some actinobacteria

3.5 Identification of selected isolates

There were 3 selected isolates had been identified by using MALDI-TOF MS. Profiles of morphology of these isolates had been checked. Result of identification showed that C15A was similar to Streptomyces aureofaciens, and C1 was similar to Streptomyces fabae, but only the isolate H6P was not identified because of having no peak found in the analysis (Figure 6).

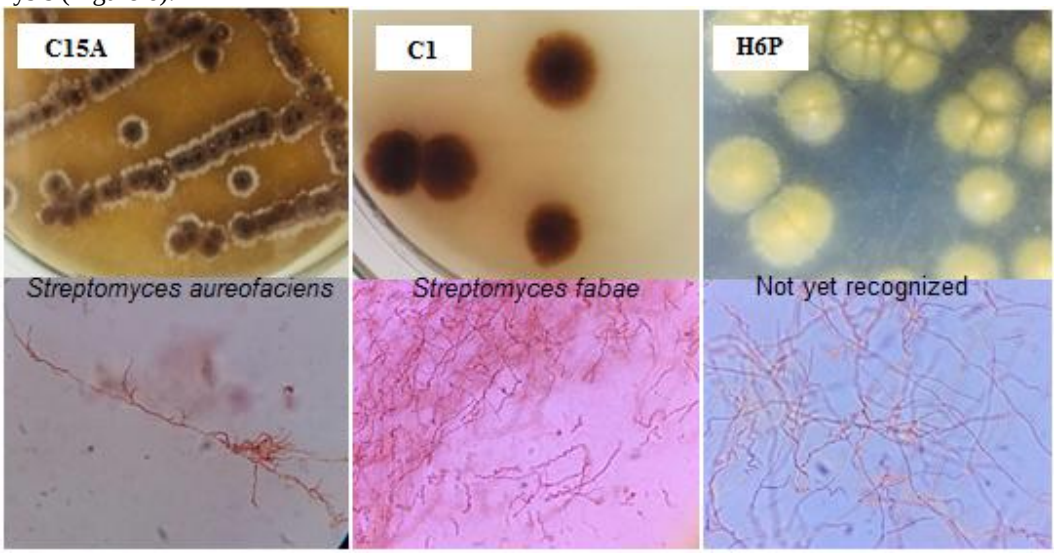

Figure 6. Morphology and name of three selected actinobacteria

Streptomyces fabae one bacterium species from the genus of Streptomyces which has been isolated from rhizosphere soil of soybean (Glycine max). It has anti-microbiology activity [37]. There is little information about Streptomyces fabae whereas Streptomyces aureofaciens is a bacterium that is more known. Streptomyces aureofaciens is a well-known actinobacteria in the field of production of antibiotics such as chlortetracycline, auricin, and antifungal activity. Recently Streptomyces aureofaciens is also isolated from the rhizosphere and and searched for PGPR characteristics Meanwhile, Streptomyces aureofaciens [30] [38] [39].

IV. CONCLUSION

A total of 68 isolates having two abilities of nitrogen fixation and phosphate solubilization were collected from 25 rhizosphere samples of Crinum latifolium cultivated in farms of Tay Ninh province. There were 40 isolates had showed the capabilities of nitrogen fixation, phosphate solubilization and IAA production. The two best isolates were identified as Streptomyces aureofaciens C15A and Streptomyces fabae C1. Both showed the siderophore production, but only Streptomyces aureofaciens C15 showed antifungal activity. These isolates are recommended to test the effectiveness in the growth of Crinum latifolium in pots experiment.

V. ACKNOWLEDGEMENT

The authors wish to thank the sponsorship of Saigon University, the support of research facilities of Ho Chi Minh City University of Science, and our colleague Truong Thi Phuong for assistance with laboratory experiments.

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