ISOLATION ENDOPHYTIC BACTERIA FROM ELEPHANT GRASS *(PENNISETUM PURPUREUM* SCHUMACH) AND THEIR POTENTIAL APPLICATION

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ABSTRACT

In this study, 25 endophytic bacteria were isolated and purified from rhizome, stem and leaf of the elephant grass, which were tested for their biological control properties. The number of living and dead brown plant hoppers were recorded and the mortality rate was analyzed by using Abbott's formula. The results indicated that three endophytic bacteria including VBL1, VBT1 and VBT5 showed the highest biological control of *Nilaparvata lugens* at the mortality rate 46.95%, 55.02% and 55.02%, respectively after 8 days of screening and significant difference compared to other isolates ($P<0.05$). Additionally, insecticidal activity of three bacterial isolates were conducted at different concentrations (10^6, 10^7, 10^8 CFU/ mL) and we found that the highest mortality rate of brown plant hopper was significantly observed at 10^8 CFU/ mL for VBL1, VBT1 and VBT5 isolates after 10 days trial ($P<0.05$). Three different isolates VBL1, VBT1 and VBT5 were similar to *Bacillus pumilus* (VBT1 and VBT5), *Bacillus thuringensis* (VBL1). This result plays an important role in understanding endophytic bacteria from elephant grass for biological control brown plant hopper in the future.

Keywords: Biological control; Endophytic bacteria; Insecticidal activity; *Nilaparvata lugens; Pennisetum purpurrerum.*

1. Introduction

Endophyte, often a bacterium or fungus, live within a plant for a least part of its life cycle, especially endophytic bacteria and fungi almost have not caused any disease symptons (Azevedo et al., 2000). Endophytes may enhance host growth by auxin synthesis (IAA) (Barbieri et al, 1986), pollutant elimination out of host (Rosenblueth and Martinez, 2006), nutrient acquisition and may improve the plant's ability to tolerate abiotic stress, enhance resistance to insects (Fahey et al., 1991). Endophytic bacteria can promote plant growth by various mechanism (Li et al., 2016). Recently, diverse endophytic bacteria are now being used worldwide as bio-inoculants to promote plant growth and development under normal and various stresses like heavy metals, herbicides, insecticides, fungicides, and salinity (Ahemad et al., 2014). Endophytic bacteria enhance the host to withstand pest attack by induced systemic resistance (ISR).

Many studies indicated the role of fungal endophytes in grasses nearly a century ago. Poaceae family is one of the most important family in plant and distributed around the world. Grass of Poaceae family are elephant grass *Pennisetum purpureum* Schumach (Poaceae), stylo grass (*Brachiaria mutica*), ect. Four selected endophytic bacterial strains were reported successfully isolated from elephant grass significantly reduced the harmful effects of salt stress, promoted plant growth and biomass yield on hybrid *Pennisetum* in vitro, which were classified into four bacterial genera such as: *Sphingomonas, Bacillus, Pantoea,* and *Enterobacterc.* Each of

the bacterial strains tested showed at least two or more PGP (plant growth promoting) properties, ability of IAA production, siderophore production, nitrogen fixation, ammonia production, inorganic phosphate solubilization, or ACC (1-aminocyclopropane-1-carboxylic acid) deaminase activity (Li et al., 2016). In addition, under greenhouse conditions, the endophytic bacteria strains EPCO 102, EPCO 16 and Pf1, which were isolated from cotton plants with chitin treated plants, showed higher growth promotion and reduced pest population. Endophyte-treated plants was greatly promoted plant growth and reduced the *Helicoverpa armigera* population compared to endosulfan treatment (Rajendran et al., 2007). Endophyte increased tolerance for abiotic and biotic stresses, produce toxin and deterrents that reduce insect herbivory on their host grasses (Takuya and Koya, 2010). Therefore, the objective of this study was to isolate endophytic bacteria from elephant grass, and to determine the susceptibility of brown plant hopper to the endophytic bacteria. The investigation would potentially offer an opportunity to exploit some valuable endophytic bacteria as biological control agents.

2. Materials and Methods

2.1. Plant material

Rhizome, sterm, leaf from elephant grass were collected from Cu Chi and Binh Duong province.

2.2. Isolation of endophytic bacteria

Rhizome, sterm, leaf were washed thoroughly under tap water for 10 min to remove any adhering soil, dipped in 10 % of commercial bleach (5.25% available chlorine) for 3 min, then transferred to a 3% hydrogen peroxide solution for 3 min, and finally rinsed three times with sterilized water. To ascertain that the surface disinfection process was successful, an aliquot of 100 µL final wash was inoculated in LB medium for sterility check. Then, tissues were macerated using a mortar and pestle in a small volume of sterile phosphate buffered saline (PBS, pH 7.4). This suspension was plated on LB medium and incubated at 28°C for 48–72 h.

2.3. Brown plant hopper biological control ability

Rice seed Nang Hoa 9 sensitive to brown plant hoppers and brown plant hopper adults were collected from Long An province. Five adults of *Nilaparvata lugens* were released into a 20 days-old rice seedlings, covered by plastic tube and muslin cloth. The isolated bacteria strains were cultured in nutrient broth (10^8 CFU/mL concentration) and 10ml bacteria broth was sprayed to the experiment continously every 24h for 3 days. Number of dead brown plant hoppers were recorded after 4, 6, 8 days. Each endophytic bacteria isolate was tested with totally 20 adults of *Nilaparvata lugens* at 10^8 CFU/mL concentration and distilled water was used as a control treatment.

2.3.1. Investigation the optimal concentration of endophytic bacteria

We selected some bacteria have the highest biological control, we examined endophytic bacteria at 3 different concentrations (10^6, 10^7, 10^8 CFU/mL). Data were recorded after 4, 6, 8, and 10 days. The number of living and dead brown plant hoppers were recorded and the mortality rate was analyzed by using Abbott's formula:

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

E (%): Efficiency percentage; C: Number of living BPH in the control treatment; T: Number of living BPH in the endophytic treatments.

2.3.2. Bacterial identification using 16S rRNA sequences

DNA extraction and PCR amplification of 16S rRNA: The DNA extraction protocol was followed Phuong et al., 2015. The amount and purity of DNA were determined by absorbance at 260 nm and 280 nm using UV-spectrophotometer. The bacterial strains were

characterized by 16S rRNA gene (rDNA) sequencing analysis. PCR were performed from overnight grown cells using universal primers (63F 5'- CAGGCCTAACACATGCAAGTC -3' and 1489R 5'-TACCTTGTTAFFACTTCA-3') (Weisburg et al., 1991; Juilian et al., 1998). The amplification was performed in a thermocycler programmed as follows: 95°C for 3 min; 34 cycles of 95°C for 30 s, 52°C for 30 s, 72°C for 2 min; 72°C for 5 min; 4°C for storage. The PCR amplicon was purified and sequenced by VNDAT company (http://vndat.com.vn/vn/). Partial 16S rDNA sequences obtained were analyzed using the BLAST tool in the NCBI website.

2.4. Statistical analysis

Data were subjected to analysis of variance (ANOVA) followed by Duncan, Statgraphics plus 3.0 software.

3. Results and discussion

3.1. Endophytic bacteria isolation

25 endophytic bacteria strains were isolated and purified (Figure.1 and Figure.2). The isolated baterial strains were tested by Gram staining and spore staining (Table 1).

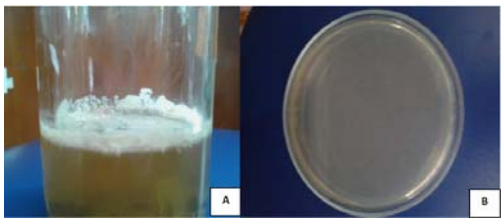

Figure 1. Soaking in TSB (A) and TSA control (B)

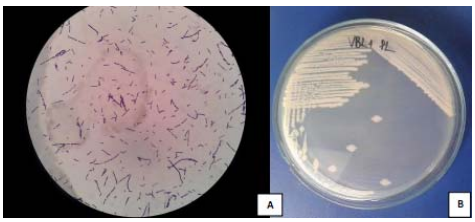

Figure 2. VBL1

Table1

Stainning results of the endophytic bacteria isolates

Collection place	No.	Strains	Gram staining	Spore stain
Cu Chi-HCM	1	VCT1	Gram (+)	+
	2	VCT2	Gram (-)	_
	3	VCT3	Gram (+)	+
	4	VCT4	Gram (+)	+
	5	VCT5	Gram (+)	+
	6	VCT6	Gram (-)	_
	7	VCL1	Gram (-)	_
	8	VCL2	Gram (+)	+
	9	VCR1	Gram (-)	_
	10	VCR2	Gram (-)	_
	11	VCR3	Gram (-)	_
	12	VCR4	Gram (-)	_
	13	VBT1	Gram (-)	_
	14	VBT2	Gram (+)	+

Collection place	No.	Strains	Gram staining	Spore stain
Binh Duong province	15	VBT3	Gram (+)	+
	16	VBT4	Gram (+)	+
	17	VBT5	Gram (+)	+
	18	VBT6	Gram (+)	+
	19	VBT7	Gram (+)	+
	20	VBL1	Gram (+)	+
	21	VBL2	Gram (+)	+
	22	VBL3	Gram (+)	+
	23	VBL4	Gram (+)	+
	24	VBR1	Gram (-)	+
	25	VBR2	Gram (+)	+

Note: Positive results: + Negative results: -

3.2. The optimal concentration of endophytic inoculants to control brown plant hopper

Table 2 indicated that the 3 isolated strains, including VBL1, VBT1 and VBT5, showed the highest biological control brown plant hopper at the mortality rate 46,95%; 55,02% and 55,02%, respectively after 8 days trial and have significant difference compare to other treatments ($P<0.05$).

Table 2

Biological control brown plant hoppers of endophytic bacteria isolates

Isolates	Days after treatments		
	4 days	6 days	8 days
VBT5	17,74 a	43,10 a	55,02 a
VBL1	8,89 a	26,58 ab	46,95 ab
VBL2	0,05 a	26,58 ab	43,10 abc
VBR2	8,89 a	26,58 ab	30,80 bcd
VBT1	8,89 a	26,58 ab	55,02 a
VCR1	8,89 a	17,74 bc	30,80 bcd
VBT3	8,89 a	17,74 bc	26,56 bcde
VCT5	0,05 a	17,74 bc	26,18 cde
VCT6	8,89 a	17,74 bc	17,74 def
VCL1	8,89 a	17,74 bc	26,58 bcde
VBL4	0,05 a	8,89 bc	8,89 ef
VBT2	0,05 a	8,89 bc	8,89 ef

Isolates	Days after treatments		
	4 days	6 days	8 days
VCR3	0,05 a	8,89 bc	8,89 ef
VCT1	8,89 a	8,89 bc	8,89 ef
VCT2	0,05 a	8,89 bc	17,74 def
VBR1	0,05 a	8,89 bc	26,56 bcde
VCL2	0,05 a	8,89 bc	8,89 ef
VBL3	0,05 a	0,05 c	26,56 bcde
VBT4	8,89 a	0,05 c	0,05 f
VCR2	0,05 a	0,05 c	0,05 f
VCR4	0,05 a	0,05 c	8,89 ef
VCT3	0,05 a	0,05 c	0,05 f
VBT6	0,05 a	0,05 c	0,05 f
VCT4	0,05 a	0,05 c	0,05 f
VBT7	0,05 a	0,05 c	8,89 ef

Data were analyzed using one-way analysis of variance (ANOVA) followed by Duncan. Different letters indicate statistically significant differences among groups ($p<0.05$).

Table 3

Investigation of the optimal concentration of three endophytic bacteria isolates

Isolates	CFU/ml concentration	Days after treatments			
		4 days	6 days	8 days	10 days
VBT5	10^8	29,24 a	36,23 a	47,97 a	60,14 a
	10^7	4,62 b	27,86 a	39,18 b	49,35 b
	10^6	0,02 b	11,26 b	29,90 c	40,69 c
VBT1	10^8	24,54 a	37,74 a	50,85 a	63,83 a
	10^7	13,83 ab	26,57 b	37,68 b	49,35 b
	10^6	4, 62 b	9,23 c	28,24 c	40,69 c
VBL1	10^8	24,54 a	39,10 a	50,74 a	61,80 a
	10^7	13,83 b	26,57 b	42,13 b	52,30 b
	10^6	0,02 c	19,44 c	31,56 c	42,13 c

Data were analyzed using one-way analysis of variance (ANOVA) followed by Duncan. Different letters indicate statistically significant differences among groups ($p<0.05$).

The result of investigation of the optimal concentration of three endophytic bacteria isolates (VBL1, VBT1 and VBT5) showed the highest biological control *Nilaparvata lugens* after 10 days of treatments at 10^8 CFU/ml concentration (Table 3). Endophytic bacteria offers an effective strategy for biological control pest. Several endophytic bacterial strains have been reported to induce systemic resistance such as ISR (induced systemic resistance), bioagents promote plant growth and reduce pest in several crops. More than 30 species of insect have been found to be combined with endophyte infected *Lolium perenne* and *Lolium arundinaceum*, or by bioassaying the compounds produced by the endophyte in those plants; however, insect species will response differently to endophyte-infected grass (Takuya and Koya, 2010). Although we have not examined in details the biological role of 3 endophytic bacteria isolates in this study but the result showed the capacity of the three endophytic bacteria isolates (VBL1, VBT1 and VBT5) to control the brown plant hoppers.

3.3. Molecular identification of effective bacterial endophytes

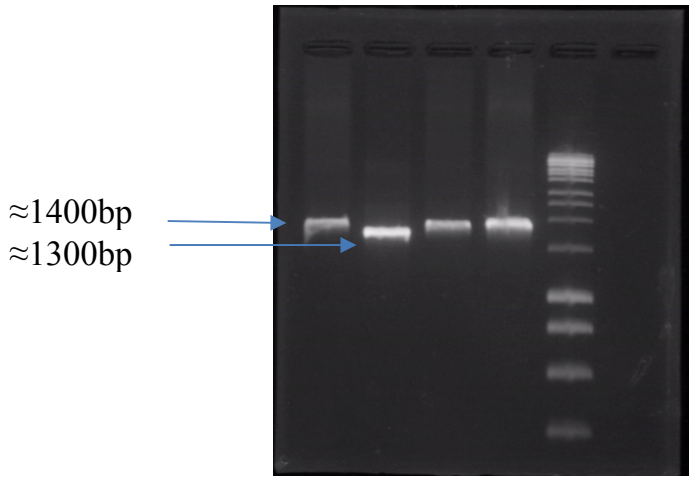

Figure 3. PCR amplification of single 1.5 kb of 16 S rDNA amplicon16S rDNA gene on an agarose gel (1%). Lane 1: VBT1 isolate- 1.4kb DNA; Lane 2: VBL5 isolate-1.3kb DNA; Lane 3, 4: VBL1-1.4 lb; Lane 5: DNA marker (1kb ladder).

The 16 S rDNA primers amplied a fragment size of 1.4kb and 1.3kb. We compared the sequence of each strain VBL1, VBT1 and VBT5 on NCBI genebank and the variable sites analyses from the alignment of the dataset were performed in MEGA 6.06. Maximum Likelihood (ML) method was used to reconstruct phylogenetic trees with value Bootstrap 1000 repeat times. The result of identification was similar to *Bacillus pumilus* (VBT1, VBT5) and *Bacillus thuringensis* (VBL1) (Fig 4). Study by Li et al. (2016) revealed the four endophytic bacteria from elephant grass which were classified into four bacterial genera: *Sphingomonas, Pantoea, Bacillus,* and *Enterobacter* significantly promoted plant growth and biomass yield, alleviated the harmful effects of salt stress on Hybrid Pennisetum. Other study on the control of insects-pests by endophytic fungi showing protection of the perennial ryegrass *Lolium perenne* L. against the sod webworm (Funk et al., 1983, Kanda et al., 1994). Also, Gaynor and Hunt (1983) observed in several ryegrasses that high fungi infection is correlated with a decrease in the attack frequency of the Argentine steem weevil *Listronotus bonariensis*.

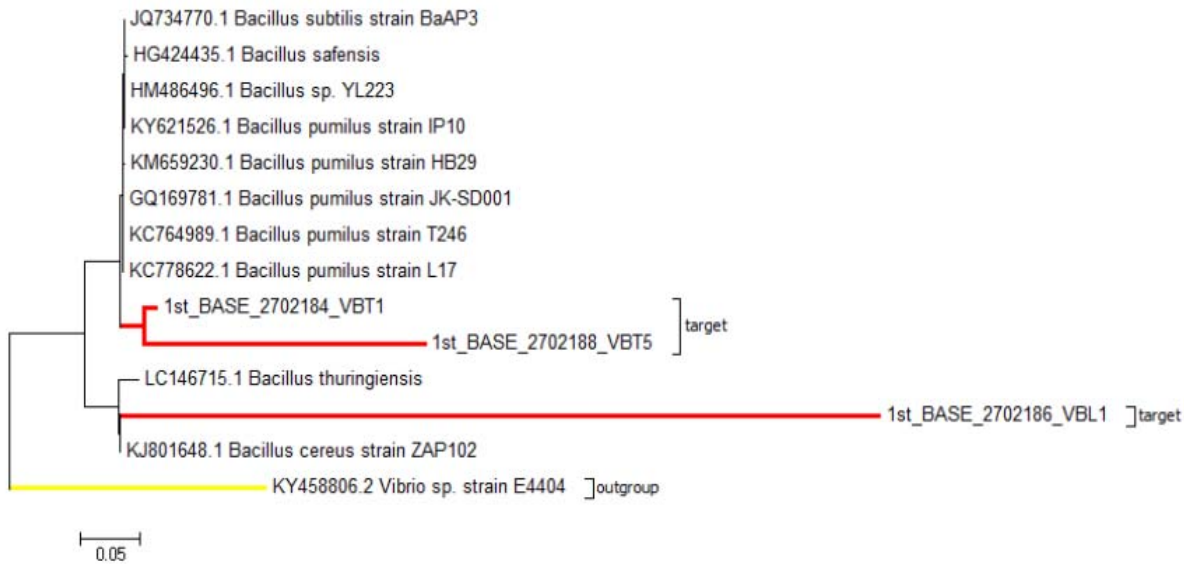

Figure 4. Maximum Likelihood tree of bacteria endophytes on 16S rRNA gene sequences.

4. Conclusion

In this study, we isolated 25 endophytic bacteria strains and selected three endophytic bacteria strains (VBL1, VBT1 and VBT5) which showed the highest biological control to brown plant hopper. Additionally, concentration of 10^8 CFU/mL is the optimal concentration in pesticide activity of these strains. Therefore, this result contributed important data for the collection of endophytic bacteria and provided potential to control brown plant hopper in the future■

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