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SCREENING OF SALT TOLERANT BACTERIA FOR PLANT GROWTH PROMOTION ACTIVITIES AND BIOLOGICAL CONTROL OF RICE BLAST AND SHEATH BLIGHT DISEASE ON MANGROVE RICE

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ABSTRACT

From 22 rice, soil and water samples collected in the field of Long An and Tien Giang provinces, we isolated and screened 87 strains of bacteria around the root zone and endophytic bacteria. Through testing the ability of plant growth stimulation, the result showed 16 strains were capable of nitrogen fixation, 13 strains were capable of phosphate solubilization, 27 strains were capable of IAA production and 2 strains had all 3 activities. By the dual testing method and the percentage of inhibition method between bacterial and fungal pathogen, LD5 and LS6 strains had the highest antifungal activity against *Rhizotocnia* sp. CR1 at 94.02 %. TS3 and TĐ13 strains had the highest antifungal activity against *Magnaporthe sp.* BP3 at 81.74 ± 0.88 % and 80 ± 0.60 %, respectively. Furthermore, there were 6 strains inhibiting both *Rhizotocnia* sp. CR1 and *Magnaporthe* sp. BP3 (LĐ5, LS4, LS6, LN1, LN6, TS3). The strains were identified by biochemical methods. The results showed that LD5, LS6 and TS3 were 70.37 % similar to *Bacillus thurigiensis*, TD13 strain was 70.37 % similar to *Bacillus pantothenticus*, TD9 strain was 72.72 % similar to *Azotobacter vinelandii* and TD6 strain was 70.37 % similar to *Bacillus subtilis*. Regarding the test of activity to stimulate growth in net house model, combination of 4-strain (TD6, TD9, TD13, TS3) had the effect of increasing the length of roots, trunk and weight of rice compared with control treatment. For evaluation of biocontrol of fungal pathogen in net house model, the abilities to control sheath blight in N-2C1 and N-LĐ5 treatment were the highest (40.59 % and 39.06 %, respectively). The ability to control rice blast in N-2C2 treatment was the highest (41.26 %). The ability to biocontrol both sheath blight and rice blast in N-4C treatment was 37.89 %.

Keywords: salt tolerant bacteria, rice blast, sheath blight, plant growth promotion activities, *Rhizoctonia* sp. CR1, *Magnaporthe* sp. BP3.

1. INTRODUCTION

Salt marsh is estimated at 380 million hectares, accounting for 1/ 3 of the world's arable areas. Salt marsh causes physiological limitation, limits the growth of trees and affects rice yield. Therefore, there is a need to do research on limiting the harmfulness levels of salt marsh. One solution to this problem is the use of bacteria capable of growing well in saline soil and stimulating the activity of plant growth such as nitrogen fixation, available phosphorus, hormone plant growth and resistance to fungal pathogens [1].

Currently, many researches have been conducted about plant growth promoting rhizobacteria (PGPR) and *endophyte* to stimulate plant growth and biocontrol of fungal pathogens such as the use of PGPR to reduce the possibility of salt stress in plants and the biological control ability on some fungal pathogens [2], as well as production of some natural compounds to plants [3]. In Viet Nam, several bacterial strains from rice rhizosphere soil and plant endophytic were recorded to have the ability of nitrogen fixtation and indole acetic acid (IAA) synthezytation [4]. Many studies were also conduted to evaluate their abilities on plant growth promoting of rice [5].

The research and application of salt tolerant bacteria on rice can be the solution to overcome the constraint low productivity and disease caused by fungus. In this research, we isolated and screened some bacteria capable of growing well in saline soil and stimulating the activity of plant growth such as nitrogen fixation, available phosphorus, indole acetic acid (IAA) production, and antifungal activity against *Magnaporthe* sp. causing rice blast and *Rhizotocnia* sp. causing sheath blight disease on rice. We aimed to make a collection of bacteria strains with capability to solve the mentioned problems and to build a foundation for later researches.

2. MATERIALS AND METHODS

2.1. Materials

For isolating rhizobacteria and endophytic bacteria, 22 samples (7 soils samples, 7 root-zone-water samples, 8 rice plant samples) collected in Tien Giang and Long An province. The fungal pathogens were isolated from 2 rice plant samples with symptoms of rice blast collected in Binh Chanh District (Ho Chi Minh City) and 2 rice plants samples with signs of sheath blight disease collected in Can Giuoc District (Long An province). Rice strain OM 6561 was provided by Dong Thap Agriculture Seed Center.

2.2. Methods

Based on the target, our research was arranged following the diagram (Figure 1).

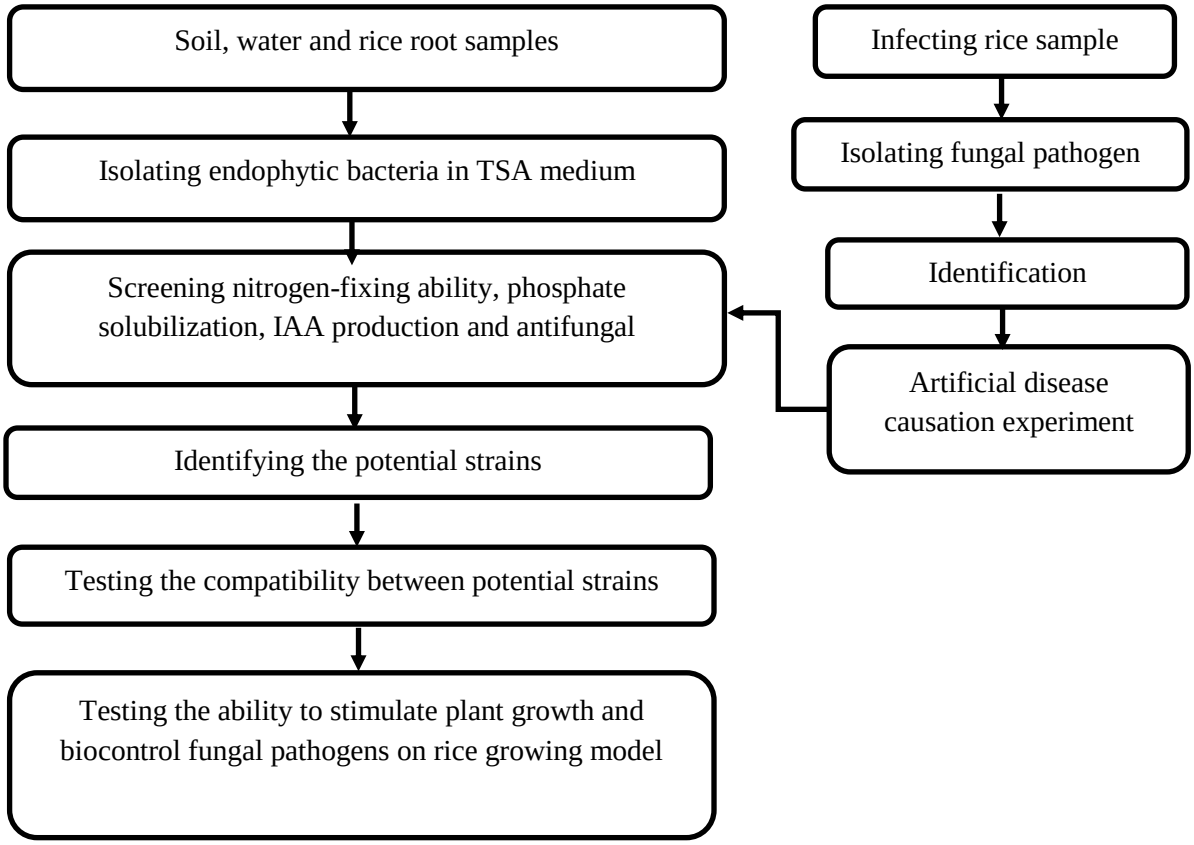

Figure 1. The *experimental arrangement.*

2.2.1. Isolation of salt tolerant bacteria

Isolation of rhizobacteria: Soil and water samples of rice growing areas were diluted 100 times, then inoculated by spreading in NA medium and incubated for 24 hours at 37 ^{o}C.

Isolation of endophytic bacteria: Surface of roots and trunks of rice were sterilized in 70% alcohol, 15 minutes. Then, all of trunks and roots were masticated, spreaded in TSA medium, and incubated in 3 days at 28 ^{o}C until appearing bacterial conlonies [6].

The pure bacterial trains were screened for salt tolerance in 2 ‰; 4 ‰; 6 ‰; 8 ‰; 10 ‰ NaCl, incubated for 24 hours at 37 ^{o}C [7]. Strains which survived in 4 ‰ NaCl were kept on test tubes with NA medium.

2.2.2. Isolation and preliminary identification of fungal pathogens and causation of artificial disease on rice

After surface sterilization, infected rice samples were put into PDA medium and covered by parafilm and incubated at 27 ± 2 ^{o}C. Preliminary identification of fungus was based on the morphological characteristics. Isolated fungus was the causation of rice artificial disease following *Koch's* Postulates [8].

2.2.3. Evaluation of plant growth promoting ability

The nitrogen-fixing ability of isolated bacteria was preliminarily screened on agar plate of nitrogen-free with 4 ‰ NaCl added [9]. The phosphate solubility of these strains was preliminarily screened on Pikovskaya's agar with 4 ‰ NaCl added. The potential strains with suitable activity should create clear zones surrounded by colonies. The quantity of available Phosphorus was determined by liquid cultivation of potential strains with tricalcium phosphate as testing chemical. The content of solubilization Phosphate in liquid culture supernatant was estimated by measuring the absorbance at a wavelength of 600 nm after giving react with the Chloromolybdic acid and Cholorostannous acid [10, 11].

IAA content created on NB medium with 4 ‰ NaCl added was determined by a colorimetric technique at 530 nm wavelength while affecting with Salkowski reagent- R2 ($FeCl_3$ – H_2SO_4) [12].

2.2.4. In vitro antifungal assay

The screening the antifungal ability of bacteria was based on the dual culture method. Both bacterium and fungus were placed on PDA with 4 ‰ NaCl added which was described by Suryadi et al. [13] and had the 3 cm distance from each other.

The antifungal activity of liquid cultivating supernatant was estimated via a growth inhibition which was described by Wang et al. [14]. The percentage of inhibition was identified by the formula:

$$I\ (\%) = (C - E)/\ C \times 100\ \%$$

I: the percentage inhibition, C: the diameter of the fungus on the control petri (cm), E: the diameter of the fungus on the petri with culture supernatant.

2.2.5. Bacteria identification

The selected strains were preliminarily identified. After that, the *Bacillus* strains classification was according to Cowan and Steel [15], while the classification of other bacteria according to Bergey's manual [16].

2.2.6. The compatibility between potential strains

The compatibility between different strains was conducted by cross streak method [17]. The compatibility between potential strains was conducted for the combination of several microbial strains when apply in field.

2.2.7. Evaluation of activity to stimulate plant growth in net house model

The ability of these strains about the nitrogen fixation, phosphate solubility and IAA production were evaluated accordingly in net house model. Seeds, culture fluid and soil were prepared according to the description of Nguyen et al. [18]. The experiment was arranged randomly (CRD) with the experiment was treated single strains/combined inoculations and control experiments untreated bacteria. Each experiment was triplicated. After 25 days, result was recorded based on trunk length, root length (cm), weight of dried and original sample (g).

Statistical analysis conducted by Microsoft Excel 2010 and Statgraphics plus 3.0. Growth promotion efficacy (GPE %) of plants was calculated by the formula below [19].

$$\text{GPE (\%)} = \frac{(1 - \text{Original weight of control treatment}) \times 100}{\text{Original weight of experiment treatment}}$$

The treatments included:

- N-DC: Control
- N-X: Rice was paralelly cultivated with single the potential bacterium or fungus as single strain (X = name of fungal or bacterial strains).
- N-4C: Rice was paralelly cultivated with the combination of 4 potential bacteria or fungi (4 strains: TĐ13, TĐ6, TĐ9 and TS3).

2.2.8. Evaluation of biocontrol of fungal pathogen in net house model

The experiment was arranged completely random. The experiment was prepared similarly to the evaluation of activity to stimulate growth on net house model**.** The treatments included:

- N-DC1: Rice was paralelly cultivated with pathogen *Rhizotocnia* sp. CR1.
- N-DC2: Rice was paralelly cultivated with pathogen *Magnaporthe* sp. BP3.
- N-DC3: Rice was paralelly cultivated with pathogens *Rhizotocnia* sp. CR1 and *Magnaporthe* sp. BP3
- N-X: Rice was paralelly cultivated with single the potential bacterium or fungus as single strain and pathogens (X = name of fungal or bacterial strains)
- N-4C: Rice was paralelly cultivated with the combination of 4 potential bacteria or fungi and pathogens (combined 4 strains: TĐ13, TĐ6, TĐ9 and TS3).
- N-2C: Rice was paralelly cultivated with the combination of 2 potential bacteria or fungi and pathogens (N-2C1 combined LD5 and LS6; N-2C2 combined TS3 and TD13)

Fungal pathogens preparation: Different fungus were plated on PDA medium, at 28 ± 1 ^{o}C for 5 days [18]. Germinated seeds were put into different sections (20 seeds/ section) with triplicates. In the adding bateria treatments, after seedlings had 1-2 cm root, they were put into bacterial culture fluid containing 10^9 CFU/ mL for 3 hours. Contemporary, NaCl was added to the treatments to create artificial salt-marsh at 0.4 % (using refractometer).

When rice reached 14 days old, 15 ml *bacterial culture fluid* containing 10^9 CFU/ ml was added accordingly to each treatment. After 5 days, 5 ml propagules were added (propagules were incubated for 5 days on PDA medium) for *Rhizotocnia* sp. CR1 and 5 mL fungal spores at $10^7 – 10^8$ CFU/ mL for *Magnaporthe* sp. BP3 in each treatment to cause disease. Moisture was kept high (> 90 %), fully covered to rice blast [20].

Disease index (%) of plants was calculated by the below formula:

$$\text{Disease index (\%)} = \frac{\Sigma\ (\text{Number of infected plants x Infected level}) \times 100}{\text{Total number of plants in the experiment} \times 4}$$

After 14 days, the ability to bicontrol disease (BD) is calculated by: BD = (A-B) / A × 100; with A: disease index of plants with fungus added. B: disease index of plants with antifungal bacteria added [20].

3. RESULT AND DISCUSSION

3.1. Bacteria isolation

From 22 rice, soil and water, we isolated 88 bacteria strains. Therein, 87/ 88 strains have ability of salt tolerance in 4 ‰ NaCl, 72/ 88 strains have ability of salt tolerance in 6 ‰ NaCl, 56/ 88 strains have ability of salt tolerance in 8 ‰ NaCl, 37/88 strains have ability of salt tolerance in 10 ‰ NaCl. This is a potential collection of salt tolerant bacteria.

3.2. Isolation of fungal pathogens

From macroscopic and microscopic observations of fungal pathogens and comparison of morphological characteristics of fungus caused rice blast and sheath blight described by Mew and Gonzales [21] and combined with artificial disease causation experiment, we found that isolated BP3 and CR1 were similar to *Magnaporthe* sp. and *Rhizotocnia* sp., respectively.

3.3. Plant growth stimulation

From 87 bacteria strains with the ability of salt tolerance in 4 ‰ NaCl, we isolated 16 strains ability of nitrogen fixation, 13 trains showing ability of phosphate solubility and 27 strains ability to produce IAA. Therein, the phosphate solubility of TD13 reached the highest (97.03 µg/ ml) and the phosphate solubility of TN4, TS1 were the lowest (19.85 µg/ ml); IAA production of TD6 reached the highest (720.00 µg/ ml), LS1 were the lowest (107.33 µg/ ml). Besides, TD9 and TS3 have multiple capabilities of nitrogen fixation and available phosphorus (34.46 and 59.6 µg/ ml, respectively), and producing IAA (237.00 µg/ ml). In the research of Tran et al. [22], the strain had high ability of soluble phosphorus produced P_2O_5 at 36.2 mg/ L, TD13 produced three times more P_2O_5 by comparison. In another research of Nguyen et al. [4], the strain with the highest ability of IAA production reached to 41.1351 µg/ ml. In comparison, TD6 produced significantly higher than the 41.1351 µg/ ml. Therefore, TĐ13, TĐ6, TĐ9 and TS3 were selected for later experiments.

3.4. The antifungal ability of bacteria

The experiment result showed that 9 strains had antifungal activity against *Rhizotocnia* sp. CR1 (LĐ5, LS4, LS6, LN1, LN3, LN6, LN8, TS3 and TS5). LD5 and LS6 had the highest antifungal activity against *Rhizotocnia* sp. (Figure 2). CR1 at 94.02 %. In addition, there were 11 strains had antifungal activity against *Magnaporthe* sp. (Figure 3). BP3 (LĐ2, LĐ5, LS4, LS6, LN1, LN6, LN10, TĐ13, TS3, TN3, TN4) and TS3, TĐ13 had the highest antifungal activity of 81.74 ± 0.88 %; 80 ± 0.60 %, respectively. Furthermore, there were 6 strains LD5, LS4, LS6, LN1, LN6, TS3 restraining both *Rhizotocni*a sp. CR1 and *Magnaporthe* sp. BP3 (Figures 2, 3). Nguyen et al. [5] screened 2 strains of *B. subtilis* and *B. macerans* capable of restraining *Rhizotocnia* sp. at 99.00 % và 98.96 %, higher than our results. Additionally, in a research by Bais et al. [23], *Pseudomonas* EA105 strain from rice root zone had activity of restraining at 76.00 %, and its activity was lower than the activitiy of TS3 strains (81.74 ± 0.88 %). For next experiment, TS3, TĐ13, LĐ5 and LS6 strains were selected.

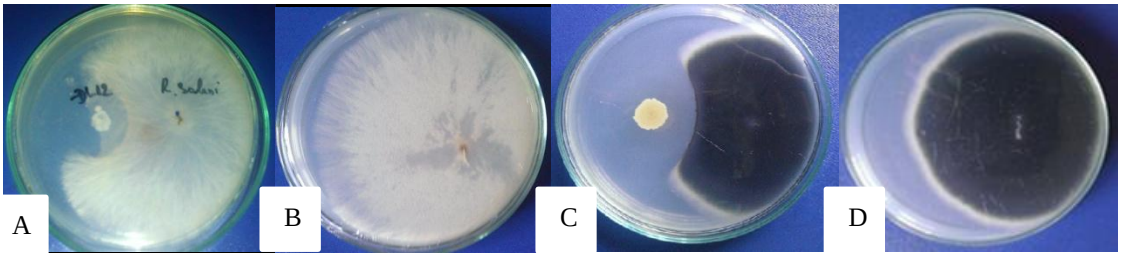

Figure 2. Dual culture between anti-phytopathogenic strains with pathogens.
A. Dual culture between CR1strain and *Rhizotocnia* sp. B. Colony of *Rhizotocnia* sp. as control. C. Dual culture between LD5 strain and and *Magnaporthe* sp. D. Cololy of *Magnaporthe* sp. as control.

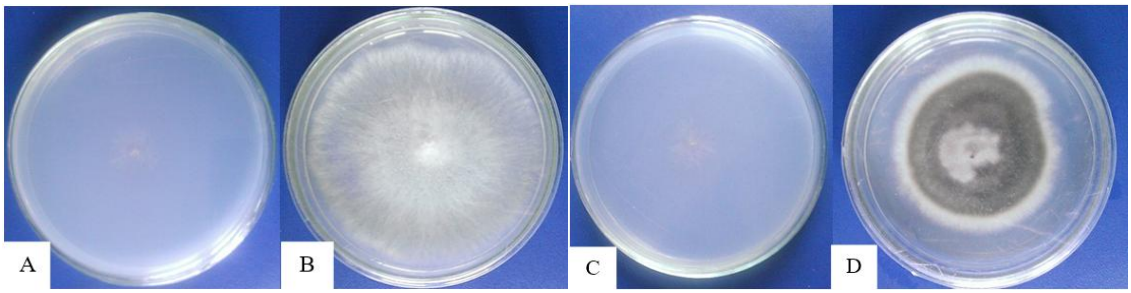

Figure 3. Plant pathogenic fungi grow in medium adding cultivation supernatant of LD5 strains.

A. *Rhizotocnia* sp. CR1 strain grows in the adding cultivation supernatant medium. B. *Rhizotocnia* sp. CR1 strain grows without adding (control). C. *Magnaporthe* sp. BP3 strain grows in the adding cultivation supernatant medium. D. *Magnaporthe* sp. BP3 strain grows without adding (control).

3.5. Identification

These bacteria were identified by their morphological, physiological and biochemical characteristics according to Bergey's Manual [15]. The rate of biochemical test was appropriate with the total biochemical test. TD9 strain was belonged to *Azotobacter* with 72.72 % similarity to *Azotobacter vinelandii*. TS3, LS6, LD5 strains were belonged to *Bacillus* with 70.37 % similarity to *Bacillus thurigiensis*; TD13 strain was belonged to *Bacillus* with 70.37 % similarity to *Bacillus pantothenticus*; and TD6 strain was also belonged to *Bacillus* with 70.37 % similarity to *Bacillus subtilis*.

3.6. Compatibility between selected strain

By the cross-streak method, the growth stimulation activity TĐ6, TĐ9, TĐ13, TS3 strain were compatible. The strains with antifungal activity LS5, LĐ6, TS3, TĐ13 were compatible. These strains can incorporate in one treatments in experiments to access the effectiveness on rice growing experiment.

3.7. Evaluation of plant growth promotion activity of the strains in net house model

Table 1. The ability to stimulate plant growth of the strains in net house.

No	Treatment	Root length (cm)	Trunk length (cm)	Original weight (g)	Fried weight (g)	GPE (%)
1	N-DC	4.07 ± 0.43^{d}	32.34 ± 0.69^{f}	1.469 ± 0.195^{d}	0.206 ± 0.023^{d}	-
2	N-TD6	8.34 ± 0.35^{c}	38.43 ± 0.68^{d}	2.308 ± 0.235^{c}	$0.346 \pm 0.,059^{c}$	36.35
3	N-TD9	9.72 ± 0.41^{b}	40.91 ± 0.51^{c}	2.404 ± 0.115^{c}	0.369 ± 0.023^{c}	38.89
4	N-TD13	7.72 ± 0.40^{c}	36.11 ± 0.60^{e}	2.110 ± 0.116^{cd}	0.300 ± 0.023^{cd}	30.37
5	N-TS3	10.48 ± 0.38^{b}	43.90 ± 0.76^{b}	4.434 ± 0.254^{b}	0.639 ± 0.021^{b}	66.86
6	N-4C	12.74 ± 0.35^{a}	49.99 ± 0.39^{a}	4.842 ± 0.392^{a}	0.659 ± 0.019^{a}	69.66

In the same column, the value with different superscript letters are significant different by Duncan test ($p \leq 0.05$).

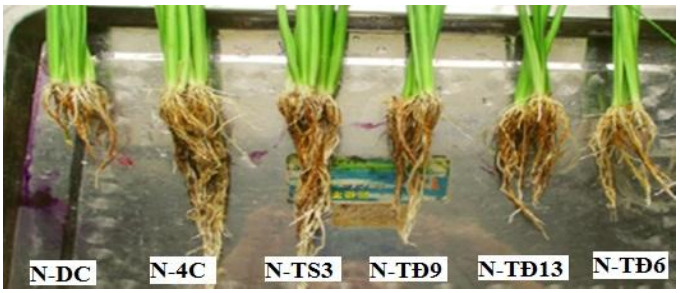

Figure 3. The length of rice roots in 6 treatments.

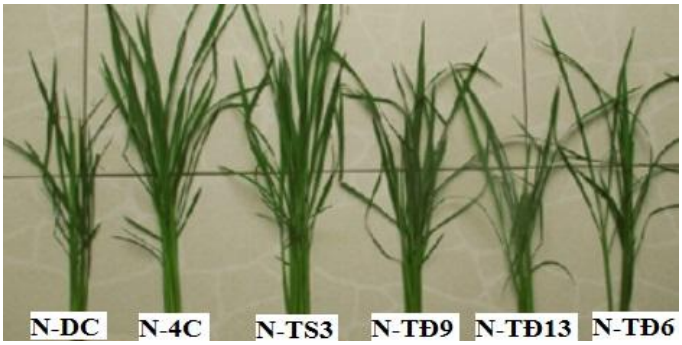

Figure 4. The length of rice trunk in 6 treatments.

From 4 strains with highest ability to stimulate plant growth, after 25 experiment days, we started accessing some agricultural characteristics such as root length, trunk length, original weight, fried weight. The result in Table 1, Figures 3 and 4 showed all 5 strains had ability to stimulate rice growth (with GPE of 69.66 % and 66.86 %, respectively). Compared to the control treatment N-DC, those all 5 treatment N-TD6, N-TD9, N-TD13, N-TS3 and N-4C can promote

rice to increase length and weight. This result also showed that the strains TD13, TD6, TD9, TS3 strains had ability to stimilate rice plant and potential of practical application for later research.

Among single strain treatment, strain TS3 has the highest capablility for stimulating rice plant. However, the combination of all strains N-4C can stimulate the growth of rice higher than single strain treatment N-TS3.

3.8. E*valuation* of biocontrol of fungal pathogen in *net house model*

Four isolates with highest antifungal activity *were evaluated* of biocontrol on rice growing experiment. The result shown in Table 2 indicated that the abilities to control sheath blight in N-2C1, N-LĐ5 treatment were the higher (40.59 % and 39.06 %, respectively) and had significant differences compared to LS6 (31.46 %). It was similar with a previous research [5] with the ability to control 39.08 % sheath blight. The ability to control rice blast in N-2C2 treatment was the highest (41.26 %), showing significant difference to other treatments. Comparing with the previous study of Lucas et al. [24], the ability to control rice blast in N-2C2 treatment is still not equal (the ability to control 50.00 % of rice blast on rice of PGPR). The ability to control to both sheath blight and rice blast diseases in treatment N-4C was achieved at 37.89 %.

Table 2. Anti-fungal experiment result.

Pathogens	Treatment	Disease index (%)	Ability to control disease (%)
Rhizotocnia sp. CR1	N-DC1	82.08 ± 1.10^{a}	-
	N-LD5	50.00 ± 0.00^{c}	39.06 ± 0.83^{a}
	N-LS6	54.58 ± 1.10^{b}	31.46 ± 0.18^{b}
	N-2C1	48.75 ± 0.00^{c}	40.59 ± 0.81^{a}
Magnaporthe sp. BP3	N-DC2	78.75 ± 0.65^{a}	-
	N-TS3	50.41 ± 0.4^{b}	34.4 ± 0.81^{b}
	N-TD13	51.67 ± 1.10^{b}	35.97 ± 0.78^{b}
	N-2C2	46.25 ± 0.00^{c}	41.26 ± 0.54^{a}
Rhizotocnia sp. CR1 + *Magnaporthe* BP3	N-DC3	79.17 ± 0.17^{a}	-
	N-4C	49.17 ± 0.72^{b}	37.89

In the same column, the value with different superscript letters are significant different by Duncan test ($p \leq 0.05$).

4. CONCLUSION

In this research, 87 bacteria strains were isolated and could survive in 4 ‰ NaCl. *Magnaporthe* sp. BP3 caused rice blast and *Rhizotocnia* sp. CR1 caused sheath blight on rice. The following experiments showed that the combination of 4 strains (TĐ6, TĐ9, TĐ13, TS3) had the potential to stimulate rice grow at the highest rate (with GPE 69.66 %). The ability to

control sheath blight in N-2C1 was the highest (40.59 %) and the ability to control rice blast in N-2C2 treatment was the highest (41.26 %). These strains are potential to produce bio-products which stimulate growth and biocontrol two fungal pathogen for rice in salt marsh. They are the foundation for further researches.

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