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BIOCONTROL POTENTIALITY OF *Burkholderia vietnamiensis* NRV12 AGAINST THE RICE BLAST FUNGUS *Magnaporthe oryzae*

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ABSTRACT

Rice blast disease, caused by the pathogenic fungus *Magnaporthe oryzae*, is a widespread infection leading to serious crop loss worldwide. In order to achieve sustainable agriculture, root-associated bacteria have been applied to manage fungal diseases and promote growth. The present study aimed to evaluate *in vitro* the growth-promoting ability and *in vivo* biocontrol activity against *M. oryzae* of rice rhizosphere bacterium. Out of sixty-eight isolates recovered from the rhizosphere of blast-infected rice plants, isolate NRV12 exhibited the highest antifungal activity against *M. oryzae* SH, with an inhibition percentage of 72.7±3.44%. By analysis of 16S rRNA sequence associated with morphology, physiological and biochemical tests, the strain was identified as *Burkholderia vietnamiensis*. In addition, NRV12 produced hydrolytic enzymes (amylase, cellulase, protease), indole acetic acid (IAA), exhibited nitrogen-fixing potential and the ability to solubilize phosphate and zinc. Innoculation with NRV12 significantly promoted *in vivo* rice seedling growth to 23.3% as compared to the non-bacteria-treated seedlings. Importantly, infected rice seedlings treated with NRV12 led to a 40% disease reduction in rice blast. These findings suggest that NRV12 is a valuable and promising isolate with biocontrol potential against rice blast caused by *M. oryzae*.

Keywords: Antifungal activity, biocontrol, *Burkholderia*, chitinase, plant growth promotion, rice blast disease.

INTRODUCTION

Rice blast caused by the ascomycete, filamentous pathogenic fungus *Magnaporthe oryzae* (syn. *Pyricularia oryzae*) can infect all growth stages and affect all above-ground parts of the plant including rice leaves, collars, nodes, necks, and parts of the panicle. The blast disease can lead to yield losses of up to 80% and cause serious threat to global food security. In Vietnam, rice blast occurred in numerous

rice-growing areas, especially under favorable weather conditions for the disease at 25 - 30°C and 80 - 95% humidity (Phi, 2023).

Various strategies have been implemented to control the blast disease, such as agricultural practices, resistant rice varieties, and chemical and biological control (Phi, 2023). Among them, chemical fungicides are easy to use and show certain effectiveness, however overuse has resulted in resistant strains and adverse effects on human health and the environment. Novel approaches based on biological fungicides to suppress the blast fungus are needed for sustainable management of the disease, for instance, using antagonistic microorganisms as alternatives to chemical fungicides. Plant growth-promoting rhizobacteria (PGPR) are known as effective biocontrol agents for the suppression of fungal phytopathogens. The common biocontrol mechanisms include competition, production of siderophores, antagonism and induced systemic resistance via metabolites such as lytic or defense enzymes/compounds e.g., protease, chitinase, peroxidase, catalase, polyphenol oxidase, phenylalanine ammonia-lyase, and cycling lipopeptides (fengycin, iturin and surfactin) (Lam *et al.*, 2021; Meng *et al.*, 2023). The characterization of plant growth promotion by rhizobacteria involves other mechanisms, including IAA production, phosphate and zinc solubilization, nitrogen fixation, and ACC deaminase activity that enhance metabolism, root development and enzyme activity of the plant. These strategies also support other beneficial microorganisms in promoting advantageous interactions with the plant and suppressing plant pathogens (Yadav *et al.*, 2017).

Burkholderia is one of the most commonly encountered PGPR genera known for its plant colonization and growth-promoting properties through direct and indirect mechanisms. A nitrogen-fixing bacterium, *B. vietnamiensis*, was first isolated from the rice plant in Vietnam (Gillis *et al.*, 1995). Following that, many study demontrated the roles of the genus *Burkholderia* in promoting rice plant growth. Mattos *et al.* (2001) reported strain *B. brasiliensis* from rice root with nitrogen fixation and exopolysaccharide synthesis. Studies by Singh and colleagues showed that *Burkholderia* sp. and *B. cepacia* RRE25 mutants from rice root overproduced IAA, exhibited growth promotion of seedlings through enhancement of plant biomass (Singh *et al.*, 2011) and improved plant height, chlorophyll content, shoot dry weight, and root dry weight (Singh *et al.*, 2013) under greenhouse conditions. Rice seedling growth and development could involve significant uptakes of phosphorous, nitrogen and potassium (Singh *et al.*, 2013). Reports from Vietnam also determined that the species *B. vietnamiensis* was able to fix nitrogen (Phong *et al.*, 2010) enhancing rice productivity (Phong *et al.*, 2018; Phong, 2018; Van *et al.*, 2000; Gillis *et al.*, 1995). Also, several *Burkholderia* spp. promote other crop plants such as maize, potato, and sugarcane through the production of auxin, nitrogen fixation, phosphate solubilization, and siderophore production (Young *et al.*, 2013; Luvizotto *et al.*, 2010; Da *et al.*, 2012).

Burkholderia species are also known for their biocontrol potential against several fungal phytopathogens. *B. phytofirmans* conferred grapevine resistance against the fungus *Botrytis cinerea* via a direct antimicrobial effect (Miotto-Vilanova *et al.*, 2016). Kim *et al.* (2020) reported that

ginseng endophyte *B. stabilis* produced antimicrobial compounds against ginseng root rot disease caused by *Cylindrocarpon destructans* (Kim *et al.*, 2020). Other *Burkholderia* presented inhibition against fungal phytopathogens such as *Macrophomina phaseolina* (charcoal rot disease in many crops) (Zaman *et al.*, 2021), *Sporisorium scitamineum* (sugarcane smut disease) (Cui *et al.*, 2020), *Phytophthora* sp. (root diseases) (Kong *et al.*, 2020), *Calonectria pseudonaviculata* (boxwood blight) (Kong and Hong, 2020), *Fusarium oxysporum* (corm-rot) (Ahmad *et al.*, 2022), and *Rhizoctonia solani* (blight disease) (Meng *et al.*, 2023). Also, several *Burkholderia* spp. have shown great efficiency in protecting host plants under abiotic stress, e.g. diesel-contaminated soil (Afzal *et al.*, 2013) and cadmium stress (Wang *et al.*, 2019).

This study had the following objectives: (i) selection of antifungal rhizobacteria against rice blast, previously isolated from rice roots, (ii) evaluation of the growth-promoting ability of the bacterial strain, and (iii) verification of blast disease tolerance in rice seedlings inoculated with the antifungal bacterium in greenhouse conditions.

MATERIALS AND METHODS

Bacterial, fungal strains, and plant materials

The bacterial isolates were previously isolated from the root (endosphere or rhizosphere or both) of *M. oryzae*-infected rice plants that were grown in the field of Nam Dinh province, Vietnam, and stored at -80ºC at the VAST Culture Collection of Microorganisms. The fungus strain *M. oryzae* SH was provided by the Laboratory of Plant Cell Biotechnology, Institute of Biotechnology. For bioassays, the rice cultivar BT7 (a rice variety commonly grown in North Vietnam, and susceptible to the rice blast fungal pathogen *M. oryzae*) was selected for the experiment.

In vitro screening of bacterial isolates for antagonistic activity against *M. oryzae* SH

Bacterial isolates were screened for antifungal activity against *M. oryzae* SH in dual culture (Lei *et al.*, 2023). An 8-mm-diameter mycelial plug of 3-day-old *M. oryzae* SH on a PDA plate was cut and placed 1 cm away from the border of the 9-cm-diameter PDA plate. Then, the bacterial isolate was streaked on the opposite side of the plate at a distance of 2 cm from the *M. oryzae* SH plug. The petri dish was inoculated with only the mycelial disc of *M. oryzae* SH alone, which served as the control. The plates were incubated for 5 - 7 days at room temperature (25±2ºC) or until the mycelium of *M. oryzae* SH covered the entire control plate. The diameter of mycelial growth was determined, and the percentage growth inhibition was calculated following the formula: $[(D - d)/D] \times 100$, in which D and d represented the diameter of fungal mycelium growth in the control and the plate with bacterial isolate (mm). The experiment was done in three replicates.

Identification of the bacterial isolate NRV12

The morphological characteristics such as shape, size, colour and opacity of the isolated colonies were determined. The Gram staining was done using a Gram staining kit (Himedia, India). The physiological tests such as effect of NaCl concentration (a range of 0.5, 1, 2, 3, 4 and 5%), medium pH (a range of 5, 6, 7 and 8)

and growth temperature (a range of 25, 30, 35 and 37ºC) of the isolate NRV12 were performed on medium MPA following standard microbiological methods. The biochemical properties of the isolate NRV12 such as catalase activity, citrate utilization, assimilation of glucose, lactose and saccarose, activity of lipase andgelatine hydrolysis were done (Gillis *et al.*, 1995).

The 16S rRNA sequence of the isolate that showed the strongest antifungal activity was performed following the description of Jing *et al.* (2020). In brief, DNA of the isolates was extracted using the commercial product QIAamp DNA mini-Kit (QIAGEN) in accordance with the manufacturer's instructions. The bacterial 16S rRNA sequence was amplified using the universal primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′) in a thermal cycler (T100™ Thermal Cycler, BIO-RAD). The PCR reaction was done with a volume of 25 µL containing 12.5 µL of MyTaq Mix 2x (Bioline), 1 µL of each primer (10 pmol/µL), 1 µL of genomic DNA (50 ng/µL and 9.5 µL of ddH_2O for 25 µL total reaction volume. The PCR reaction was performed following the protocol: initial denaturation for 5 min at 95ºC, followed by 35 cycles of denaturation for 30 s at 94ºC, annealing for 45 s at 55ºC, DNA extension for 60 s at 72ºC and a final extension for 10 min at 72ºC. The PCR product was analyzed via electrophoresis on a 1% agarose gel and purified by the PCR GeneJET™ Gel Extraction Kit (Thermo Scientific). The purified PCR product was sequenced at First Base (Malaysia). The bacterial isolate was identified by comparing the 16S rRNA sequence with those available in the GenBank database using the BLASTN program of the NCBI (http://www.ncbi.nlm.nih.gov). The phylogenetic tree was constructed by the neighbor-joining method with 1000 bootstraps using Kimura 2-parameter distances in MEGA v7.0 (Kumar *et al.*, 2016).

Characterization of extracellular enzyme activity

The activity of enzymes including amylase, cellulase, chitinase and protease was determined by culturing the tested bacteria on medium plates with corresponding substrates. For amylase, cellulase and protease tests, a single colony of the NRV12 culture was placed on the MPA medium plate (5 g/L peptone, 3 meat extracts, 5 g/L NaCl, (pH = 7) and 17 g/L agar) with 0.2% starch, 0.5% carboxymethyl cellulose (CMC) and 1% casein, respectively. The plates were incubated at 30ºC for 24 – 48 h. For chitinase test, the NRV12 was cultured on a medium plate containing 3 g/L meat extract, 5 g/L peptone, 0.01 g/L $FeSO_4.7H_2O$, 0.5 g/L $MgSO_4 \cdot 5H_2O$, 0.3 g/L KH_2PO_4, 0.7 g/L K_2HPO_4, 2 g/L chitin colloidal at pH 7, 17 g/L agar and incubated at 30ºC for 24-48 h (Murthy and Bleakley, 2012). For detecting enzyme activities, the culture plates were overlaid with a layer of 1% Lugol's solution for amylase, cellulase and chitinase, 1% TCA solution for protease. The development of a clear zone around the bacterial colony was an indication of hydrolytic enzymes. The diameter of the clear zone was determined. Each test was replicated three times.

Growth promoting traits of *Burkholderia vietnamiensis* NRV12

Nitrogen fixating ability

A bacterial isolate NRV12 exhibiting the highest antifungal activity as a result of the previous experiments was identified as *Burkholderia vietnamiensis* by the 16S rRNA sequence. The isolate was selected for the nitrogen-fixing test on Ashby agar medium. The NRV12 was grown on a nitrogen-free Ashby agar plate (20 g/L glucose, 0.1 g/L $CaSO_4$, 0.1 g/L KH_2PO_4, 5 g/L $CaCO_3$, 0.2 g/L $MgSO_4.7H_2O$, 20 g/L NaCl, 18 g/L agar, adjusted for a final volume of 1000 mL with water, and at pH = 7.0) at 30ºC for 5-7 days (Richard *et al.*, 2009). The nitrogen fixation capacity of the bacteria was determined by the presence of a bacterial colony on the Ashby plate. The experiment was done in three replicates.

IAA production

The IAA concentration was determined by the color reaction of the Salkovski reagent following the method of Glickmann and Dessau (1995). The isolate NRV12 was cultured in MPA broth supplemented with 100 mg/L tryptophan (3 g/L meat extract, 5 g/L peptone, 5 g/L NaCl, pH = 7) then incubated at 30ºC, with shaking at 200 rpm for 3 days. After each 24h interval, a 1-mL bacterial suspension was centrifuged for 5 min. The cell-free supernatant was added to 0.5 mL of Salkowski coloring reagent (35% HClO4 50 mL, 0.5 M FeCl3 1 mL). The mixture was incubated in the dark for 30 min at room temperature. After the reaction, absorbance at 530 nm was measured and IAA concentration was calculated according to the standard curve.

Phosphate and zinc solubilization

The ability of NRV12 to solubilize phosphate and zinc was tested using the Pikovskaya's agar plate culturing method. The spot NRV12 inoculation was placed sequentially on the P1 medium plate (10 g/L glucose, 1 g/L NaCl, 5 g/L NH_4Cl, 1 g/L $MgSO_4 \cdot 7H_2O$, 2 g/L $Ca_3(PO_4)_2$ at pH 7 and 15 g/L agar) and the Z1 medium plate (10 g/L glucose, 1 g/L NaCl, 1 g/L $(NH_4)_2SO_4$, 0.2 g/L KCl, 0.1 g/L K_2HPO_4, 1 g/L $MgSO_4.7H_2O$, 2 g/L ZnO, at pH 7 and 15 g/L agar) at 30ºC for 5-7 days. The diameter of clear halos around the colony was determined. The experiment was done in three replicates.

In vivo assay for plant growth promotion of the strain *Burkholderia vietnamiensis* NRV12

The plant growth-promotion assay was conducted following the description of Riaz *et al.* (2021) with modifications. Rice seeds of cultivar BT7 were surface sterilized for five minutes using 70% alcohol, then added to 20 mL of Javen 60% and shaken at 190 rpm for 45 minutes. Then, the rice seeds were rinsed five times in sterile distilled water. After that, sterilized seeds were soaked in *Burkholderia vietnamiensis* NRV12 suspension (OD_{600} of 0.1) for 16 hours at 28ºC with gentle shaking at 50 rpm. The seeds incubated with sterile distilled water were used as a control. The bacterial inoculated seeds were placed in the box containing sterilized paper for 6 days in a dark room, at 26ºC and 80% humidity. Ten of 7-day-old rice seedlings were transferred to hydroponic boxes with 1/10 MS solution (1650 mg/L NH_4NO_3, 440 mg/L $CaCl_2.2H_2O$, 180.7 mg/L $MgSO_4.7H_2O$, 170 mg/L KH_2PO_4, 1900 mg/L KNO_3, trace elements 6.2 mg/L H_3BO_3, 0.025 mg/L $CoCl_2.6H_2O$, 27.8 mg/L $FeSO_4.7H_2O$, 22.3 mg/L $MnSO_4.4H_2O$, 0.83 mg/L KI, 0.25 mg/L $Na_2MoO_4.2H_2O$, 8.6 mg/L $ZnSO_4.7H_2O$, 36.7 mg/L FeNaEDTA, 0.025

mg/L $CuSO_4.5H_2O$, vitamins of 100 mg/L Myo-Inositol, 0.5 mg/L Nicotinic Acid, 0.5 mg/L Pyridoxine.HCl, 0.1 mg/L Thiamine.HCl, 2 mg/L Glycine). All the rice seedlings were placed in greenhouse conditions for 12/12 h (light/dark) with 80% relative humidity and 26°C. After 45 days of growth, the fresh weight of the rice seedlings was measured in milligrams. The experiment was done in three replicates.

In vivo assay for antagonism of the strain *Burkholderia vietnamiensis* NRV12

For the pathogenicity assay, one-month-old rice seedlings were infected with *M. oryzae* SH, as described by Lam *et al.* (2021). In detail, rice seeds (BT7 cultivar) were first sterilized and germinated as described above. Subsequently, the 7-day-old seedlings were transferred to hydroponic boxes supplemented with 1/10 MS (the same component listed for the plant growth promotion assay). Furthermore, *M. oryzae* SH was grown on PDA plates for 10 days under UV light (12/12 h of light/dark) to induce sporulation. Then, the spores were obtained by adding 10 mL of 0.01% Tween 20 to the *M. oryzae* petri dish and wiping the spores loose with a paint brush. The suspension was filtered using gauze cloth to collect the spore suspension and remove mycelium. The spore suspension was diluted to get 1 x 10^5 spores/mL. After that, the spore suspension was sprayed on one-month-old rice seedlings and kept in an infection room for 24 h at 26ºC, 100% humidity, and dark conditions. Afterward, infected seedlings were transferred to the greenhouse for three weeks. Assessment of the antagonistic effect was defined according to Sha *et al.* (2020) with modifications as follows: counting diseased lesions (equivalent to disease scales of 1, 2 and 3), measuring the diseased area on the leaves (comprising the scales of 4, 5, 6, 7, 8 and 9) and photographing the leaves after evaluation. The experiment was done in three replicates.

Statistical analysis

The data from all experiments was presented as the means ± standard deviation of three replicates. The statistical analysis was done using a *t*-test with significant differences at $p < 0.05$.

RESULTS AND DISCUSSION

Screening of antifungal bacterial isolates against *M. oryzae* SH

A total of 68 bacterial isolates were previously isolated from the roots of *M. oryzae*-infected plants and tested for antagonistic activity against *M. oryzae* SH *in vitro*. Among them, nine isolates exhibited strong inhibition against mycelial growth, ranging from 35.1 to 72.7% (Table 1). The result also showed that NRV12 displayed the highest antagonistic activity (72.7 ± 3.44 %) against *M. oryzae* SH followed by NRV30 (71.9 ± 0.75%) and NRV40 (70.1 ± 3.44%). In addition, the isolate NRV12 was capable of fixing nitrogen as a strong growth on Ashby agar plates (Table 1).

Table 1. The antagonistic activity of nine bacterial isolates against the fungus *M. oryzae* SH.

No.	Bacterial isolates	Mycelial inhibition (%)	Nitrogen fixation
1	NRV1	35.1 ± 2.25	+

2	NRV4	41.6 ± 1.30	-
3	NRV5	48.1 ± 1.30	+
4	NRV6	48.5 ± 4.92	+
5	NRV12	72.7 ± 3.44	++
6	NRV16	39.4 ± 1.98	+
7	NRV30	71.9 ± 0.75	+
8	NRV39	67.5 ± 2.60	+
9	NRV40	70.1 ± 3.44	-

Note: ++, strong growth; +, slow growth; -; no growth.

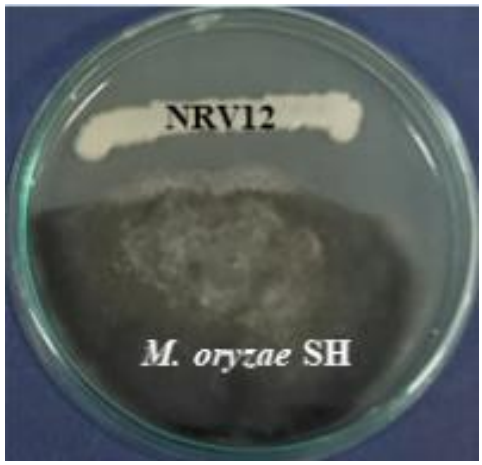

Figure 1. Antagonistic activity against rice blast fungi *M. oryzae* of NRV12 isolate.

Identification of the antagonistic bacterial isolate

The isolate NRV12 was further studied for its morphological characteristics. On MPA medium, most colonies were light brown in color, circular in shape, opaque, and about 1-2 mm in size (Fig. 2a). Under the light microscope, the NRV12 cells are rod-shaped, Gram-negative, aerobic, and motile (Fig. 2b). Isolate NRV12 grew in the presence of 0-2% (w/v) NaCl and optimally at 30°C and pH 7.0 (Table 2). The biochemical analyses showed the ability to ferment glucose, sucrose, and itaconate. Further 16S rRNA sequence analysis revealed that the isolate NRV12 showed 99.9% similarity with *B. vietnamiensis* LMG 10929^T (NR 041720). A phylogenetic tree supported that the NRV12 and *B. vietnamiensis* LMG 10929^T were clustered in the same group of *B. vietnamiensis* with a bootstrap value of 71% (Fig. 2c). *B. vietnamiensis* LMG 10929^T isolated from rice rhizosphere soil, Binh Thanh, Vietnam was previously proposed to be a novel species (Gillis *et al.*, 1995). Moreover, itaconate was utilized by *B. vietnamiensis*, but not by *Burkholderia cepacia* or *Burkholderia gladioli* (Gillis *et al.*, 1995). Taken together, the isolate NRV12 was assigned to the species *B. vietnamiensis* based on the morphological

and biochemical characteristics as well as 16S rRNA gene sequence analysis. The 16S rRNA gene sequence of *B. vietnamiensis* NRV12 was deposited into the GenBank database under the accession number OR584139.

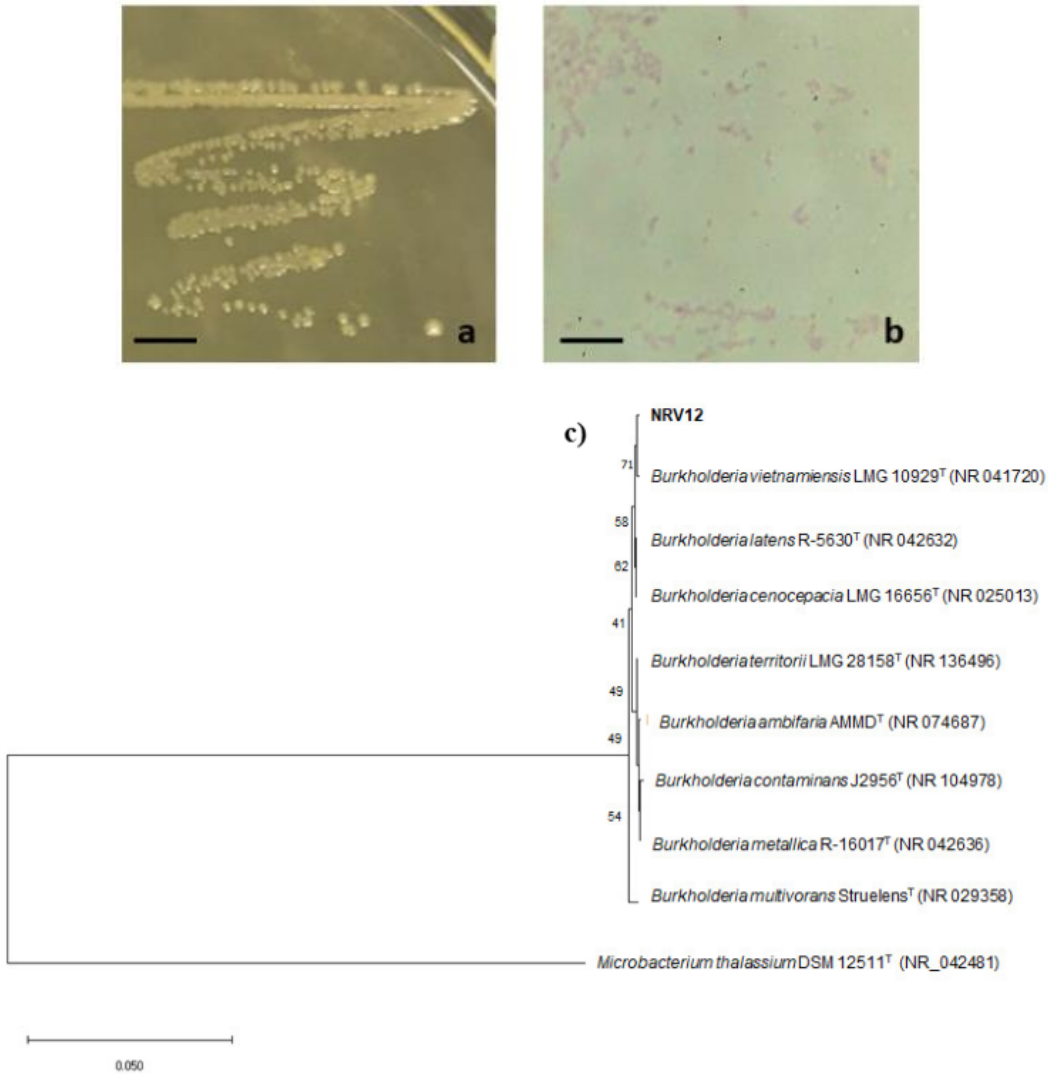

Figure 2. Morphological and molecular identification of the isolate NRV12. Colony grown on the MPA plate. (a) and cell morphology (b) of the isolate NRV12 with scale bars of 5 mm and 5 µm, respectively. (c) Phylogenetic tree based on 16S rRNA gene sequences from the NRV12 and type strains of closely-related *Burkholderia* species.

Table 2. Biochemical and physiological characteristics of the isolate NRV12.

No.	Tests	Results
1	Maximum salt tolerance	1 – 2%
2	Optimal pH for growth	7.0

3	Optimal temperature for growth	30°C
4	Catalase activity	positive
5	Citrate utilization	positive
6	Gelatin hydrolysis	negative
7	Lipase activity	negative
8	Glucose utilization	positive
9	Sucrose utilization	positive
10	Itaconate utilization	positive

Plant growth-promoting traits of *B. vietnamiensis* NRV12

B. vietnamiensis NRV12 was tested *in vitro* for traits related to plant growth-promotion including enzymatic production, IAA secretion, phosphate and zinc solubilization, and nitrogen fixation. The enzymatic tests showed that the halo zones formed around the test colony were determined for amylase (18.7 ± 1.15 mm), cellulase (18 ± 1 mm), and protease (18.3 ± 0.58 mm) (Table 3, Fig. 3a, 3b, 3c). However, chitinase activity was not observed. The IAA content in the NRV12 culture broth reached a maximum level of 14.8 ± 0.49 µg/mL after 48 h. The phosphate and zinc solubilization abilities of NRV12 were determined by the diameter of the clear zone of 7 ± 0.82 mm and 13 ± 0.54 mm, respectively (Table 3, Fig. 3e, 3f). The nitrogen fixation capacity of NRV12 showed a positive result with the colonies presence on the nitrogen-free Ashby medium plate after 5 days (Fig. 3g).

IAA production, phosphate and zinc solubilization and biological nitrogen fixation by rhizospheric and endophytic bacteria play an important role in plant growth and development (Duca *et al.*, 2020; Spaepen *et al.*, 2007; Ngalimat *et al.*, 2021). *Burkholderia* isolates from rice, maize, and sugarcane plants contributed to crop growth via the secretion of growth-promoting substances such as IAA and gibberellic acid, siderophore, and ACC deaminase. These isolates also exhibited apparent colony growth on nitrogen-free medium (Aroumougame *et al.*, 2020). The nitrogen fixation ability of the NRV12 was also consistent with earlier studies of *B. vietnamiensis* capable of fixing nitrogen (Van *et al.*, 2000; Gillis *et al.*, 1995; Phong *et al.*, 2010). The metagenomic analysis of soil in Taoyuan, China for super rice yield revealed the taxa diversity with nitrogen metabolism functions and an abundance of genes involved in the nitrification process (Zhong *et al.*, 2020).

Extracellular hydrolytic enzymes such as amylase, cellulase, chitinase, and protease play a key role in the survival of microbes and contribute to plant pathogenic suppression (Bardin *et al.*, 2015; Rais *et al.*, 2016). For complex organic matters composed of cellulose, hemicelluloses, pectins, starch, chitin, glycoprotein, and lignins, extracellular degradation requires the simultaneous and sequential activities of different types of enzymes (multi-enzymes). Thanks to hydrolytic enzymes, polymeric compounds are degraded into monomeric and oligomeric molecules, which could be

directly taken up by cells. Given that chitinase-producing bacteria have the potential to control phytopathogenic fungi (Veliz *et al.*, 2017), *B. vietnamiensis* NRV12 was not found to produce chitinase. A previous study revealed that *B. vietnamiensis* WPB showed antifungal activity against *Rhizoctonia solani* AG-8, *Fusarium culmorum* 70110023, and *Gaemannomyces graminis* var. *tritici* ARS-A1 due to the presence of biosynthetic gene clusters encoding for antimicrobial compounds including occidiofungin A, cepacin A, and ornibactin (Doty *et al.*, 2022). These results indicated that *B. vietnamiensis* NRV12 might produce secondary metabolites against *M. oryzae*, which will be an interesting subject for future study.

Table 3. Characterization of the antifungal bacterial isolate NRV12.

Extracellular enzyme activity	**Results**
Amylase	18.7 ± 1.15 mm
Cellulase	18 ± 1 mm
Chitinase	0
Protease	18.3 ± 0.58 mm
Growth promotion traits	
Phosphate solubilization	7 ± 0.82 mm
Zinc solubilization	13 ± 0.54 mm
IAA production	14.8 ± 0.49 µg/mL

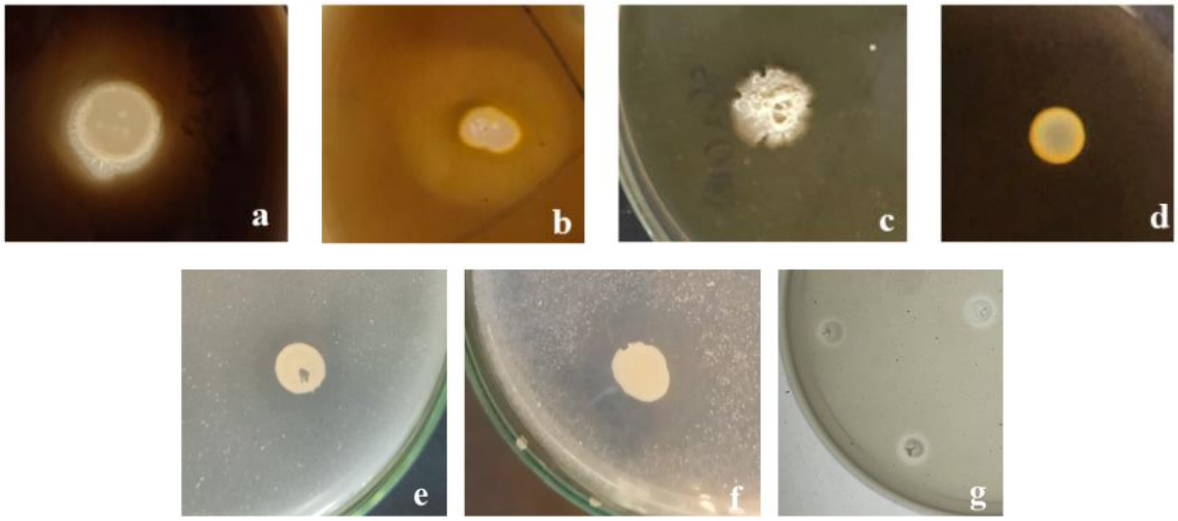

Figure 3. Characterization of the NRV12: extracellular enzyme activity of amylase (a), cellulase (b), protease (c), and chitinase (d); solubilization of phosphate (e) and zinc (f); and growth on Ashby agar plate (g) at 5 days after incubation.

Effect of *B. vietnamiensis* NRV12 on the growth of rice seedlings

In order to identify the growth promotion of NRV12 isolate in greenhouse conditions, a hydroponic system was used to perform this

assay. The result showed that NRV12 could promote the growth of rice seedlings under *in vivo* conditions. A significant increase (23.3%) in fresh weight was observed in the NRV12-inoculated seedlings (4206 ± 231 mg) compared to the control (3227 ± 222 mg) ($p < 0.05$) (Fig. 4). The increase in fresh weight of NRV12-inoculated seedlings might be attributed to several plant-growth promotion traits, such as IAA production, phosphate and zinc solubilization, or nitrogen fixation of *B. vietnamiensis* NRV12. For instance, Meng *et al.* (2023) reported that *B. vietnamiensis* C12 exhibited plant-promoting abilities on rice via IAA production, nitrogen fixation and phosphate solubilization. The previous study by King *et al.* (2019) showed that *Paraburkholderia kururiensis* M130 and *B. vietnamiensis* LMG 10929^T exhibited significant growth promotion in rice plant through fixing nitrogen and stimulating a higher transcriptional level in the aerial parts of leaves than in roots at 7 days post-inoculation. Moreover, *B. vietnamiensis* LMG 10929^T significantly increased shoot and root weights, leaf surface, and grain yield without adding nitrogen fertilizers (Van *et al.*, 2000). Another study by Mattos *et al.* (2008) illustrated that IAA-producing *B. kururiensis* could promote both plant growth and grain yield in rice. As a result, the isolated *B. vietnamiensis* NRV12 strain could become a potential candidate for growth promotion in rice plants.

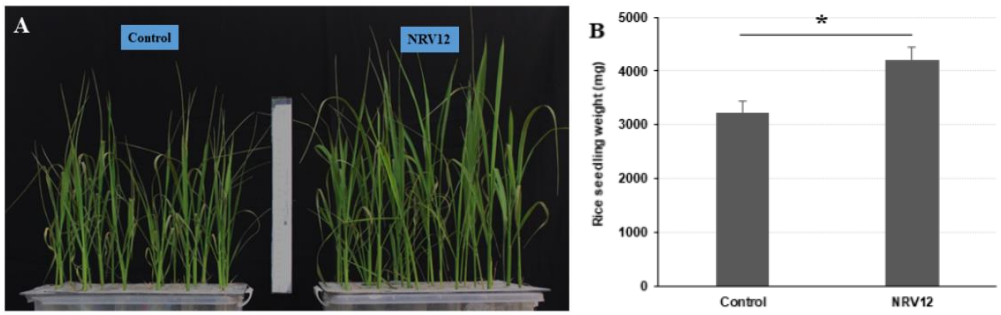

Figure 4. The effect of bacterial strain *B. vietnamiensis* NRV12 on the growth of rice seedlings in greenhouse conditions. (A) NRV12 inoculated seedlings and a control box were grown in a hydroponic system after 45 days of growth. (B) Rice plant fresh weight comparison between inoculated and control seedlings after 45 days of growth. An asterisk represents a statistically significant difference between the 2 groups at $p < 0.05$ (*t*-test). A ruler is 30 cm in length.

Effect of *B. vietnamiensis* NRV12 on rice blast development under greenhouse conditions

After observing the growth promotion capacity of NRV2 on rice seedlings, we want to test its blast disease tolerance on rice seedlings in greenhouse conditions. The results showed that strain NRV12 exhibited an inhibitory effect on the incidence of rice blast to the rice seedlings infected with the pathogenic fungus *M. oryzae* SH (Table 4). In details, the number of diseased lesions was significantly reduced by 40% in the NRV12-inoculated seedlings compared with control groups ($p < 0.05$). In addition, the rate of infected leaves with more than 25% of the diseased area in the control seedling group was greater than that of the NRV12-inoculated seedling (Table 4). Especially in the presence of NRV12, the percentage of the healthy leaves accounted for 20.16 ±

3.57%, whereas none was found in the control group (Table 4; Fig. 5). Inoculating rice seeds with *B. vietnamiensis* NRV12 not only resulted in a significant reduction in the number of diseased lesions on leaves and the number of leaves with diseased areas larger than 25%, but also an increase in healthy leaves.

The biocontrol of *M. oryzae* by plant growth-promoting bacteria serves as an alternative to synthetic fungicides. Bacteria such as *Bacillus*, *Burkholderia*, and *Streptomyces* genera have been reported as effective biocontrol agents to control rice blast (Zeng *et al.*, 2023). The application of *Bacillus subtilis* G5 reduced the disease index of rice blast to 28.2% in a greenhouse (Lei *et al.*, 2023), which was higher than that of *B. vietnamiensis* NRV12. *Streptomyces globisporus* JK-1 was shown to provide 88.3% protection against rice blast as compared to rice plants treated with *M. oryzae* alone (Law *et al.*, 2017). However, *Streptomyces* spp. and *Burkholderia* spp. have not been formulated to be commercial biocontrol products yet. It raises a chance to develop *B. vietnamiensis* NRV12 as a biocontrol or bio-fertilizing agent.

To date, only a few studies have reported the biocontrol efficacy of *B. vietnamiensis* in controlling rice blast disease. Endophytic *B. vietnamiensis* C12 isolated from *Ficus tikoua* Bur acted as a biocontrol agent for rice sheath blight caused by *Rhizoctonia solani* because of its ability to induce enzymatic antioxidant defense systems such as superoxide dismutase, phenylalanine ammonia lysae, lactoperoxidase, catalase, and catechol oxidase (Meng *et al.*, 2023). In support of this report, *B. gladioli* KRS027 promoted the expression of genes related to inducing systemic resistance in cotton plants and produced extracellular metabolites and volatile organic compounds conferring fungal cell wall degradation, inhibition of melanin biosynthesis, and disturbing the autophagy process (Wang *et al.*, 2023). Plant growth-promoting rhizobacteria *B. vietnamiensis* B418 exhibited potent biocontrol effects against root-knot nematodes on watermelon through the modulation of the rhizosphere microbial community (Liu *et al.*, 2022). In contrast, a previous study proved that *Burkholderia* sp. BV6 significantly decreased the disease index of rice blast via the production of small-molecule secondary metabolites (Xue *et al.*, 2022). Thus, it is required to shed light on a possible mode of action of *B. vietnamiensis* NRV12 in future studies.

Table 4. Biocontrol effect of isolate *B. vietnamiensis* NRV12 against rice blast disease *in vivo*

Treatment	Number of diseased lesions	Non diseased leaf (%)	Diseased leaf area with more than 25% (%)
DC	114 ± 23.5*	0	24.2 ± 4.29
NRV12	68 ± 16.1	20.16 ± 3.57	16.1 ± 2.57

Values are presented as mean ± standard deviation and representative of three replicates.

The asterisk * indicates significant differences ($p < 0.05$).

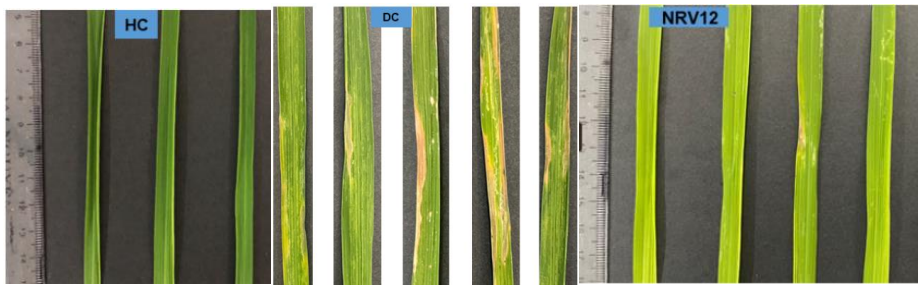

Figure 5. Effect of *B. vietnamiensis* NRV12 on rice seedlings at 3 weeks after infecting with *M. oryzae* SH fungus in a greenhouse condition. HC – Heathy control; DC – Diseased control and NRV12 – the inoculated group with NRV12.

CONCLUSION

The obtained results indicated that the plant-growth promotion bacterial isolate, *B. vietnamiensis* NRV12, showed efficient suppression of blast disease under greenhouse conditions. Inoculation of rice seeds with the strain NRV12 significantly enhanced plant growth through IAA production, phosphate and zinc solubilizations, and nitrogen fixation. The presence of NRV12 reduced the blast disease incidence (number of diseased lesions and diseased leaf area with more than 25%) and increased the number of healthy leaves in rice seedlings after infecting with *M. oryzae*. These findings present the antagonistic *B. vietnamiensis* NRV12 as a biologically potential agent for the control of rice blast disease. However, rice blast resistant effect of the bacterial strain on rice plants should be further evaluated under field conditions and in combination with other antagonistic strains or control strategies.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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