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Original Paper

Molecular Identification and In-Vitro Screening of the Isolated Rhizobacteria in Organic Farm Against Rhizoctonia solani Kuhn

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***Abstract*— this study determined the in vitro effect of isolated rhizobacteria from rice rhizosphere against the rice fungal pathogen Rhizoctonia solani. The antagonistic effects of rhizobacteria against R. solani were assessed using the dual culture technique and scanning electron microscopy for the mode of action and interaction. Among the nine rhizobacteria isolates, seven belong to the genus Bacillus and one belongs to the genus Lysinibacillus, while one isolate, RM-W3-5, and is unknown. The isolated rhizobacteria were identified as B. pumilus, B. megaterium, B. cereus, B. amyloliquefaciens, Bacillus sp., B. rhizosphaerae, B. clausii, and L. macroides. The results from the dual culture method showed that the rhizobacterial isolates reduced the growth of R. solani by between 8.9% and 82.61%. The highest mycelial growth inhibition (MGI) of 82.61% was obtained from R. solani paired with B. pumilus, while the lowest MGI of 8.9% was obtained from B. clausii. The interactions between RM-W3-5 and B. pumilus against R. solani were examined using a scanning electron microscope (SEM) because these two isolates performed well in the dual culture test. Isolate RM-W3-5 paired to R. solani showed the structural alterations such as shrinking, shriveling of the hyphae, and hyphal disintegration. A similar occurrence was observed when R. solani was paired with B. pumilus. The results of the present study suggest that B. pumilus and RM-W3-5 are potential biological control agents against R. solani. However, supplementary analysis has to be done to validate the potential of these rhizobacteria.**

Keywords*— *Dual culture technique, Mycelial growth inhibition, Rhizobacteria, Rhizoctonia solani, Scanning electron microscope s)

I. INTRODUCTION

Rice is one of the most extensively cultivated food crops worldwide; however, its production is constrained by many fungal, viral, and bacterial diseases [1]. Among the fungal diseases, sheath blight and rice blast have been reported as two of the most economically important diseases affecting rice production [2]. Sheath blight (ShB), caused by the fungus R. solani, is a universal soil saprotrophic and facultative plant parasite. This fungus has limited movement due to a lack of spores and can only survive in unfavorable conditions by forming sclerotia or dormant mycelia. The sclerotia of R. solani in soil can survive for about 2 years and spread during field preparation. Sclerotial bodies or the hyphae can spread by attaching to the plant, thus infecting and causing ShB disease [3]. Sheath blight can also transmit via seeds and can drastically reduce the yield of lowland rice by about 30%. It can also seriously affect seed quality by producing discolored and unfilled grains. Currently, no rice blast resistance is available for commercial production due to the lack of sources for resistance in cultivated and wild rice species possessing ShB resistance traits [4].

To control various diseases, including ShB, effective methods include seed treatment, soil application, and foliar sprays using systemic fungicides and antibiotics [5]. However, the growing cost of these inorganic pesticides and the consumer's demand for pesticide-free products led to the search for more sustainable and environmentally friendly pest control systems [6]. Biological control is increasingly viewed as an alternative treatment for sustainable agricultural management that does not harm the environment and is utilized to target pathogens or diseases [7]. Among the biological control agents, root-associated bacteria, or rhizobacteria, have recently become more popular because they can effectively live on plant roots, which helps them fight off various soil-borne plant diseases [8]. . Also, rhizobacteria make a wide range of substances like antibiotics, lytic enzymes, siderophores, and phytohormones, which help fight diseases and promote plant growth.

The rhizosphere is inhabited by a diverse range of microorganisms, and the bacteria colonizing the roots are those whose study was intended to determine the mode of interaction and antagonistic abilities of rice rhizospheric bacteria isolated from organic farm areas that inhibit the growth of R. solani. Additionally, the results generated in this study can be used to improve rice sheath blight control using antagonistic rhizospheric bacteria with the aim of helping farmers enhance crop yields without harming the environment or health.

II. METHODOLOGY

A. Isolation of Bacteria from Rice Rhizosphere

Bacteria were isolated from rhizospheric soil samples obtained from the organic rice area of Ramon Magsaysay-Center for Agricultural Resources and Environment Studies (RM-CARES). Five rice plant samples were randomly collected from the site. To isolate bacteria from the rhizosphere, the entire root system was collected and carefully tapped to remove soil adhering to the roots. Ten grams of roots were placed in 90 ml of sterile distilled water and shaken thoroughly on a wrist action shaker. Soil suspension was diluted from 10^{-1} to 10^{-4} dilutions. From these dilutions, 0.1 ml was inoculated in plates with Nutrient Agar (NA) medium and then incubated at room temperature (28-30°C). Bacterial colonies showing different morphologies were selected and purified on NA plates. To determine the initial classification of the isolated rhizobacteria, gram staining was performed to characterize the isolates of physical properties of the cell walls.

B. Genomic DNA Isolation

The The rhizobacterial strains were grown in nutrient agar plates for 24 hours at room temperature. The genomic DNA of the isolates was extracted using the NucleoSpin® Microbial DNA kit (Macherey-Nagel) following the manufacturer's instructions. Briefly, cells were harvested from the culture by centrifugation in a microcentrifuge tube. The supernatant was discarded, and 100 µl of the elution buffer was added to the pellet. The cell suspension was then transferred into the Nucleospin bead tube, and 40 µl of buffer MG and 10 µl of liquid proteinase K were added. The NucleoSpin tube was centrifuged for 30 seconds at 11,000 rpm. About 600 µL of buffer MG was added to the NucleoSpin tube and then centrifuged to clean the lid, sediment, glass beads, and cell debris. The supernatant (500-600 µl) was transferred onto the NucleoSpin DNA column placed in a 2 ml collection tube. After centrifugation, the collection tube was discarded, and the column was placed in a new collection tube. In washing the silica membrane, the first wash involved the addition of 500 ul buffer BW, and the second wash involved 500 ul buffer B5. After the second wash, the flowthrough was discarded, and the column was placed back in the new collection tube. Centrifugation was then performed to dry the binding column. Finally, the highly pure DNA was eluted from the column.

Two microliters of the DNA samples were placed into a 1.0% agarose gel mixed with 0.5X Tris-Acetate-EDTA (TAE) buffer and tested at 100 V for about 30 minutes using a gel electrophoresis system. The stained gel was then viewed under UV light using the BIO-RAD gel viewer and Image Lab Software from BIO-RAD.

C. Sequencing and phylogenetic analysis

The PCR run was performed using a BIO-RAD thermal cycler. The genomic DNA from rhizobacterial isolates was copied using PCR with universal primers 27F (5-AGAGTTTGATCMTGGCTCAG-3) and 1494R (5-TACGGCTACCTTGTTACGAC-3) in a 60-µL mixture that included 180 µL of nuclease-free water, 120 µL of PCR buffer, 48 µL of MgCl2, 60 µL of dNTPs, 60 µL of forward primer, 60 µL of reverse primer, 60 µL of Taq DNA polymerase, and 6 µL of template DNA. The thermocycling program consists of one cycle of initial denaturation at 95°C for 2 minutes, 30 cycles of denaturation at 95°C for 1 minute, annealing at 52°C for 30 seconds, and extension at 72°C for 1 minute, and one cycle of final extension at 72°C for 5 minutes with a holding temperature of 4°C for temporary storage of the reaction.

Two microliter aliquots of all PCR products were run in 1.0% agarose gel in 0.5X Tris-Acetate-EDTA (TAE) buffer at 100 V for approximately 30 minutes using a gel electrophoresis system and were viewed under UV using a BIO-RAD gel viewer and Image Lab Software by BIO-RAD after staining with SYBR Safe DNA gel stain. The DNA concentration of the PCR products was determined by a NanoDrop spectrophotometer.

D. Sequencing and phylogenetic analysis

PCR products were prepared and sent for sequencing at Apical Scientific Sequencing, Malaysia, through Asiagel Corporation, Quezon City, Metro Manila. The DNA sequences were then assessed for similarities using the BLAST program of the NCBI-BLAST website (http://blast.ncbi.nlm.nih.gov). Sequences were obtained and compared with the known isolates. The phylogenetic tree was constructed using MEGA7 (Molecular Evolutionary Genetics Analysis) software.

To calculate identities, similarities, and differences, the sequences were aligned using the ClustalW algorithm. The evolutionary history was inferred using the neighbor-joining method. The bootstrap consensus tree, created from 1000 tests, was used to show the evolutionary history of the studied groups. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) was shown next to the branches. The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Jukes-Cantor method and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated.

E. In vitro Screening of Antagonistic Rhizobacteria

Pure culture of R. solani was obtained from the Crop Protection Division, Philippine Rice Research Institute—Central Experimental Station (PhilRice—CES).

The antagonistic activity of all isolated rhizobacteria was tested in vitro using a dual culture test. A 6 mm agar block of R. solani was inoculated in petri plates containing sterilized Potato Dextrose Agar (PDA) with peptone. Each of the isolated rhizobacteria was inoculated at a distance of approximately 5 cm opposite to the phytopathogenic fungi. Plates containing the pathogen were used as the control. All treatments and controls were replicated three times. The experimental plates were incubated at room temperature (28-30°C), and the diameter of growth of R. solani was measured every 24 hours using a digital vernier caliper.

Percentage of mycelial growth inhibition was calculated according to the formula:

$$MGI\% = \frac{dc-dt}{dc}\ X\ 100 \quad \text{.....(1)}$$

Where:

dc= colony diameter of the pathogen in control sets
dt= colony diameter of the pathogen in treatment sets

F. Interaction between Rhizobacteria and R. solani in Scanning Electron Microscope (SEM)

The interaction of R. solani with the most promising antagonistic isolates based on the dual culture assay results was studied in SEM. An agar block of R. solani was grown on a glass slide containing a thin film of sterilized PDA with peptone. Selected rhizobacteria isolates were spot-inoculated with a distance of approximately 3 cm away from the pathogen. The slides were then placed into a sterilized petri plate dish in an elevated manner; each plate was lined with moist sterile filter paper to serve as a moist chamber. The interactions between rhizobacteria and phytopathogenic fungi were documented using a Hitachi SU1510 scanning electron microscope.

G. Statistical Analysis

Statistical analyses were performed using SAS 9.1.3 portable. All data gathered were analyzed using one-way analysis of variance (ANOVA), and Fisher's Least Significant Difference (LSD) at $p<0.05$ was used to assess significant differences between treatments.

III. Results And Discussion

A. Isolation and identification of Rhizobacteria isolates

A total of nine isolates were collected from the rice rhizosphere. These rhizobacteria were coded as RM-W4-1, RM-W4-4, RM-Y3-7, RM-W4-2, RM-W3-5, RM-W4-8, RM-W3-3, RM-W3-6, and RM-C4-9. All isolates are motile, producing fast-growing round to irregular colonies. Morphological characteristics and Gram staining were performed, and 8 out of 9 rhizobacteria are gram positive, except for isolate RM-W3-5 (Table I). Gram-positive bacteria are stained blue to purple because of their thick peptidoglycan layer, while gram-negative bacteria have a thin peptidoglycan layer and are stained red to pink [9]. According to [10] Gram-positive bacteria are reported to have a plant growth-promoting rhizobacteria effect and exhibit biological control against pathogens. Gram-positive bacteria cell wall structures differ from those of gram-negative bacteria. Many of the gram-positive bacteria produce several active compounds that are important in agricultural applications such as natural ways of improving productivity in crop productions through the use of bio fertilizers or plant growth promoting rhizobacteria that can stimulate plant growth and development [27].

Among the nine rhizobacteria isolated from the rhizopheric soil of rice in organic farm, seven belong to the genus Bacillus and the other one belongs to the genus Lysinibacillus, while RM-W3-5 is unknown. The rhizobacterium isolate RM-W3-3 had a close relation to Bacillus cereus strain 1 with 90% identity. Moreover, RM-W4-8 is closely related to Bacillus clausii with 96% certainty; RM-W3-6 is related to Bacillus sp. strain Z20 with 99% certainty; RM-W4-1 is related to Bacillus pumilus strain E6 with 99% certainty; RM-W4-4 is related to Bacillus amyloliquefaciens strain PHYDB6 with 98% certainty; RM-W4-2 is related to Bacillus megaterium strain HX-2 with 99% certainty; RM-Y3-7 is related to Bacillus rhizosphaerae strain TB-134 with 99% certainty; and RM-C4-9 is related to Lysinibacillus macroides strain LNHL43 with 95% certainty (Table II)

TABLE I. Identities Of Antagonistic Rhizobacteria Based On 16s Rna Sequencing.

Isolate	Colony Color	Gram Stain
RM-W4-1	White	Positive
RM-W4-2	White	Positive
RM-W3-3	White	Positive
RM-W4-4	White	Positive
RM-W3-5	White	Negative
RM-W3-6	Yellowish	Positive
RM-Y3-7	White	Positive
RM-W4-8	White	Positive

According to [11], bacteria of diverse genera have been identified as beneficial rhizobacteria, or PGPR, of which *Bacillus* and *Pseudomonas spp*. are predominant while Lysinibacillus from the family of Bacillaceae is well-known for its insecticidal and larvacidal effect that use mainly as biological control of diseases and entomopathogenic potentials. [12]. Most of the soil rhizosphere possesses a large number and diverse population of microorganisms, including Gram-positive bacteria [13]. Many of the microorganisms from the rhizosphere have the potential to solubilize unavailable phosphate, increase nitrogen uptake, and act as growth promoters [14] [15]. The study of [16] confirmed that bacterial consortia isolated from the organic farm soil produced plant growth promoting activities and showed antagonistic activities against plant pathogens and increase crop yield of rice plant under organic farming conditions in which various rhizobacteria were used and marketed as plant growth promoters and biological control agents because of their beneficial effects on plants, and among the isolated rhizobacteria from the rice rhizosphere while gram-positive bacteria are common, in the study of [17]. While study of [18] showed that different morphological variation of Rhizobacteia produces ability to produce phytohormones in the soil.

TABLE II. IDENTITIES OF ANTAGONISTIC RHIZOBATERIA BASED ON 16S RRNA SEQUENCING.

Rhizobacterial isolates	Closest related species	Match accession number	No. of base pairs amplified	Maximum Identity %
RM-W3-3	*Bacillus cereus* (strain 1)	MH734616	1260 bp	90%
RM-W4-8	*Bacillus clausii*	AF172603	1283 bp	96%
RM-W3-6	*Bacillus sp*. (strain Z20)	MG470672	1294 bp	99%
RM-W4-1	*Bacillus pumilus* (strain E6)	MG594850	1285 bp	99%
RM-W4-4	*Bacillus amyloliquefaciens* (strain PHYDB6)	KY784657	1264 bp	98%
RM-W4-2	*Bacillus megaterium* (strain HX-2)	MH930825	1253 bp	99%
RM-Y3-7	*Bacillus rhizosphaerae* (strain TB-134	KF843727	1261 bp	99%
RM-C4-9	*Lysinibacillus macroides* (strain LNHL43)	MG008673	1292 bp	95%
RM-W3-5	unknown	-	-	-

To determine the potential of the nine isolated bacteria taken from the rice rhizosphere to control *R. solani*, which causes leaf sheath and leaf blades in rice panicles, there were tested using a dual culture assay. Table III shows the daily growth diameter of *R. solani* as affected by the presence of isolated rhizobacteria after 3 days of incubation; it was observed that *R. solani* in the control set colonized nearly the entire plate. Hence, the mycelial growth diameter of *R. solani* grown with *B. pumilus* (24.5 mm), RM-W3-5 (21.5 mm), *Bacillus* sp. (24.83 mm), and *B. rhizosphaerae* (35.83 mm) was significantly lessen its mycelial growth compared to the growth diameter of *R. solani* in the control set (90 mm). The results of the study can be compared to the previous study of [7] in which it was found that out of 220 bacterial samples taken from the potato roots, only 11% were very effective at stopping the growth of *R. solani*, and just 3 of those samples worked well against *R. solani* in lab tests. Meanwhile study of [19] showed the ability of the rhizobacteria from organic rice field to produce enzymes that carry out hyperparasitic effect that can hydrolize and attack the pathogenic cell wall of *R. solani*, in vitro, thus volatile organic compounds (VOCs) exhibited by some rhizobacteria against *R. solani* showed potential use for biological control of rice sheath blight [20].

TABLE III. MORPHOLOGICAL CLASSIFICATION OF THE NINE ISOLATED RHIZOBACTERIA.

Treatment	Mycelial Growth of *R. solani* (mm)		
	Day1	***Day 2***	***Day 3***
Control	47.83 a	70.17 a	90.00 a
RM-W4-1	24.50 ^{e}	25.33 ^{d}	24.50 ^{d}
RM-W4-2	42.50 ^{bcd}	61.67 ^{abc}	67.50 ^{ab}
RM-W3-3	40.67 ^{d}	49.17 ^{c}	53.83 ^{ab}
RM-W4-4	45.17 ^{abc}	66.67 ^{ab}	77.00 ^{a}
RM-W3-5	23.00 ^{e}	22.50 ^{d}	21.50 ^{d}
RM-W3-6	25.17 ^{e}	25.17 ^{d}	24.83 ^{cd}
RM-Y3-7	33.83 ^{e}	35.17 ^{d}	35.83 ^{bc}
RM-W4-8	43.17 ^{ab}	63.83 ^{ab}	82.00 ^{a}
RM-C4-9	43.17 ^{cd}	56.33 ^{bc}	63.33 ^{a}

Note: *Values with the same letter within a column are not significantly different at P<0.05 according to LSD.

After three days of incubation, the percentage of mycelial growth inhibition (MGI) was measured as shown in Figure 1. The results indicated that the 9 rhizobacterial isolates can inhibit the mycelial growth of R. solani from 8.9% up to 82.61%. R. solani paired with B. pumilus in a dual culture plate yielded the highest MGI, while B. pumilus inhibited the mycelial growth of R. solani by 82.61%. However, a comparison of the means revealed that it is not significantly different from RM-W3-5 (76.11% MGI) and Bacillus sp. (72.41% MGI). On the other hand, the lowest MGI (8.9%) was observed in R. solani paired with B. clausii. The study referenced in [21] on the in vitro inhibition of mycelium growth of *R. solani* by the rhizobacterial isolates showed maximum inhibitions of 55% and 75%, while one bacterial isolate achieved a 99% inhibition, indicating complete suppression of fungal growth. Other studies revealed that some bacterial isolates significantly inhibited the growth of R. solani, while others showed no reaction at all (21). These findings could be attributed to the mechanism of growth inhibition in one organism that may contribute to competition for a limited supply of nutrients, the production of antibiotics and enzymes, and other volatile substances, resulting in the reduction of fungal growth [22].

In Figure 2 illustrates the interaction of R. solani and rhizobacteria, showing that not all rhizobacteria produced clear inhibition zones but profuse growth that inhibited the mycelial growth of R. solani. It was observed that some of the isolates ramified the agar surface faster, therefore suppressing the further growth of the pathogen. According to [1], biocontrol agents, specifically bacteria, perform several modes of action, such as antibiosis, competition for iron through siderophores, parasitism that may involve production of extracellular enzymes, and induction of plant resistance mechanisms. Moreover, a study [23] found that bacteria taken from the area around plant roots and from many different groups can effectively reduce diseases caused by various soil-borne plant pathogens. Thus, representatives of these genera are effective competitors in the rhizosphere because of their ability to produce antimicrobial compounds, rapidly colonize plant roots, and, in some cases, produce biofilms [24].

In this study, the majority of the isolated rhizobacteria exhibited high motility when paired with R. solani. A similar study [25] suggested that this activity might be due to competition for nutrients and space rather than inhibition by antimicrobial secretion. These bacteria are known to colonize

microsites faster than the surface fungi [26]. The results suggest that rhizobacterial isolates used in this study might be effective nutrient competitors and, consequently, can be efficient biocontrol agents of *R. solani*.

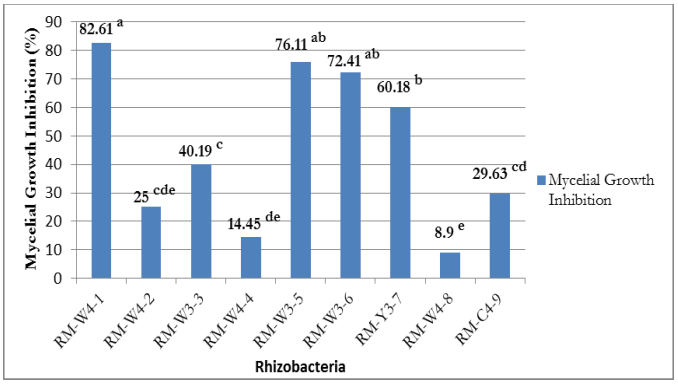

Fig. 1. Percentage of mycelial growth inhibition of R. solani by rhizobacteria after 3 days of incubation period.

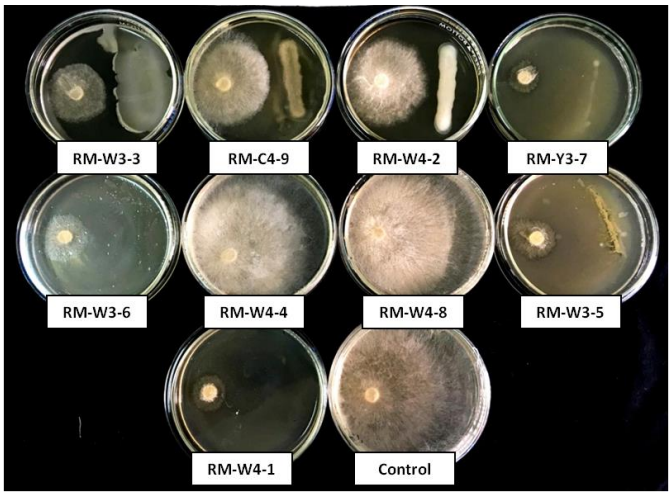

Fig. 2. Dual culture plates of R. solani and rhizobacteria at 3 days of incubation.

The most promising rhizobacteria were selected based on their performance on dual culture assay. The interactions of these rhizobacteria, *B. pumilus* and RM-W3-5, with *R. solani* were studied under the SEM. Figure 3 shows the scanning electron photomicrographs of the mycoparasitism of rhizobacteria on *R. solani* hyphae. When paired with RM-W3-5, the hyphae of *R. solani* showed structural alterations. Malformation of fungal structure leading to shrinking and shrivelling of hyphae was observed (Fig. 3c). Hyphal disintegration and lysis were also evident in R. solani (Fig. 3d). These observations were different compared to the hyphae of *R. solani* free from RM-W3-5 in which it showed normal morphology (Fig 3 a&b).

Similarly, abnormalities in the morphological structures of *R. solani* hyphae were observed when grown with *B. pumilus*. The hyphae of the pathogen paired with *B. pumilus* lost their structural integrity, leading to deterioration of the hyphae (Fig. 3e). According to [16], hyphal deterioration leading to breakage and lysis was the final step in the phenomenon of biological control of fungal pathogens using bacteria. Additionally, stress in the development of the pathogen hyphae was observed with the evidence of the deformation of the hyphae leading to shrinking and coiling (Fig. 4f). In contrast, the hyphae of *R. solani* in the control plates exhibited structural integrity, with no noticeable deformities (Fig. 4 a & b).

These observations concurred with the results obtained by [16] in their study on *B. subtilis – R. solani* interaction. SEM interaction studies reported similar findings, except for the engulfing activity of bacteria on R. solani. Based on the results, it could be inferred that *B. pumilus* and RM-W3-5 may produce compounds that could cause damage to the hyphae of R. solani [16] demonstrated that the mode of action of B. subtilis against *R. solani* involves two forms of antagonism: antibiosis and parasitism. Antibiosis causes the loss of structural integrity in the hyphae of the pathogen. Parasitism, on the other hand, can be seen through shrinking, shriveling, abnormal coiling, and lysis of hyphal filaments.

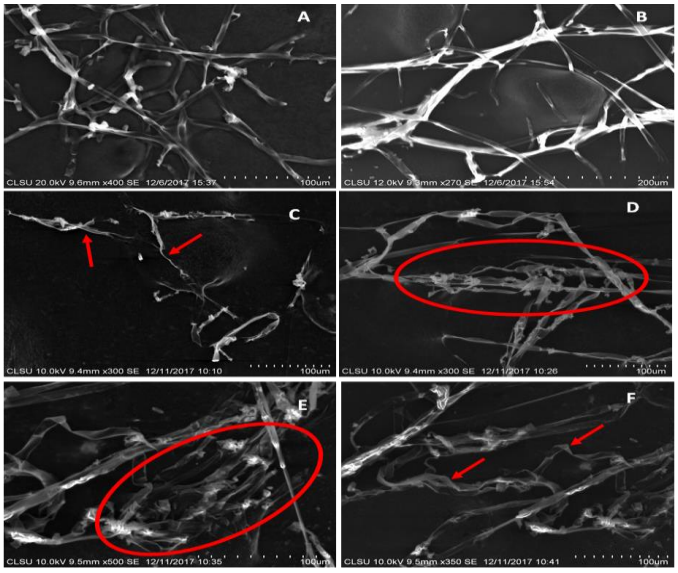

Fig. 3. Scanning electron photomicrographs showing the interaction of rhizobacteria on the hyphae of R. solani. Normal hyphal branching of R. solani (a&b). Shrinking and shrivelling of hyphae caused by RM-W3-5 (c). Hyphal disintegration and lysis due to RM-W3-5 (d). Deterioration of the hyphae due to RM-W4-1 (e). Shrinking, shrivelling and adherent caused by RM-W4-1 (f).

IV. CONCLUSION

In this study, rhizobacteria from the rice roots could be a good source of bacteria that fight against R. solani, and among the tested samples, B. pumilus and RM-W3-5 were the most promising options for biological control. They effectively stopped the growth of R. solani on dual-culture plates. Additionally, B. pumilus and RM-W3-5 damaged the structure of R. solani hyphae, leading to shrinkage, shriveling, and unusual twisting of the hyphal filaments. They were effective in inhibiting the mycelial growth of R. solani on dual-culture plates. Moreover, B. pumilus and RM-W3-5 cause damage to the structure of R. solani hyphae, causing the shrinkage, shriveling, and abnormal coiling of the hyphal filaments of R. solani. These suggest that B. pumilus and Bacillus sp. are potential biological control agents against R. solani. However,

further analysis and molecular identification are still required to confirm the potential of these rhizobacteria.

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