Rhodospirillum centenum, A New Growth Stimulant and Antagonistic Bacteria Against Leaf Spot of Rice Caused by Curvularia lunata

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ABSTRACT

The research objectives were to find Rhodospirillum spp. to promote plant growth and as antagonists to control leaf spot of rice. The results showed that rice var. RD41 and Pitsanulok 2 (PL2) had leaf spot caused by Curvularia lunata. R. centenum is gram negative, mobile cell motility and negative of gelatin activity test. SM41 and 61 showed glucose and lactose and/or sucrose fermentation activity, but SM72 and 92 were non-fermented activity. Isolates of R. centenum were antagonized C. lunata, a leaf spot pathogen. R. centenum expressed for ability to produce amylase, protease and lipase. R. centenum isolates SM41, SM61, SM72, and SM92 with C. lunata causing leaf spot and seed-borne fungus. In addition, the inoculated seeds var. RD 41 and Pitsanulok 2 (PL2) applied R. centenum 41, 61, 72 and 79 gave significantly better seed germination, shoot and root length. Isolate SM41 reduced disease incidence in rice var. RD41 which the disease index was on level 2 when the inoculated one was level 5. Isolate SM61 showed the disease index of level 2 but the inoculated control was level 5. It is the first report that R. centenum plays the role for growth stimulant and biological control agents against leaf spot of rice caused by C. lunata.

INTRODUCTION

Rice (Oryza sativa L) is the most widely consumed as staple food for a large part of the world’s human population, especially in Asia. It is the food commodity with the third-highest worldwide production, after sugarcane and maize, according to 2015 FAOstat data. Rice is the most important crop of human nutrition and caloric intake, providing more than one-fifth of the calories consumed worldwide by human (Smith, 1998). Rice is served to be one of the staple foods to increase in world population, especially in Asia. There are more than 70 diseases caused by pathogene have been recorded on rice (Manandhar, Jørgensen, Mathur, & Smedegaard-Petersen, 1998). The important problem encountered in rice production concerning disease and insect pests, especially diseases caused by Curvularia lunata (leaf spot) and Pyricularia oryzae (blast) etc., leading to yield loss, low quality and quantity. These diseases are the limiting factors to affect rice cultivation, causing annual yield losses which estimated at 5% (Song & Goodman, 2001).

C. lunata is an invader of monocotyledon plant (Domsch, Gams, & Anderson, 1980), the most common ones are rice, barley, wheat, maize and sorghum (Fakhrunnisa, Hashmi, & Ghaffar, 2006; Pitt & Hocking, 1997). In Thailand, C. lunata is one of the most prevalent pathogen in paddy rice fields, where infection of 28% in the fields and 3% of all grains (Pitt et al., 1994). Leaf spot disease becomes the most common symptom of rice diseases caused by phytopathogenic fungi, especially C. lunata. The symptoms are recognized by circular necrotic lesion on rice leaves from seedlings until mature stage. Rice infected with leaf spot pathogen can significantly affect yield and productivity. This disease damages rice yield and reduces seed
quality and usually infects rice plants before and after harvest. The disease severity depends on the surrounding environmental and pathogen race (Thavong, 2002).

The farmers have been applied chemical fungicides for years and faced the pathogens become resistant to those chemical fungicides and much disease epidemic throughout the rice production areas. The effective microbial antagonists are proved to solve these problems. Recently, Tongon & Soytong (2015) stated that nanoparticles obtained from Chaetomium sp gave a good control leaf spot of rice caused by C. lunata.

Photosynthetic bacteria become widely applied in Asia for various biotechnological applications in agriculture. It is used for water purification, bioremediation of chemical, wastewater purifiers, animal feed and aquaculture supplements, biological hydrogen production and coenzyme Q production, and bio-fertilizers for plant growth etc. The purple nonsulfur bacteria are the most study and being diversified group of the phototrophic bacteria. The purple nonsulfur bacteria are member of Rhodopseudomonas, Rhodobacter and Rhodospirillum which known to eliminate the H₂S odor nuisance from the facultative ponds of waste ponds by oxidizing to sulfur or sulfate (Kim et al., 2004; Tadesse, Isoaho, Green, & Puhakka, 2003; Veenstra, Al-Nozaily, Mondol, Bose, & Mandal (2016) stated that Rhodospirillum rubrum is a good mineral solubilizing and plant growth promoting activities on fly-ash. It is the best biofertilizer which has ability in co-operated on fly-ash leading to highest Vigor-Index (990) value. Plant hormones from biomass of the purple non-sulfur bacterium, R. rubrum is reported to isolate and found bioactive compounds which expressed high physiological activities (300–330%) in the cytokinin bioassay. Three cytokinins were also found in the cells of R. rubrum, one of these identified as 6-(4-hydroxy-3-methyl-2-trans-2-bytenylamino)-9-β-D-ribofuranosylpurine zeatinriboside (Serdyuk, Smolygina, Kobzar, & Gogotov, 1993).

The researcher want to isolate rice leaf spot pathogen and photosynthesizing bacteria, screening for enzyme production and antagonistic activities against leaf spot pathogen, so the objectives of this research were to find Rhodospirillum spp. to promote plant growth and as antagonists to control leaf spot of rice.

MATERIALS AND METHODS

Sample Collection, Isolation and Identification
The research was conducted at Faculty of Agricultural Technology, King Mongkut’s Institute of Technology Ladkrabang, Bangkok, Thailand during 2016-2018. The samples were collected in the rice fields at Chachengsao province, Thailand. The disease samples of leaf spot of rice var. RD41 and Pitsanulok 2 (PL2) were collected and isolated to pure culture using tissue transplanting technique followed the method from Soytong (1992). Pure culture of pathogen was proved pathogenicity to pure culture using tissue transplanting technique. The samples were collected from rice fields in Samutprakan, Thailand and brought to the laboratory. The samples were then isolated to be pure culture by dilution plate method. It is diluted and inoculated to soft-agar tubes G-5 medium that consisted of peptone (5.0 g), yeast extract (5.0 g), L-glutamic acid (4.0 g), malic acid (3.5 g), KH₂PO₄ (0.12 g), and K₂HPO₄ (0.18 g) which modified...
from Kohlmiller Jr. & Gest (1951). The tubes were incubated under anaerobic condition with the illumination level of 1,000 until 1,500 lux at room temperature (28-30°C) for 7-12 days. Then, a full loop of pinkish, reddish or brownish culture both was streaked onto G-5 agar and incubated for 7 days. Each single colony was transferred to new plate and subsequently culture until obtain pure culture.

Species of bacteria were morphological identified by followed Bergey’s Manual of Systematic Bacteriology (Imhoff & Trüper, 1989). Gram-reaction, mortality, shape and color of colony were determined using light microscopy and scanning electroscope including gelatin hydrolysis.

Molecular phylogenetic identification was done using 16S rDNA amplification. Genomic DNA of the bacterium was extracted using Alkaline Lysis Method according to manufacturer’s protocol (Sambrook, Fritsch, & Maniatis, 1989). The primers used for 16S rDNA gene region amplification were 557F (5’-CGCACCTGGACTGGAC-3’) and 750R (5’-CCCATGTCGCCAGCAGAA-3’). The PCR products were analyzed by electrophoresis with 1% agarose gel in 1xTAE buffer at 100 V for purity and size of approximately 500 bp. Nucleotide sequence and phylogenetic analysis was done by purification. The PCR products were subjected to sequence and compared with those in the National Center for Biotechnology Information (NCBI) Genbank and compared with those in the National Center for Biotechnology Information (NCBI) Genbank and compared with those in the National Center for Biotechnology Information (NCBI) Genbank and compared with those in the National Center for Biotechnology Information (NCBI) Genbank and compared with those in the National Center for Biotechnology Information (NCBI) Genbank and compared with those in the National Center for Biotechnology Information (NCBI) Genbank.

**Screening of Antagonistic Bacteria Against Leaf Spot Pathogen**

Dual culture assay for in vitro inhibition of mycelial growth of Curvularia sp. was done with antagonistic bacteria isolates. Curvularia sp. was cultured on potato dextrose agar plates at 25°C for five days, then a mycelial disc was cut and placed in the center of the petri plate containing nutrient agar medium. Bacterial isolates were transferred to medium at a distance of 1.5 cm from the opposite edge of the same plate, then inhibition zones were measured after incubation at 25°C for 7 days.

**Photosynthesizing Bacteria for Stimulating Plant Growth and Disease Control**

Seeds of RD41 and Pitsanulok 2 (PL2) were surface sterilized with 95% ethanol for 5 minutes, then with 2% sodium hypochlorite for 2 minutes, and rinsed thoroughly in sterilized distilled water, air dried for 1 h, then inoculated with pathogen by soaked into spore suspension of 1 x 10⁶ spore/ml for 20 minutes before testing. All bacteria isolates were grown in nutrient (NA) medium. Bacterial cells were washed twice with sterile distilled water, and the inoculation density of the bacterial suspension was adjusted to 1 x 10⁶ cells/ml. Seeds were inoculated by overnight (more than 12 h) by soaking with suspension of bacteria (10⁵ cfu/ml). The soaked seeds in sterilized distilled water were used as the control. The research was designed as two factors experiment in Completely Randomized Design (CRD) and four replications. Factor A was rice varieties (RD41 and Pitsanulok 2 (PL2)) and Factor B represented bacterial isolates SM41, SM61, SM72, and SM92. Each treatment was ten seeds that put in sterilized Petri dishes containing filter paper (Whatman # 102). Seed germination was examined in 3 days after inoculation, and the root and shoot lengths of the germinated rice

**Enzyme Production Properties**

The bacterial isolates coded as SM41, SM61, SM72, SM92 and SM93 were tested for ability to produce extracellular degradative enzymes such as amylase, protease and lipase. Completely Randomized Design (CRD) was performed with three replication. Each treatment were assayed and non-transferred plates served as negative control. Amylase activity was done as follows: starch agar medium was streaked the bacterial culture by a drop of bacterial suspension onto the starch agar plate, then incubated at 35-37°C for 18-24 h. When colonies were visible, then flooded the plates with Lugol’s iodine solution, and observed the clear zone surrounding the colony. If the starch was hydrolyzed by the excreted amylase, a clear zone around the bacterial colony appeared. A blue or purple zone indicated that starch was not hydrolyzed (Harrigan & McCance, 1976). Protease evaluation was used skim milk agar medium, streaked bacterial culture on the plates, incubated at 35-37°C for 18-24 h, then observed the clear zone surrounding the colony. When colonies were visible, then observed and the plates for clear zones around that implies positive reaction. Lipase activity test was determined by growing the isolates on Polysorbate 80 agar. Cultural characteristics were observed after incubated at 35-37°C for 18-24 h. A positive test appeared as the occurrence of precipitated fatty acid crystals around the colony.
seedlings were measured after 7 days incubation at 25°C beginning with a cycle of 10 h dark followed by 14 h of light fluorescent.

**Statistical Analysis**

The emergence of seedling was calculated with the following formula:

\[
\text{Emergence} (\%) = \frac{\text{Number of emerged seedlings}}{\text{Number of seeds grown}} \times 100
\]

Data were computed analysis of variance and treatment means were compared using Duncan's Multiple Range Test at \( P = 0.05 \) and \( 0.01 \) by Statistical Package for Social Sciences (IBM SPSS Statistics, ver. 21.0) software program.

**RESULTS AND DISCUSSION**

**Identification Result of Rice Leaf Spot Pathogen and Photosynthesizing Bacteria**

Based on the results showed that leaf spot var. RD41 and Pitsanulok 2 (PL2) caused by *Curvularia lunata* and both isolates were proved to be aggressive isolates causing leaf spot in each rice variety. Similar results were reported by Tann & Soytong (2017) found brown leaf spot caused by *C. lunata* of rice variety IR66 in Cambodia. Four bacterial isolates coded as SM41, SM61, SM72, SM92 were preliminary identify by morphological characters. These isolates were cultured in soft-agar tubes of G-5 medium. The bacterial isolates of SM41, SM61, SM72 and SM92 were morphological and physiological identified as red and orange colonies using *Bergey's Manual of Systematic Bacteriology* (Imhoff & Trüper, 1989) as seen in Fig. 1. Physiological characteristics of cultures SM41, SM61, SM72 and SM92 were studied. All isolates were gram negative, mobile cell motility and negative of gelatin activity test. TSI activity revealed that isolates SM41 and SM61 expressed glucose and lactose and/or sucrose fermentation activity but isolates SM72 and SM92 were non-fermented activity (Imhoff & Trüper, 1989).

Scanning electron microscopy was shown in Fig. 2. The bacterial isolates coded as SM41, SM61, SM72 and SM92 were used for phylogenic analysis. The 16S rRNA gene sequence analysis showed 100% similarity with *Rhodospirillum centenum*. Moreover, the sequences of the SM41, SM61, SM72 and SM92 and closely related isolates were aligned using MEGA6 and phylogenetic analysis exhibited that the isolate SM41, SM61, SM72 and SM92 were also aligned within the same clade with *Rhodospirillum centenum* (Fig. 3) which supported the Blast search result. As result, Berleman & Bauer (2004) reported that *R. centenum* is an anoxygenic photosynthetic bacterium. It can grow in phototrophically liquid, a single polar flagellum expressed heat tolerance and drought resistance.

**Enzymatic Ability of Photosynthesizing Bacteria Against Leaf Spot Pathogen**

The photosynthesizing bacterium, *Rhodospirillum centenum* for ability to produce extracellular degradative enzymes was evaluated. All isolates were proved to produce amylase, protease and lipase. Anderson & Fuller (1969) stated that *Rhodospirillum rubrum* produced ribulose 1,5-diphosphate carboxylase which subjected to control the facultative photoautotroph. Rana, Meikap, Mondol, Bose, & Mandal (2016) reported that *R. rubrum* can be used as green fertilizer for agriculture.

In addition, the result test for antagonistic activity against *C. lunata* showed that all isolates of *Rhodospirillum centenum* showed the inhibition zone in dual culture assays after 10 days. Yet in controls were no inhibition zone. It was shown that the number of spore in dual culture plates were higher than in control plates. Similar result was reported by Vareeket & Soytong (2017) which phytosynthesize bacteria expressed to inhibit *C. lunata* causing rice leaf spot.

**Photosynthesizing Bacteria for Stimulating Plant Growth and Disease Control**

*Rhodospirillum centenum* isolates SM41, SM61, SM72, and SM92 are photosynthesizing bacteria which were tested to inoculate seed germination of rice var. RD41 with *C. lunata* causing leaf spot and seed-borne fungus of rice var. Pitsanulok 2 (PL2). The results showed that the inoculated seeds var. RD 41 and applied *R. centenum* 41, 61, 72 and 79 gave significant better seed germination shoot and root length than the non-treated control. Similar report by Rana, Meikap, Mondol, Bose, & Mandal (2016) stated that *Rhodospirillum rubrum* increased rice seed germination, sprout growth promoting ability. The inoculated seeds var. RD 41 with *C. lunata* and applied *R. centenum* isolates 41, 61 and 71 gave the highest significance in seed germination, followed by isolate SM92 when compared to the non-treated control.
Fig 1. *Rhodospirillum centenum* isolates: A. Bacteria isolates in media slant; B. SM41; C. SM61; D. SM72; E. SM92

Fig 2. SEM image of *Rhodospirillum centenum*
The research found that the inoculated seeds var. RD 41 incorporated with *R. centenum* isolates SM41, SM61, SM72, and SM92 significantly gave better shoot and root length than the non-treated control (Table 1). Isolate SM41 incorporated with rice var RD41 and isolate SM61 mixed with rice var. PL2 were the lowest disease incidence when compared to the other isolates and inoculated control. Rana, Meikap, Mondol, Bose, & Mandal (2016) stated that some isolate of *Rhodospirillum rubrum* had ability to decrease disease incidence in rice. The other report for biocontrol of rice leaf spot by Tann & Soytong (2016) who found that metabolites derived from *C. cupreum* gave a good control *C. lunata* causing leaf spot of rice var. Sen Pidao in Cambodia. The research proved that *R. centenum* as a new promising biocontrol agent and bio-stimulant for the first time in rice growth.

**CONCLUSION**

Leaf spot pathogen (*C. lunata*) of rice var. RD41 and Pitsanulok 2 (PL2) proved to be suppressed by *Rhodospirillum centenum*. *R. Centenum* was identified by morphological and molecular phylogeny. It was proved to produce amylase, protease and...
lipase act as control mechanism. Isolate SM41 and Isolate SM61 gave a good result to reduce leaf spot incidence and promote plant growth. *R. centenum* is a new growth stimulant and antagonistic bacteria against leaf spot of rice caused by *Curvularia lunata*.

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Rungrat Vareeket and Kasem Soytong: Rice Stimulant and Antagonistic Bacteria

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