RICE HULL IMMOBILIZED INDIGENOUS BACTERIA AS A NOVEL BIOFUNGICIDE AGAINST RICE BLAST DISEASE

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ABSTRACT

Plant root – rhizospheric microbe natural interactions are essential for plant health, plant growth and disease suppression. Exploring the abilities of naturally occurring rice soil bacteria to reduce rice blast disease, caused by the fungal pathogen *Magnaporthe oryzae*, could provide a sustainable solution to minimize the yield loss due to rice blast disease. In the Philippines alone, yield losses ranging from 50-85% have been reported. Rhizobacteria were isolated from the rice field soil in University of the Philippines Los Banos and were tested for their effectiveness to inhibit the growth of M. oryzae. The bacteria were identified as Bacillus subtilis 5, Bacillus cereus 3S5 and Pseudomonas fluorecens 10S2. The biofungicide was formulated with both rice hull mixture and liquid media which contained bacterial suspension of Bacillus subtilis 5, Bacillus cereus 3S5 and Pseudomonas fluorecens 10S2. The biofungicide was tested on rice cultivar UPLRi-5 under controlled conditions and the results showed decrease in rice blast disease intensity, which is 33% higher compared to the control.

Keywords: rhizobacteria, rice blast disease, biocontrol, biofungicide

1. Introduction

Rice is the staple food for 2.7 billion people in Asia where 90% of the world's rice is grown and eaten [1]. One of the top five rice crop disease being addressed in the Philippines today is rice blast disease. The disease is caused by the ascomycete fungus *Pyricularia grisea* [2]. The disease poses a significant threat to food security, damaging as much as 30% of the global rice harvest and yield losses of 11.9 kg/ha in South and Southeast Asia. In the Philippines, it was reported that the yield losses are within the range of 50-85% [3].

Different approaches were developed and are in place to increase productivity and overcome such challenges. Developing resistant rice crop varieties to control blast only had a partial success because of the ability of the rice blast fungus to evolve to new races and the resistant cultivar is dependent on the favorability of the environment [1,4]. Chemical pesticide and fertilizer application are still widely used to control rice blast disease [1,5]. Due to the ability of rice blast fungus to get around synthetics, many fungicides have been developed to control blast [6,7]. Extensive use of fungicides is not advisable because it increases the emergence of resistant populations of the pathogen [7]. It is expected that usage and associated risk to human health and the environment will increase rather than decrease in the near future unless peripheral interference provoke alterations towards more sustainable forms, such as pesticide free, of crop cultivation with very minimal economic loss [8]. Among such new strategies, biocontrol agents appear to hold promise in blast management [5]. This process introduces natural enemies to undergo a population-levelling process in which the population of one species decreases the numbers of another species by mechanisms such as predation, parasitism, pathogenicity or competition [1,9,10].

Therefore, reduction or elimination of synthetic fungicide applications in agriculture is highly desirable because of its alarming impact in public health and environmental degradation.

The research and development activities on production, formulation, and use of microbes as biocontrol agents have gained impetus in recent years for the sake of sustainable agriculture [10]. Biocontrol agents have emerged as an alternative or as a supplemental form for the reduction of fungicide input or in certain instances, biological substitutes to these chemical poisons [11].

Recent studies have shown the potential of plant root – rhizospheric microbe natural interactions, which are essential for plant health, plant growth and disease suppression. This study intends to explore the abilities of naturally occurring rice soil bacteria to inhibit the growth of rice blast fungal pathogen, M. oryzae, to develop a sustainable, non-polluting and cost effective biofungicide formulation to control rice blast disease.

2. Materials and Methods

2.1. Preparation of culture media

The preparation procedure and formulation of the agar found in the bottle packaging were followed. The different culture media used in this study are: Trypticase Soy Agar (TSA), LB Agar (LBA), Potato Dextrose Agar (PDA) and LB Broth (LBB).

2.2. Isolation of rice blast fungus (M. oryzae)

The pure culture of M. oryzae (obtained from UPLB College of Agriculture – Crop Protection Cluster) was maintained on potato dextrose agar (PDA).

2.3. Isolating the biocontrol agent bacteria from the rice soil sample

Soil samples were collected 8 inches from the crop roots in various points of the rice field in the University of the Philippines Los Banos (UPLB), College of Agriculture, under the Crop Protection Cluster. The samples were air-dried at room temperature for 8 days and then passed through a mesh sieve and were preserved in ziplock bags at room temperature before use. The samples (1g) were dissolved in 10mL sterile 1M PBS buffer solution. Serial dilution technique was used and three replicates were considered for each dilution. Plates were incubated for 16-48 hours at 25 ± 3°C. Rhizobacteria colonies were isolated in TSA and LBA, incubated at 28°C for 2 days and refrigerated as pure cultures before use. For the inhibition test 300 pure Rhizobacteria isolates were collected.

2.4. Inhibition test

The antifungal activity of the isolated soil microorganisms against the disease pathogen were evaluated using a modified agar disc method suggested by Ebrahimi Zarandi *et al*. (2009)[12] and dual culture technique suggested by Naureen *et al*. (2009)[13]. Three replications were made for each strain.

The diameter of the cleared zone, the inhibition of mycelia growth, was measured. The results were expressed in inhibition diameter (mm). Antifungal activity around the soil microorganism was evaluated using the ratings suggested by Ebrahimi Zarandi *et al*. (2009)[12]: (1) no inhibition = mycelial growth not different from control (-); (2) weak inhibition = partial inhibition of mycelia growth, measured as a diameter of 5-9 mm (+); (3) moderate inhibition = almost complete inhibition of mycelial growth, measured as a diameter of 10-19 mm (++); 4) strong inhibition = complete inhibition, in which most mycelia did not grow, measured as a diameter of >20 mm (+++). Blank agar disc was used as control.

2.5 Speciation of the bacterial colony

DNA Extraction: The pure culture bacteria were grown in liquid media containing LBB and incubate for 1 day at 37°C. Bacterial solution (1mL) was transferred in an Eppendorf tube, centrifuged for 1min at 12000 rpm and the supernatant was discarded. STE (Sodium Chloride-Tris-EDTA) buffer (1mL)

was added to the bacteria pellets, vortex, centrifuged for 1min at 12000 rpm and the buffer solution was discarded slowly. STE buffer (200µL) was added to the solution and vortex. SDS (sodium dodecyl sulfate) buffer (20 µL, 10%) was added to the solution and vortex. The solution was then suspended in a water bath for 20-30 minutes at 65ºC. The desired product is a clear solution. After the water bath, 4µL of Protein ASEK (10mg/mL Proteinase K) was added to the solution, the solution was mixed and it was again suspended in a water bath overnight at 56ºC. After the second water bath, the solution volume was increased by adding 400µL STE buffer and it was gently mixed. The solution was washed using phenol and chloroform. Following the standard laboratory practice in handling carcinogenic substances, 10 drops of phenol and 10 drops of chloroform were added to the solution. The solution was mixed vigorously. The solution was placed in a centrifuge for 5min at 12000 rpm. The phenol and chloroform wash was repeated until a clear solution is obtained. Equal parts chloroform was added to the extracted solution and it was centrifuged at 12000 rpm for 5min. The desired product is a clear solution. The upper solution was carefully extracted and it was transferred to a new Eppendorf tube. Three molar of NaOAc (sodium acetate) of pH 5.2 was added, 1/10 parts of the extracted solution volume; and it was mixed slowly. Ethanol (95%) was added to increase the solution volume to 1mL and it was mixed slowly. The solution was allowed to sit in the freezer for 10min. It was then centrifuged at 12000 rpm for 15min and the alcohol was gently discarded. The solution was placed in the centrifuge for a few seconds and the excess alcohol was removed. The Eppendorf tube was dried in the oven for 5min at 60ºC to evaporate any remaining alcohol. Distilled water (20µL) was added to the dry DNA pellets, the solution was then given a quick spin in the centrifuge and it was allowed sit for 5min to make sure the DNA was dissolved in water.

Preparation of PCR solution: Fifty µL PCR solution was prepared by adding the following reaction solutions (exact order) in the PCR tube: 34.5µL ddH2O, 5µL 10xPCR Buffer, 8µL dNTP, 0.5µL forward primer, 0.5µL reverse primer, 1µL Temp DNA (1µL DNA extraction product + 99µL ddH2O), and 0.5µL enzyme (rTaq, DNA Polymerase). The solution was mixed in the centrifuge for 5s and the tube was quickly loaded in the PCR machine.

Agarose Electrophoresis (Mupid-2 plus, submarine electrophoresis system) was used in this study to check the number of base pairs.

The biocontrol agent (BCA) bacteria genomic analysis for speciation, 16s rRNA Gene Sequence Analysis, was conducted by Genomics, a service laboratory located in 14F, No. 100, Sec. 1, Xintai 5th Road, Xizhi District, New Taipei City, Taiwan. The PCR product was sent to Genomics for analysis. The species DNA sequence data were matched on the BLAST database (http://www.ncbi.nlm.nih.gov/BLAST/) to identify the rhizobacteria strain.

2.6. Preparation of the biofungicide

Rice Hull Mix biofungicide formula: 100mL LBB in a 500mL conical flask were prepared. The LBB was inoculated with the biocontrol agent bacteria and it was incubated in the dark for 5 days at 25 ± 3°C. The rice hull and rice grain mixture was prepared in a 500mL conical flask as suggested by Nagarajkumara *et al*. (2005)[14] (300g rice hull; 100g rice grain; and 200mL distilled water) and the mixture was sterilized in an autoclave for 30 minutes at 121°C. LBB (80mL) inoculated with biocontrol agent bacteria were aseptically transferred to the mixture and it was incubated for 2 weeks at 25 ± 3°C.

Liquid biofungicide formula: 120mL LBB in a 500mL conical flask were prepared. Five mL 1M glucose solution was added to the sterile LBB and it was inoculated with the biocontrol agent bacteria and incubated in the dark for 5 days at 25 ± 3°C.

3. Results and Discussion

3.1. Selection of bacterial strains

A variety of bioantagonistic bacteria, including members of the genera Azospirillum, Azotobacter, Azoarcus, Pseudomonas, Bacillus and Enterobacter, are known to colonize the rhizosphere of rice and can be used as plant growth promoters and biocontrol agents [13].

Six percent of the tested soil microorganisms gave a positive result in the inhibition test. The strains identified as Bacillus and Pseudomonas fluorescens, were considered to be most effective. The data in Table 1 shows the clearing zone diameter (mm) and the antifungal activity of the indentified bacteria against rice blast disease.

Table 1. Degree of inhibition of the different soil bacteria

Soil Bacteria Strain*	Soil sample source**	Inhibition Diameter[A] (mm)	Antifungal activity[B]
5	Soil with rice blast infected crops (UPLB)	18	++
4	Soil with healthy crops (UPLB)	16	++
3S5	Soil with bacterial blight & sheath blight infected crops (UPLB)	18.1	++
6	Soil with healthy crops (UPLB)	15	++
11	Soil with bacterial blight & sheath blight infected crops (UPLB)	14.5	++
10S2	Soil with rice blast infected crops (UPLB)	24.3	+++
12	Soil with tungro infected crops (North Cotabato)	16.1	++

*Identification label used during the experiment

**Specific point in the rice field the soil sample was collected and its location

A: Mean value, B: Inhibition: No difference with control (-), 5-9mm (+), 10-19mm (++), >20mm (+++)

The data in Table 1 was obtained by calculating the mean value of the three replicates of each soil bacterium. In the plate test, Bacillus isolate 5, Bacillus isolate 3S5 and Pseudomonas fluorescens isolate 10S2; gave the maximum inhibition of the rice blast fungus.

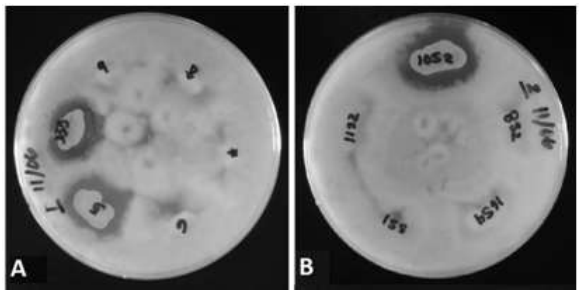

Figure 1: Inhibition of rice blast fungus in PDA**

A: (5) Bacillus subtilis strain S12; (3S5) Bacillus cereus NC77401, B. (10S2) Pseudomonas fluorescens Pf0-1_, **Fungal inhibition was observed after 3 days of incubation

3.2. Speciation of the soil bacteria Biological Control Agent (BCA)

The top 3 soil bacteria strains, based on the inhibition diameter, were identified using PCR and 16s rRNA Gene Sequence Analysis. Since the soil bacterium is unknown and there is limited information on its specie family, the universal primer for bacteria was used for the PCR reaction. The Reverse primer (1492r): 5' TAC GGT TAC CTT GTT ACG ACT T 3' and Forward primer (27f): 5' GAG TTT

GAT CAT GGC TCA G 3', were used in this study. The PCR primers were combined to amplify the16s rRNA genome sequence from nucleotide 27 to nucleotide 1492.

The Agarose Electrophoresis result of the PCR products is shown in Figure 2. Based on DNA maker ladder (column M), the results displayed bands with 1500bp (base pairs). The length of the DNA molecule is measured based on the number of nucleotide pairs that it contains. This represents many identical copies of a particular size fragment of DNA.

The PCR product of soil bacteria labelled 5, 10S2 and 3S5; were identified using 16s rRNA gene sequencing. The sequences obtained were compared with public database, BLAST. The 3 isolates were identified at least to the genus level, as shown in Table 2.

Table 2. 16s rRNA Gene Sequencing Results

Soil Bacteria	Inhibition Diameter (mm)	Lineage	Identity	Max Score	Total Score	Ident	Identities
5	18	Bacteria, Bacillus	Bacillus subtilis strain S12	1925	1925	99%	1046/1048 (99%)
3S5	18.1	Bacteria, Bacillus	Bacillus cereus NC7401	1873	1873	99%	1018/1020 (99%)
10S2	24.3	Bacteria, Pseudomonas	Pseudomonas fluorescens Pf0-1	1834	2373	99%	1007/1014 (99%)

3.3. Biofungicide formulation

The treatment formula used for the study are as follows: A – Bacillus subtilis 5 (Bacillus subtilis strain S12) inoculated rice hull biofungicide + Bacillus subtilis 5 (Bacillus subtilis strain S12) grown in LBB with glucose (liquid biofungicide); B – Bacillus cereus 3S5 (Bacillus cereus NC7401) inoculated rice hull biofungicide + Bacillus cereus 3S5 (Bacillus cereus NC7401) grown in LBB with glucose (liquid biofungicide); and C – Pseudomonas fluorecens 10S2 (Pseudomonas fluorecens Pf0-1) inoculated rice hull biofungicide + Pseudomonas fluorecens 10S2 (Pseudomonas fluorecens Pf0-1) grown in LBB with glucose (liquid biofungicide).

The biofungicide formulations were tested on rice cultivar UPLRi-5 under controlled conditions and the results showed decrease in rice blast disease intensity, which is 33% higher compared to the control.

3.4. Discussion

The results indicate a significant degree of inhibition in rice blast development. The different levels of disease suppression might be due to the different colonization pattern and secretion of secondary metabolites by the rhizobacteria [13].

Antibiotic compounds produced by antagonist bacteria contribute to the growth suppression against plant pathogen [16]. Previous studies have reported that enzymes increase after treatment of biocontrol agent such as peroxidise [17]. Peroxidase is an enzyme which acts as catalisator on the final stage of lignin and hydrogen peroxidise biosynthesis [18]. It is a pathogenesis-related protein (PR-protein) that is toxic to pathogen and increases the resistance of the cell wall against degrading enzymes produced by the pathogen [15,18]. It is reported to be seen in healthy plant tissue and its concentration remarkably increased when the plant was infected with the pathogen [15]. Bacillus polymyxa and Pseudomonas fluorescens were reported to increase plant health through increase of peroxidise activity in the plant [4].

In sustainable agriculture, in order to make a significant impact on global rice production and food security, a biocontrol solution for rice blast disease must be developed while reducing or eliminating

the need for synthetic chemical fungicides. The work on the isolation of antiblast microorganisms and substances suggest that these have an essential role in the biological control of rice blast disease. The results collected in this study can be used to improve rice blast control using antagonistic bacteria. Biological control as disease management proved to be an additional measure even if no single cultivar may be suitable for all upland rice ecologies. Cultivar varieties can be improved through genetic manipulation but its feasibility will require further assessment and comprehensive understanding of pathosystems and the biological control agents. The antifungal activity of the microorganisms found in this study (Bacillus subtilis 5, Bacillus cereus 3S5 and Pseudomonas fluorecens 10S2) demonstrated the ability to restrict the growth of rice pathogen (M. oryzae) and is a strong candidate for a biocontrol agent for rice blast disease. In contrast to chemical fungicides, biocontrol bacteria produce a mixture of antifungal compounds which can fluctuate based on environmental cues [6]. The antifungal activity of Bacillus subtilis 5, Bacillus cereus 3S5 and Pseudomonas fluorecens 10S2 could lead to a long term solution and more effective strategy for reducing rice blast disease than present chemical fungicides, which can lead to the emergence of resistant populations of M. oryzae and raises public health concerns if used extensively. Furthermore, as living organisms, these biocontrol microbes are continuing to evolve with their rhizospheric neighbours ensuring a more sustainable solution. Effective plant defense may be due to an ability of the host plant to modulate the composition of root exudates, attracting microbes which can trigger plant resistance. The recruitment of beneficial microbes can also alter physiological functions in plants to resist aerial pathogens [6]. Therefore, the direct antifungal activity of Bacillus subtilis 5, Bacillus cereus 3S5 and Pseudomonas fluorecens 10S2 against M. oryzae could have ecologically relevant implications in preventing blast infections. The precise mechanism by which rice rhizospheric microbes induce physiological effects on the host (rice crop) is not known, but the identified microorganisms in this study were able to trigger a defense mechanism and reduce disease symptoms during pathogen infection. To my knowledge, this is the first report of a biofungicide using a mixture of rice hull and biocontrol agent (bacteria), which can protect rice cultivar against rice blast disease and shows direct antifungal activity and stimulation of resistance in the host (rice crop). There are compelling reasons to believe that root associated microbes are equally important to plants as they are to animals. Plant roots encounter diverse microbial populations in soil and generate a unique ecological niche for microbes by the secretion of resources into the rhizosphere. These rhizospheric resources are limited and some microbes have evolved antimicrobial traits to reduce competition from other microbes and to bolster the health of their plant host [6]. However, this study lacks a comprehensive understanding of the contribution presented by individual microbial strains to plant growth and protection. Since biocontrol have established to be a plausible approach to crop protection, further studies are needed to classify probable biocontrol agents from the diverse pool of soil microorganisms and to understand the mechanisms by which they influence plant health, pathogen resistance and productivity.

4. Conclusion

The rhizobacteria Bacillus isolate 5, Bacillus isolate 3S5 and Pseudomonas fluorescens isolate 10S2; gave the maximum inhibition of the rice blast fungus in the plate test. The strains were subjected to PCR analysis and 16s rRNA Gene Sequence Analysis. Using the BLAST database, the strains were classified as follows: Bacillus subtilis 5 (Bacillus subtilis strain S12); Bacillus cereus 3S5 (Bacillus cereus NC7401); and Pseudomonas fluorecens 10S2 (Pseudomonas fluorecens Pf0-1). Three biofungicide formulations were developed. It contained both rice hull and liquid media (LBB + glucose) mixed with rhizobacteria isolated from rhizospheres of rice which respectively contained bacterial suspension of Bacillus subtilis 5, Bacillus cereus 3S5 and Pseudomonas fluorecens 10S2. The biofungicide was tested on rice cultivar UPLRi-5 under controlled conditions and the results showed decrease in rice blast disease intensity, which is 33% higher compared to the control.

5. Recommendation

The antifungal activity of the isolates found in this study highlights its importance as a candidate for further investigation as biological control of rice blast disease. Recommendations for further research are as follows: (1) Field trial should be conducted in order to further evaluate the stability and effectiveness of the biofungicide formula under field conditions in different locations; (2) Isolation and identification of antifungal metabolites produced by the antagonistic bacteria isolates; (3) Isolate and utilize the gene coding of the antifungal characteristics of the antagonistic bacteria to develop resistant transgenic-rice crops bearing elevated resistance to infections by M. oryzae with recombinant DNA having antifungal genes cloned from biologically active rhizobacteria isolates.

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