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Molecular Identification of Endophytic Fungi from Rice (Oryza sativa L.) and its Anatagonistic Effect Against Staphylococcus aureus and Escherichia coli

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Abstract

Endophytes are organisms living inside the tissue of the host organism. Most of them usually do not cause detrimental effect to its host species. The study aimed to isolate and identify endophytic fungi from the leaves and roots of rice plant, and evaluates their antagonistic effect against Staphylococcus aureus BIOTECH 158 and Escherichia coli BIOTECH 1634 bacteria. Identification of the collected endophytic fungi was done through PCR based approach using the internal transcribed spacer region of the ITS gene sequence. Three fungi were collected, isolated and were identified as Anthostomella leucospermi (EU552100.1), Ceriporia lacerata (KP689233.1) and Fusarium equiseti (KT277307.1). The isolates Anthostomella leucospermi and Fusarium equiseti have growth inhibition against Staphylococcus aureus and Escherichia coli bacteria. On the otherhand, the growth of Ceriporia lacerata was not inhibited by E. coli but inhibited by S. aureus. The potentials of endophytes that have antibacterial property in this study are good source of important bioactive compounds and are important for discovery of drug components also important for industry and in agricultural and biotechnological research.

Keywords: Anthostomella leucospermi; bioactive compounds; endophytic; Ceriporia lacerata; Fusarium equiseti.

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1. Introduction

Rice (Oryza sativa L.) is well known to be a staple food essential in Asian countries and leading out as third crop that is being produced worldwide after corn and wheat. The high demand for this food includes all social classes and majority on rural households [1]. But as a living organism, such as rice plant also has enemies including pest, bacteria and fungi that cause diseases that tend to threaten the food security for rice production. Fungi, which is considered to be the most diverse and important group of organism has dual impact on humanity [2]. They are good source of food and drinks, medicine and in agriculture, as biological control organisms. They are popular on the production of wine, beer, bread, cheese and other popular food we eat [2]. But then some of the species that belongs to this kingdom tend to cause life-threatening diseases in human, animals and plants. There are also fungi called endophytes that live inside the tissues of the plants without manifestation of diseases in the plants [3]. Endophytes are those fungi that grow inside the tissue of its host and contribute to the recycling of nutrient in ecosystem within its host plant [4]. These fungi were believed to grow within the plant cell and above the ground surface level of the plant and numerously colonizers [5]. The interesting part about endophytes is that their known producers of natural bioactive compounds that potentially offer a breakthrough in the field of medicine, agriculture and even industrial utilization [6]. This study aims to isolate and molecularly identify colonistic endophytic fungi present in the healthy leaves and roots of rice plant and to determine the antagonistic effect or antibacterial potential of the fungal isolates against the presence of Staphylococcus aureus and Escherichia coli in dual culture assay.

2. Materials and methods

2.1Plant Sample Collection

Collections of healthy rice plants at vegetative stage were obtained from the rice field based on the procedures of Atugala and Deshappriya [7]. The segmented leaf plant samples were washed and immersed to 70% ethanol for one minute, soaked into 0.25% sodium hypochlorite (NaOCl) for fifteen minutes and to 70% ethanol for thirty seconds and washed with distilled water for three times. The segmented plant samples were placed inside the petri dishes with sterilized and cooled malt extract agar with 50 mg/L of Tetracycline. Five replications were set for both roots and leaves samples. The petri plates were sealed afterwards and were incubated for ten days at a room temperature.

2.2 Isolation and purification

Isolation of fungi from the cultured petri plates was subjected for sub-culturing and further purification. A block of agar with fungi from culture plates was transferred ascetically into newly prepared MHA media in petri plates with 50 mg/L of Tetracycline to inhibit bacterial growth, the fungi was then used for the antibacterial assay. To prepare pure culture stock, about 10 mm^2 of pure culture of each fungus was then inoculated at the test tube with Potato Dextrose Agar (PDA).

2.3 Molecular identification of endophytic fungi

The isolated fungi were subjected to DNA extraction using CTAB method (with minor modifications) [8]. DNA amplification was performed using the following: a total of 20 µL was prepared per reaction, 2µL of 10x PCR Buffer, 0.5 µL dNTP mix, 1.0 µL of ITS1-F (5'-CTTGGTCATTTAGAGGAAGTAA-3') and 1.0 µL of ITS4-R (5'-TCCTCCGCTTATTGATATGC-3'), 13.46 µL of sterile distilled water, 0.04 µL Taq-polymerase and 2 µL of genomic DNA. PCR components were subjected to PCR using the following PCR profile; initial denaturation 94 ºC for 5 minutes, denaturation at 94 ºC for 30 seconds, annealing at 55 ºC for 45 seconds and extension at 72 ºC for 30 seconds for 35 cycles. The final extension was at 72 ºC for 7 minutes. The size of amplified DNA for each sample was analyzed using Gel Documentation System based on 1kb ladder (KAPA^{TM} Universal Ladder, Kapa Biosystems). The PCR products were then sent to Apical Scientific Sequencing in Malaysia for sequencing. The resulting nucleotide sequence was subjected to BLAST analysis from NCBI [9] for identification.

2.4 Antibacterial assay

Pure culture of Staphylococcus aureus BIOTECH 158 and Escherichia coli BIOTECH 1634 were obtained from Philippine National Collection of Microorganism, National Institute of Molecular Biology and Biotechnology, University of the Philippines, Los Baños, Philippines. A loopful of bacteria was placed in sterilized test tubes with nutrient broth. The tubes then were kept overnight at room temperature for antibacterial assay. One hundred µL of the nutrient broth with the inoculant was pipetted and was spread plated evenly in the plates with PDA media using the sterile bacterial cell spreader. Afterwards, using a sterile cork borer, an agar block was aseptically and carefully pulled out and was place in the middle of the media with bacteria. The plates were sealed and incubate for 12 hours. The mean growth was measured from day 1 up to day 5 of observation using a digital caliper.

2.5 Data analysis

The data were analyzed using student t-test comparing the mean growth observed in with and without the test organism.

3. Results

Table 1: Identity of the collected endophytic fungi using the partial rDNA-ITS sequence

Sample Code	Identity	% Sequence Identity	Accession No.
Sample A	Anthostomella leucospermi	99%	EU552100.1
Sample B	Ceriporia lacerata	100%	KP689233.1
Sample C	Fusarium equiseti	100%	KT277307.1

A total of three endophytic fungi were isolated from the rice plants. Using the partial rDNA-ITS region, the Sample A was identified as Anthostomella leucospermi (EU552100.1) with 99% identity. The Sample B was identified as Ceriporia lacerata (KP689233.1) with 100% identity. On the other hand, the Sample C was identified as Fusarium equiseti (K277307.1) with 100% sequence identity (Table 1).

The growth pattern of the isolated fungi was tested and determine against E. coli and S. aureus bacteria. The E. coli significantly suppressed the growth of the Anthostomella leucospermi and Fusarium equiseti during second day to fifth day of observation. On the other hand, Cereporia lacerata was not inhibited by the presence of E. coli based on the growth mean of Cereporia lacerata without and with the presence of E. coli until five days of observation (Table 2). It shows that the presence of E. coli exhibit antagonistic effect to Anthostomella leucospermi and Fusarium equiseti but not affecting the growth of Cereporia lacerata.

Table 2: Mean growth (mm^2) of the identified endophytic fungi against E. Coli for five days.

	Day 1		**Day 2**		**Day 3**		**Day 4**		**Day 5**	
Identified species	–	+	–	+	–	+	–	+	–	+
Anthostomella leucospermi	0.72	0.58 ns	3.88	1.32 **	10.48	2.63**	16.07	4.74**	23.60	6.68**
Ceriporia lacerata	4.85	4.88 ns	24.63	20.27 ns	43.63	33.68 ns	62.05	48.03 ns	73.59	60.66 ns
Fusarium equiseti	1.65	0.52 ns	9.25	2.11**	14.52	2.62**	19.36	2.97**	22.62	3.15**

** Growth means were significantly diferent between without and with E. coli with respect to nth days of observation (– = without E. coli; + = with E. coli).

ns = not significant

The mean growth of Anthostomella leucospermi, Ceriporia lacerata and Fusarium equiseti against S. aureus were not significant compared to the growth of the endophytic fungi without the test organism (Table 3). The presence of S. aureus did not inhibit the growth of three endophytic fungal isolates from Day 1 to Day 5. The growth of the endophytic fungi have no antagonistic effect with the presence of S. aureus.

Table 3: Mean growth (mm^2) of the identified endophytic fungi against S. aureus for five days.

	Day 1		**Day 2**		**Day 3**		**Day 4**		**Day 5**	
Identified species	-	+	-	+	-	+	-	+	-	+
Anthostomella leucospermi	0.72	0.81 ns	3.88	3.37 ns	10.48	11.73 ns	16.07	18.43 ns	23.60	23.90 ns
Ceriporia lacerate	4.85	6.19 ns	24.63	25.31 ns	43.63	44.27 ns	62.05	62.21 ns	73.59	73.59 ns
Fusarium equiseti	1.65	2.03 ns	9.25	8.91 ns	14.51	14.53 ns	19.36	20.46 ns	22.62	25.08 ns

** Growth means were significantly diferent between without and with S. aureus with respect to nth days of observation (– = without E. coli; + = with E. coli).

ns = not significant

4. Discussion

Among the fungal isolates, only the growth of Ceriporia lacerata showed antagonistic effect with E. coli. The

new strain of Ceriporia lacerata was first reported on 2012 during the field survey in Korea University Experimental Forests and was based on morphological characteristics and 28S, 18S, and internal transcribed spacer rDNA sequences [10]. However it was first described in 2003 [11] using morphological approach and was reported in China [12, 13]. This species was classified as new endophytic fungi. Ceripori lacerata isolated from the bud of C. operculatus produces a bioactive flavonoid called DMC or 2', 4'-Dihydroxy-6'-methoxy-3', 5'-dimethylchalcone which has an anti-cancer effect [14]. Interestingly, extracts from Ceriporia lacerata found to have protective effect on dexamethasone-induced cytoxicity in INS-1 cells [15].

This suggests that Ceriporia lacerata is composed of several metabolites that need to study further. In this study we also found out that the growth of Anthostomella leucospermi, Ceriporia lacerata and Fusarium equiseti was not affected in the presence of S. auresus. A study in 2016 of the crude extract of Anthostomella species, an endophytic fungus specifically Anthostomella brabeji, was reported to have antibacterial effect against Staphylococcus aureus, Staphylococcus sebutal and Candida albicans but none in E. coli [16].

Similar antibacterial study in 1974, showed that Fusarium equiseti NRRL 5537 strain produced substance that exhibit antibacterial activity against gram positive bacteria including S. aureus but not in gram negative bacteria like E. coli [17]. E. coli showed inhibition in the growth of the two fungal isolates Anthostomella leucospermi and Fusarium equiseti however, there is still increasing growth observed from Day 1 up to Day 5. This suggests that E. coli did not suppress the growth of the fungal isolates but instead decreased the rate of their growth.

5. Conclusion

In this study, three endophytic fungi were isolated from rice plants and was identified Anthostomella leucospermi, Ceriporia lacerata and Fusarium equiseti based on the internal transcribed spacer rDNA sequences by direct PCR. Ceriporia lacerata was found to have antagonistic effect to E. coli and showed similar growth with or without the presence of the bacteria.

On the other hand, Anthostomella leucospermi, Ceriporia lacerata and Fusarium equiseti have no antagonistic effect against S. aureus since the bacteria did not affect the growth of the fungal isolates. Endophytic fungi are very diverse group of organisms. Many endophytic fungi contain secondary metabolites with important biological activity.

Secondary metabolites are important defense mechanism of plants and fungi and also their means of communication, thus these secondary metabolites are important and must discover for its potential use.

6. Recommendation

Based on the outcome of the study, further research is needed to determine the presence and properties of bioactive compounds from the endophytic fungi isolates. Also, studies related to agricultural application are interesting promising since these endophytic fungi are not just potentially involve in the defense mechanism of plants against microbial outbreaks and stresses but also ideal biological agent organisms that promote growth and development to the host plant.

References

[1] Gumapac M., 2011. RICE: A Filipino constant. BAR Digest. 13 (4).

[2] Perlin, D. S., 2015. Mechanisms of echinocandin antifungal drug resistance. Annals of the New York Academy of Sciences. 1354(1): 1-11.

[3] Schulz B., & Boyle C., 2005. The endophyte continuum. Mycological Research, 661-686.

[4] Sun X., Guo, LD., & Hyde, K. D., 2010. Community composition of endophytic fungi in Acer truncatum and their role in decomposition. Fungal Diversity, 47: 85-95.

[5] Blackwell M., 2011. The Fungi: 1, 2, 3… 5.1 million species? American Journal of Botany 98:936-948.

[6] Strobel G., & Daisy B., (2003). Bioprospecting for microbial endophytes and their natural products. Microbiology and Molecular Review, 419-502.

[7] Atugala D.M., & Deshappriya D.M., 2015. Effect of endophytic fungi on plant growth and blast disease. Journal of National Science Foundation Sri Lanka 43: 173-187.

[8] Healey A., Furtado A., Cooper T., & Henry R.J., 2014. Protocol: a simple method for extracting next-generation sequencing quality genomic DNA from recalcitrant plant species, Plant Methods 10:21.

[9] Geer L.Y., Marchler-Bauer A., Geer R.C., Han L., He J., He S., Liu C., Shi W., & Bryant SH., 2010. The NCBI BioSystems database. Nucleic Acids Res. (Database issue):D492-6.

[10] Jang Y., Choi H.E., & Lim Y.W., 2012. The first report of Ceriporia lacerata (Phanerochaetaceae, Basidiomycota) in Korea. Mycotaxon, 119 (7): 397-403.

[11] Suhara H., Maekawa N., Kaneko S., Hattori T., Sakai K., & Kondo R., 2003. A new species, Ceriporia lacerata, isolated from white-rotted wood, Mycotaxon, 86:335–347.

[12] Cui B., Wei Y., & Dai Y., 2006. Polypores from Zijin Mountain, Jiangsu province. Mycosystema, 25:9–14.

[13] Dai Y.C., 2012. Polypore diversity in China with an annotated checklist of Chinese polypores. Mycoscience 53: 49–80.

[14] Wang J., Yao L.-Y., & Lu Y.H., 2013. Ceriporia lacerata DMC1106, a new endophytic fungus: Isolation, identification, and optimal medium for 2. Biotechnology and Bioprocess Engineering , 669-678.

[15] Kim J., Park Y., Kim J., Lee S., Kim B., & Jang B., 2013. Crude extract of Ceriporia lacerata has a

protective effect on dexamethasone-induced cytotoxicity in INS-1 cells via the modulation of PI3K/PKB activity. International Journal of Molecular Medicine, 32, 179-186.

[16] De Amorium, M.R., Somensi, A., Araujo, A.R., Bonifacio, B.V., Bauab, T.M., & dos Santos, L.C., 2016. Compounds of Anthostomella brabeji, an Endophytic Fungus Isolated from Paepalanthus planifolius (Eriocaulaceae). Journal of Brazil Chemical Society, 1-7.

[17] Burmeister, H.R., Benneth, G.A., Vesonder, R.F., & Hesseltine, C.W., 1974. Antibiotic Produced by Fusarium equiseti NRRL 5537. American Society for Microbiology, 634-639.