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Alginate Encapsulation of *Trichoderma harzianum* as Biocontrol Agent against Brown spot Disease on Rice (*Oryza sativa*) in Vitro Assays

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***Abstract*— Brown spot caused by *Bipolaris oryzae* is an important rice disease which can cause a reduction in yield and grain quality in Malaysia. The application of chemical agents in controlling the brown spot disease can cause undesirable effects such as residual toxicity, environmental pollution, and development of pathogen resistance to fungicides. Instead, biocontrol methods have to be developed to ensure environmental safety, the longevity of usage while effective methods of plant disease management. However, to the best of our knowledge, no research has been conducted about alginate encapsulation of *Trichoderma harzianum*. Therefore, the objectives for this study is to measure the performance of encapsulation of *Trichoderma harzianum* isolates in controlling the brown spot disease on rice. Alginate encapsulation was produced from conidial suspension by adding sodium alginate and calcium chloride which result in small beads. The isolate was isolated and characterized based on morphological characters and have been proved by pathogenicity tests. Based on the results dual culture test showed the highest percentage inhibition of radial growth which was 85.07% with 10 replication of *Bipolaris oryzae*. The findings from this study can be used as an alternative method to control brown spot disease which will render easier application to rice plantation in the future which suit for commercial purposes and contamination-free environment.**

***Keywords*— *Bipolaris oryzae*; *Trichoderma harzianum*; alginate encapsulation.**

I. INTRODUCTION

Rice is the most important food sources and as an economical crop to the farmers and workers through the region of Asia countries [1]. Rice also essential to provide employment and helped the million small of farmers to generate their income. Rice supplied 21% of energy and 15% of protein to human, from that the quantity and the quality needed to give more attention to ensure the two factors could be improved [1]. In the latter half of the 1960s, the green revolution has made the improvement of yield per hectare increased from 2014kg/ha to 3960kg/ha [2].

Rice (*Oryza sativa*) is the staple food and the most important cereal in Malaysia which is consumed by more than 2,500 million people. Rice production is increased from the present 560 to 750 million tons by 2020 since the increasing populations of consumers [3]. Rice is third-highest production after oil palm and rubber in the major crops of Malaysia. The total rice production in Malaysia contributed up to 85.5% where the rice is planted area Peninsular Malaysia which mainly in Kedah, Perlis, Kelantan, Terengganu, Pulau Pinang, Perak and Selangor [4].

Rice cultivation is usually planted in the rural area and traditional farmers. The area involves eight main rice producing regions of Malaysian. These area are: 1) the Muda Agricultural Development Authority (MADA); 2) Kemubu Agricultural Development Authority (KADA); 3) Barat Laut Selangor Integrated Agriculture Development Area (IADA Barat Laut Selangor); 4) Penang Integrated Agriculture Development Area (IADA Penang); 5) North Terengganu Integrated Agriculture Development (KETARA); 6) Kerian Sungai Manik; 7) Seberang Perak; and 8) Kemasin-Semerak. Traditional farmer need to know how to increase their production to help maintain the quality and quantity of rice. Not only that, but the government also need to give full support to the farmers and establish any program that can help the farmers to get the information or any new technology regarding the paddy cultivation [5].

Malaysia cover about 600,000 Ha of rice cultivation which located in Peninsular Malaysia. The production of rice for the country's annual production of nearly two million tons of rice. About 400 rice miles of varying capacity are located in and around the granaries as well as the other rice areas. The rice mills which periodically import about 10

percent to fulfill the national requirement in the public sector [6].

The average of rice mills usually is small, seldom larger than 1.5 Ha. Besides that, the holding is fragmented, land parcels being quite irregular and miniature. The rice cultivation has remained restricted for a small field. Free fertilizer and price support give to farmers who cultivate less than 2.5 Ha. Besides that, the effort to extend rice farming on a larger scale through enlarged farm size is also in progress. Through group farming and locational land consolidation, the efforts can be achieved [7]. Nowadays, several thousand hectares are being farmed of rice extensively to exploit the rice economy fully and to commercialize its production efficiently [7].

A good variety will help to increase the production of rice. The variety needs to be in good quality and at the same time must have a good of characteristics such as drought tolerance and resistant to pest and disease. With the advanced of technology in agricultural, new varieties replaced the traditional crop that can help to improve the yield performance [8]. Poor of grain quality will cause a lower demand from the consumer. In Ghana, the consumer more prefers imported rice about 70% compared to the local rice [9]. Malaysia started produced its owned variety such as Mahsuri, Mahsuri Mutant, NS 9192 and Putri (Q-50) [10]. In 2015, the Malaysian Agricultural Research and Development Institute (MARDI) was introduced the new variety of rice is MR284.

Like other major crops, rice is also prone to several diseases that affect the growth of rice production. Every year, about 10-20% of the total world of food production becomes decreased due to plant disease and thus contributed to the loss of billions of dollars [11]. Rice brown spot is a prevalent disease caused by fungus *Bipolaris oryzae*, and it occurs in all rice-growing areas worldwide. *Bipolaris oryzae* can cause disease on all growth stages of rice plants. The infection shows significant yield and grain quality losses to the country's economy as well to consumers [12].

Fungus *Bipolaris oryzae* is the causal agent for brown spot disease. That fungus also is known as *Helminthosporium oryzae*. The fungas can infect all part of paddy plants. The life cycle and morphogy of the fungus are very complicated. However, based on plant pathologists usually, the causal agent can be identified by looking at the conidia (spore) microscopically. The conidia of the *Bipolaris oryzae* are very small, generally curved, and cylindrical and light brown to golden brown [13].

Bipolaris oryzae is a seed-borne pathogen [14]. The pathogen can survive it life within the seed for four years. When the scientist studied the samples collected from the different agro-climatic region of Bahar, Jammu, Andhra Pradesh and Orissa, they found that *Bipolaris oryzae* is to be the most predominant seed-borne fungi. The prevalence of *Bipolaris oryzae* is from Assam [15]. By using genetic fingerprint analysis, the diversity and pathogenicity of the rice brown spot pathogen have been investigated in Bangladesh [15].

This disease usually will become serious when at the maturity stage. Symptoms of brown spot occur on leaves, young seedling, panicle branches and glumes of maturing plants. It showed dark brown to reddish brown, and become larger spots shown a dark brown margin and reddish brown to gray centers. The symptom is circular to oval in shape and varies in size are typically 1/8 inch in diameter. On the first seedling leaves also this disease will start to appear as small circular to oval spots if the planted sees are a disease (air-borne) and by the spores, the disease can be spread from plant to plant in the field [15].

The symptoms of brown spot disease may be evident shortly after seedling emergence and continue to grow until the symptom at the maturity stage. The leaf spot that appears on the paddy vary in size, typically 1/8 inch in diameter and the shape is circular to oval. The size of smaller spots shown dark brown to reddish brown become dark brown margin and reddish brown to grey at centers when large [16].

For the disease cycle, the most common source of primary infection is infected seeds and stubbles. The conidia that have on mycelium in the infected tissues and on infected grain are viable for 2 to 3 years. The airborne conidia can infect the infected leaves in the nursery and in the main field. Usually, the fungus can survive on the collateral host like *Echinochloa colonum* and *Leersia hexandra*. The brown spot fungus is normally present in areas with a long history of rice culture. In infested debris and older lesions, an airborne spore that is capable of causing infection are produced [17].

In the present investigation of rice brown spot disease, about more than 50% losses of paddy due to brown spot disease in Malaysia have been reported. Thus, Malaysia needs effective alternative disease management strategies in controlling the disease without causing side effect to the environment. Beneficial microorganisms as a bio-control agent to suppress plant disease is one alternative that can save our environment. Besides being the antagonist to the disease, it can also act as a bio fertilizer which is very useful for the growth and development of the plant itself. Common beneficial microorganisms used as the bio-control agent is *Trichoderma harzianum, Trichoderma polysprum, Trichoderma pseudokoningii, Gliocladium virens, Poecilomyces variotii* and *Poecilomyces lilacinus* [18]. *Trichoderma* spp. is a very effective biocontrol agent to control fungal diseases especially brown spot disease as they colonize the root surface and rhizosphere from the infected seeds. It can also stimulate plant growth and productivity of the plant [19], [20].

Biological control is the eco-friendly approach and innovation method against diseases. *Trichoderma* spp. Is known as a biological control agent for control of fungal diseases which are the mycoparasitic and antagonistic mechanism. *Trichoderma* is present in all soil, and they are the most cultural fungi which are strongly antagonistic to other phytopathogenic fungi. *Trichoderma* produces hydrolytic enzymes which are responsible for playing an important role in the phytopathogenic fungi. The diffusion of the enzymes dissolves cell fragments of host cells. Based on the study, *Trichoderma harzianum* was the most antagonistic and inhibited the radial growth of the *Bipolaris oryzae* while *A.flavus* was the least antagonist [21].

Certain microorganisms are produced to protect plants from the disease usually by producing them in large quantities. However, large production for various commercial uses may be difficult. Therefore, the formulation

of microbial products must be produced to suit commercial purposes, ensure a contamination-free environment for the product while maintaining a high quantity of viable microorganisms. *Trichoderma* spp. have been formulated for commercial uses by encapsulating the *Trichoderma* spp. Encapsulating the microorganisms provides many advantages than chemical pesticides and other biological products [22].

Nowadays, application methods for controlling disease has become the main problem due to the cost, maintenance and handling methods. Since formulations of microbial products are the effective method to control the disease, this study aims to investigate biocontrol activities against *B. oryzae* from rice using the encapsulation of *Trichoderma* isolates.

II. Materials and Methods

A. Isolation of B. oryzae

Several samples with brown spot disease symptoms on rice were collected randomly from the area of Merlimau, Melaka. Direct isolation of *B. oryzae* can be obtained from infected brown spot disease on rice. The samples were cultured on Potato Dextrose Agar (PDA) for 7 days.

B. Isolation of Biocontrol Agent

Pure cultures of *T. harzianum* were re-cultured from stock slants onto PDA medium. Mycelium will form as compact or loose tufts in shades of green or yellow or less frequently white within three or four days. The yellow pigment may be secreted into the agar. All these observations, especially on PDA, were recorded and radial growth of *T. harzianum* colony on PDA was observed daily until the plate is covered.

C. Morphological Identification of B. oryzae Isolates

Pure culture obtained was used for identification purposes. The isolates were then examined using morphological characteristics [23], [3].

D. Pathogenicity tests

Isolate of *B. oryzae* was randomly selected and cultured on PDA medium at room temperature 27 °C for 7 days. After that, scraping the surface of *B. oryzae* to prepare the conidial suspensions and washing in sterile water. The fungal suspension was filtered and adjusted to 5×10^4 using a hemocytometer for conidial suspension. Then, the pathogenicity test was carried out in the greenhouse. Forty two days old plants were rubbing using an autoclaved cotton bud with a conidial suspension of isolates with three replicates. Control seedlings were rubbing with distilled water containing distilled water. The infected leaves were observed after 7 days and data were recorded and scored using a rating of 0-9 [24].

E. Dual Culture Technique

Isolates of *B. oryzae* were placed at the circumference of an 8.5 cm diameter PDA culture plate for 7 days. After that, a 5 mm disc was taken from the edge of a 2 days old pure culture of each *Trichoderma* spp. Isolates and placed on the periphery on the opposite side of the same plate of PDA. *Bipolaris oryzae* isolates were cultured on PDA plates as a control.

The result was recorded for the mean of colony growth of the causal pathogen and in the presence of the antagonistic pathogen. Then, the results growth of *B. oryzae* on the control plate also recorded. The result from two readings was incorporated into the formula as below:

$$\%ofPIRG = \frac{R1 - R2}{R2}(100) \quad (1)$$

PIRG = percentage inhibition of radial growth
R1 = radial growth of *B. oryzae* in the absence of the antagonist (control)
R2 = radial growth of *B. oryzae* in the presence of the antagonist.

F. Preparation of Alginate Encapsulation of T. harzianum

Trichoderma harzianum were grown onto PDA medium for 1 week at 28 ◦C. Then, the agar surface submerged in sterile water was gently scraped with a cotton-tipped applicator for fungal suspension. To determine the number of viable conidia, dilutions of the spore suspensions were plated directly onto PDA medium. Conidial suspensions were used to prepare alginate-cellulose or without cellulose pellets.

10 g/l of sodium alginate was added to the fungal conidial suspension after adding the food base material (10% cellulose) in some cases under sterile conditions. Then, to allow a homogenous dissolution of the polysaccharides the mixture was vigorously stirred. After that, the mixture was extruded through sterile plastic nozzles with a diameter of 1 mm. Then, 6g/l $CaCl^2$ solution where gelation of sodium alginate into a calcium alginate matrix occurred. As a result, a small bead (2 mm, mean diameter) containing entrapped fungal conidia was obtained. Then, the beads were maintained in the $CaCl^2$ solution at room temperature for 1 to 2 hours. The $CaCl^2$ solution was pumped out and the beads are washed twice with sterilized distilled water. The fresh beads can be used directly or kept at 4–5 ◦C in sealed flasks for several days.

III. Results and Discussions

A. Morphological characterization and Pathogenicity tests

Macroscopic and microscopic features are observed for identification of *B. oryzae* isolates [23], [25]. In the present study, isolate was identified as *B. oryzae* based on morphological characteristics. *Bipolaris oryzae* in this study showed the upper and lower colony was grey colour (Fig. 1 and Fig. 2). The result was similar with study on the colony color upper and lower surface of *B. oryzae* were ranged in white to grey color [26]. The shape of conidia were oval-shaped with brown pigmentation and had one to three septations (Fig. 3). Conidia shape was similar to the shape [27]. All the characteristics were identical on the isolates that were classified in the first group which had grey to dark grey with curved conidia, golden brown and three septa [28].

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Fig. 1: Colony color on the upper surface

Fig. 2: Colony color on the lower surface

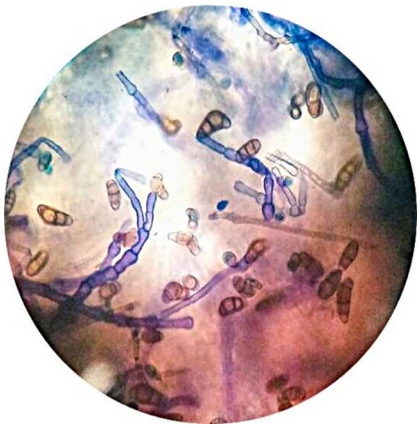

Fig. 3: Conidia

B. *Pathogenicity tests*

During the inoculation time, the age of the rice was 42 days (Fig. 4). After inoculation, data were collected for 7 days and observe the severity of the disease [24]. The disease severity showed lesion develop from day 3 until day 7 (Fig. 5). The percentage disease severity showed 15% with score 1 to 83% with score 5. The isolate was confirmed as *B. oryzae* which causal agent on brown spot disease by pathogenicity tests followed by Koch Postulates. According to [28], *B. oryzae* was the most virulent species compare than other species of *Bipolaris*

Fig. 4: The leaves before inoculation

Fig. 5: The leaves after inoculation on 7th day

C. *Dual culture test*

Result showed that *T. harzianum* exhibited antagonistic activities against *B. oryzae* isolates by highest percentage inhibition was 85.07% after 7 days. The mycelium of *T. harzianum* were found growing over the *B. oryzae* (Figure 6). *T. harzianum* has highest inhibition zone against *B. oryzae* compare the different species of *Aspergillus* and *Pencillium* [29]. *Trichoderma* spp. have three ways to inhibit the growth of fungus such as by competition to get space and nutrients, by production of an inhibitory metabolite or antibiotic and by parasitism to get nutrient from the host [30]. *Trichoderma* spp. have been reported can produce enzyme amylase which responsible for vigorously growth of *Trichoderma* spp. [31]. Besides amylase, *T. harzianum* also produce extracellular cellulose and pectinase enzyme which can hydrolytic the cell wall of other fungi [32]. *T. harzianum* was antifungal metabolites which can suppresses the growth of *B. oryzae* [30]. The interaction between *B. oryzae* and *T. harzianum* in dual culture test were shown in Table 1.

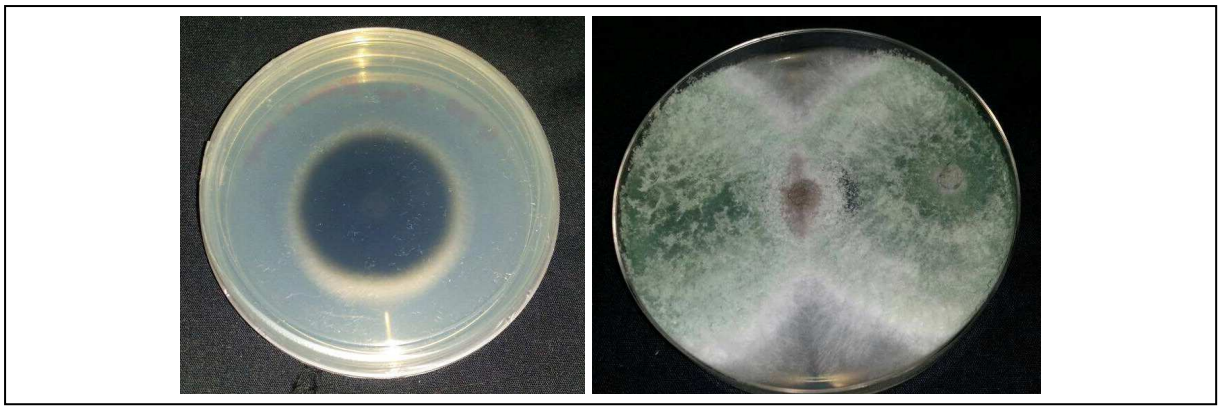

Fig. 6: Different Growth of *B. oryzae* and the Interaction between *T. harzianum* vs. *B. oryzae* after 7 Days

TABLE I
RESULT ON THE GROWTH OF B. ORYZAE AND THE INTERACTION BETWEEN T. HARZIANUM VS. B. ORYZAE AFTER 7 DAYS

Replication	Control (mm)	*T. harzianum* vs *B. oryzae* (mm)
R1	3.5	0.6
R2	3.5	0.5
R3	3.6	0.6
R4	3.6	0.5
R5	3.6	0.6
R6	3.6	0.5
R7	3.5	0.6
R8	3.5	0.4
R9	3.5	0.5
R10	3.6	0.5

D. Alginate encapsulation of T. harzianum

Trichoderma harzianum was encapsulated with alginate for further study on effectiveness towards rice (Fig. 7). Application of alginate encapsulation technology become attention among several investigators which in the form of containing spores, mycelia or mixtures of both [33]. Alginate composed of two repeating carboxylated monosaccharide units which are mannuronic and guluronic acids and the ratio depends on the properties of the biopolymer. Besides that, alginate also is biopolymers in the form of capsules which an active ingredient can be incorporated using an aqueous system at ambient temperature [33].

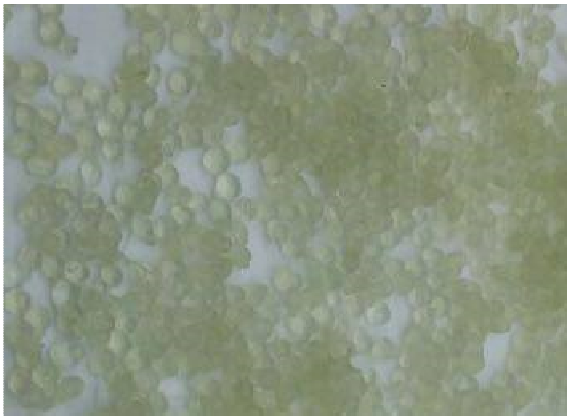

Fig. 7: Alginate encapsulation of *T. harzianum* in the form of beads

IV. CONCLUSIONS

The finding study showed an isolate was identified *as B. oryzae* which causal agent of brown spot disease on rice. Nowadays, the application of chemical control needs to reduce due to effect to environmental and health of human. Therefore, application of *algimate encapsulation* from *T. harzianum* was developed to control brown spot disease on rice. *Trichoderma harzianum* can be effective potential microorganism to against *B. oryzae* and can be commercialized for future use to reduce the application of fungicides which can give result in sustainable cropland usage in the future. Further field work is needed to determine the effectiveness of the alginate encapsulation under field conditions to consider them as potential bio-control agents as an alternative to chemical pesticides in the future.

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