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Efficiency of Locally Isolated *Trichoderma virens* for Controlling Rice Brown Leaf Spot Disease Caused by *Bipolaris oryzae*

Tikka Dewage Chamarika Priyadarshani[1*], Dinayaduru Dulanji Naveesha Karunarathna[1], Dirukshi Nadeeshani Menike[2], Prathiba Aruni Weerasinghe[1], Manawaduge Ayesha Madushani[1] and Kammala Waththe Asanka Madhushan[1]

[1]Department of Plant Sciences, Faculty of Agriculture, Rajarata University of Sri Lanka, Anuradhapura, Sri Lanka.
[2]National Institute of Post-Harvest Management, Anuradhapura, Sri Lanka.

Abstract

Rice brown leaf spot disease caused by *Bipolaris oryzae* is one of the major rice diseases in Sri Lanka and which effects the rising concerns of food safety. Biological control of brown spot disease is an alternative method for synthetic fungicide. *Trichoderma* is a widely studied bio-control agent and it is proven to be effective against a wide range of plant pathogens. In the present study a locally isolated *Trichoderma virens* was tested against the brown spot pathogen, *Bipolaris oryzae* in *in-vitro* conditions. Identity confirmation was done by using morphological methods and molecular through Polymerase Chain Reaction (PCR) with ITS 1 and ITS 4 primers and subsequent homology search. Antagonism of *T. virens* against *B. oryzae* was tested using dual culture technique. Locally isolated *Trichoderma virens* had an inhibition ability of 75 % in dual culture test. Finally, it is evident that *Trichoderma virens* can successfully inhibit growth of the pathogen under *in vitro* conditions.

Key Words: *Biological control*, *Polymerase Chain Reaction*, *Inhibition ability*

Introduction

RBLS caused by *Bipolaris oryzae* has been drastically impacting the rice production. Most of the farmers rely mainly on chemical control strategies to control RBLS disease, and there is an increasing inclination to reduce the use of chemical applications as it can have negative effects on the environment and human and animal health (Tariq et al., 2002). Therefore, in the new era of sustainable agriculture, a shift from chemical towards integrated methods have been promoted. Hence control of the pathogen through biological control is a good alternative approach to minimize the abusive use of chemicals.

Biological control is a mechanism in which the natural enemies diminish pathogens (Kamal et al., 2015; Pal and Gardener, 2006). Moreover, the introduction of micro-organisms into the phylloplane, the rhizosphere, or soil, stimulating indigenous antagonist, induced resistance, and bio-rational approaches are the main concepts in bio-control (Van Lenteren, 1988; IRRI knowledge Bank, 2020). Therefore, bio-control assumes special significance being an eco-friendly and cost-effective strategy that can be used in integration with other strategies for a greater level of protection with sustained rice yields (IRRI knowledge Bank, 2020). There are diverse groups of antagonistic micro-organisms such as bacteria and fungi that exist in nature.

Various species of fungi and bacteria have been proven effective as bio-control agents. Namely, *Pseudomonas, Serratia* and Bacillus-like bacterial species (Ganamanickam, 2009; Jaignesh et al., 2007; Harman, 2006; Nandakumar et al., 2001), *Trichoderma* spp. (De Franca et al., 2015; Khalil et al., 2011; Ganamanickam, 2009), *Aspergillu* spp., and *Penicillum* spp. (Kunyosying et al., 2018; Alam et al., 2011; Ade-bola and Amad, 2010). Moreover, antagonistic micro-organisms possess multiple beneficial characters such as competence in the rhizosphere, antagonistic potential, ability to produce antibiotics, lytic enzymes, and toxins (Mukherjee and Maheswari, 2018). According to the Verma et al., (2007), among all antagonistic micro-organisms, most studied members include those belonging to the genus *Trichoderma. Trichoderma* spp. grow in the rhizosphere and are capable of penetrating and internally colonizing plant roots. This symbiosis is driven by the ability of *Trichoderma* to derive sucrose or other nutrients from plants, in return for boosting plant immunity against invading pathogens as well as stimulating the growth of plants (Harman, 2006). *Trichoderma virens* in the rhizosphere is providing opportunities to plants by producing plant growth-promoting hormones (e.g. IAA). Accumulation of auxins or increased responses to auxins might lead to diverse outcomes on the plant side, such as enhancing root depth and root concentration; in turn, increased the water and nutrient uptake capacity of plants. Also, that promotes plant defense mechanisms (Contreras-Cornejo et al., 2009; Navarro et al., 2006). Nevertheless, *T. virens* is providing external protection to the plant as well by using its own mechanisms. *Trichoderma* gains more attention because of its effectiveness and ease of production for commercialization. *Trichoderma* species have been utilized to control rice diseases. For example, *T. viride* was used against rice

brown spot (Gomathinayajam et al., 2010), sheath blight (Mathivanan et al., 2005), and *T. harzianum* against brown spot (Khalil, 2011; Abdel- Fattah et al., 2007). Moreover, in the Sri Lankan context, *T. herinaceum* was used against *Rhizoctonia solani* (Herath et al., 2015). Therefore, this research was carried out with the objective of isolation and efficiency testing of locally isolated *Trichoderma* species against the RBLS disease.

Materials and Methods

This study was conducted from December 2019/2020 *Maha* season (wet season) including a field sample collection in the research field, Faculty of Agriculture (FoA), Rajarata University of Sri Lanka (RUSL).

Isolation and identification of rice brown spot pathogen using conventional methods
The brown spot-infected leaf samples were collected from the rice field maintained by the faculty for research purpose. Isolation and purification of the pathogen responsible for brown spot using the samples were done at the Plant Pathology Laboratory, Faculty of Agriculture, Rajarata University of Sri Lanka. Rice brown leaf spot infected leaves were surface sterilized using 1 % sodium hypochlorite solution and 70 % ethanol for 1 min. followed by a washing step with distilled water. Potato Dextrose Agar (PDA) medium was used for culturing the microorganisms. Surface sterilized samples were dried after placing them between two sterile filter papers. Then the samples were placed on PDA medium under aseptic conditions. Subsequently, the samples were incubated at 28 ± 1 °C for 72 h. Identification of fungi associated with brown spot lesions was done on the basis of spore size and shape, culture characteristics, other microscopic observations such as mycelial structure variations, types of conidia, and molecular techniques. Koch postulate was performed and confirmed the pathogen.

Molecular confirmation of the isolated rice brown spot pathogen
Fungal DNA extraction was done according to Mc Gravey and Kaper (1991) method with some modifications. Briefly, mycelia were scraped off from 7-day-old culture grown at room temperature and ground in the extraction buffer with the help of a pre-cooled motor and pestle. The macerate was mixed with 2 mL of homogenization buffer followed by incubation at 37 ºC for 15 min. After the incubation, 1 mL of extraction buffer, 300 µL of 20 % SDS and 0.5 % of mercaptoethanol (v/v) were added and mixed by inverting. Then the solution was incubated at 65 ºC for 1 h. Homogenate was extracted twice with the addition of equal volume from a mixture of phenol: chloroform: isoamyl alcohol (25:24:1 v/v/v). Phenol and aqueous phases were separated by centrifugation at 12000 rpm for 5 min. The aqueous phase was treated with RNase A (100 mg/mL) (Sigma Aldrich, USA) and Proteinase K (10mg / mL) (Sigma Aldrich, USA) and incubated for 15 min at 37 º C. DNA was extracted by using equal volume of chloroform: isoamyl alcohol (24 :1 v/v) and centrifuged at 12000 rpm for 5 min and precipitated by adding 0.6 x vol. of isopropyl alcohol followed by incubation on ice for 30 min. DNA was purified by re-precipitating with 2.5 x vol. ice-cold 70 % ethanol and 0.5 x vol. 7.5 M ammonium acetate (pH 7.7) and incubated at room temperature for 30 min. and centrifuged at 12000 rpm for 20 min. The final pellet was washed with 70 % ethanol, air-dried, and re-suspended in 15 µL TE buffer pH 7.5.
The resulted DNA was used for subsequent Polymerase Chain Reaction (PCR) assays using BIO-RAD My Cycler PCR machine with primers targeting the Internal Transcribed Spacer Region (ITS) of fungi. The nucleotide sequences of these spacer regions are often much more polymorphic between species than within species. Therefore, polymorphisms in ribosomal DNA (rDNA) and sequence data of the internal transcribed spacer 1 and 2 regions are widely used for differentiation of fungal pathogens. ITS1 (3' GCC GTA GGT GAA CCT GCG G 5') and ITS4 (3' GCC TCC GCT TAT TGA TAT GC 5') universal primer pair was used in the PCR (White et al., 1990). The PCR products were visualized by gel electrophoresis in a 1.5 % agarose gel and sent to Macrogen Inc. Korea for DNA sequencing. DNA sequence data of *B. oryzae*, generated from this study was subjected to homology search using the NCBI_BLAST software (National Center for Biotechnology Information – The Basic Local Alignment Search Tool) for the identification purpose and the sequence was deposited in the NCBI gene bank under the accession number of MZ618926.

Isolation and confirmation of locally isolated *Trichoderma* species
Soil samples were collected from various locations in the faculty farm premises. Old mushroom litter samples were collected from the mushroom unit in FoA, RUSL. Root samples of *Imperata cylindica* (*Illuk*) and *Megathyrsus maximus* (Guinea grass) were also collected from the particular field. All soil samples that were being taken using a soil auger of 2.5 cm diameter from the 0-30 cm top layer, were mixed to make a composite sample of around 0.5 kg. After placing the samples in sterilized plastic bags, they were brought to the laboratory and kept under cool condition. From each composite sample, 10 g of soil was weighed and mixed with 90 mL sterile distilled water and two drops of Tween 20. The mixture was vortexed (3500 RPM) and allowed to stand for 5 min to settle the soil particles. Serial dilutions were done up to 10^{-3} concentration. 100 µL from each dilution was plated onto PDA media. Plates were maintained at 28 ± 1 °C, for 6 days in dark and observed daily

(Montoya-Gonzalez et al., 2016). *Trichoderma* species was isolated from the old mushroom litter samples. Thirty-five grams from each of the fifteen samples of old litters were taken and mixed to make a composite sample of around 0.5 kg. Samples were cultured using the Serial Dilution Technique (Aneja, 2002). Dilution series were prepared up to 10^{-3} level in sterilized Petri plats and 1 mL from each dilution was cultured. Plates were maintained at 28 ± 1 °C for 6 days in darkness and observed daily. *Imperata cylindica* and *M. maximus* plants were uprooted and root sections of 0.5 kg were collected in plastic bags. Excess soil particles adhere to roots were removed by using distilled water. Subsequently, they were cut into small portions and placed in PDA plates. Plates were maintained at 28 ± 1°C up to 6 days in darkness and observed daily. Identification of antagonistic fungi was done on the basis of spore morphology and culture characteristics.

Molecular confirmation of the isolated *Trichoderma* species

Molecular identification was as same as described above procedure follow for the *B. oryzae*. The sequence was deposited in the NCBI gene bank under the accession number of MT256290.1.

Efficiency of *Trichoderma virens* against *Bipolaris oryzae*

Dual Culture Test (Dennis and Webster, 1971) was used to study the reduction in the growth of pathogens and inhibition zone formed due to the antagonistic activity of the bio-control agents**.** Five mm diameters gel plugs of the pure culture of each bio-control agent were placed on the PDA medium in opposite direction against pathogenic fungi. The plates were incubated at 28 ± 1°C, and the results were noted from the day after culturing until 6 days. The length of the mycelium growth was measured. Data were analyzed using proc mixed model in ANOVA followed by LSD mean separation by using SAS version 9.0 software.

Results and Discussion

Isolation and identification of brown spot pathogen using conventional methods

During the study after 72 h of incubation; initially, the molecularly confirmed *B. oryzae* colony appeared whitish in color. As the colony matures, it gradually turned grey to dark grey with a whitish margin. The fully matured culture was black in color (Figure 1). Similar observations have been made by Monisha et al. (2019) and Nayak and Hiremath (2019). Furthermore, the pathogen was microscopically confirmed by the presence of characteristic conidiophore that arises singly or groups, multi-septate, and brown in color. Conidia were curved or slightly curved; initially hyaline and later on maturity turns brown in color, fusiform with hilum at the base. Similar observations have been made by Monisha et al. (2019).

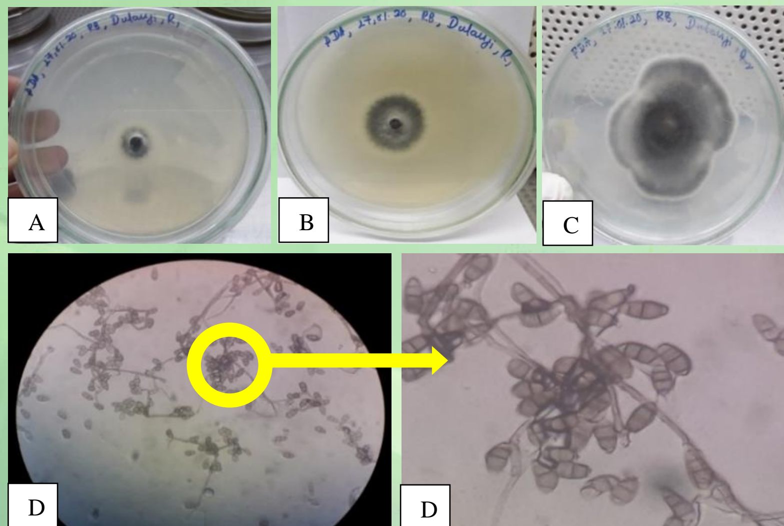

Figure 1: Macroscopic and microscopic features of *B. oryzae* (A) One-day old culture, (B) Three-days old culture, (C) Four-days old culture, (D) Three-celled, pyriform, hyaline conidia

Molecular confirmation of the isolated *Bipolaris oryzae*

Bipolaris oryzae used in this study gave 100 % maximum identity with a query cover of 100 % to the sample (MT446115.1) NCBI gene bank accessions.

Morphological basis confirmation of isolated *Trichoderma* species

The isolated three *Trichoderma* species appeared to be filamentous and white initially, turning into green to dark green colour. The growth was fast and it covered the plate just in 4 days. These observations were similar to the discription of Samuels (2006) and Druzhinia et al. (2006). Based on the microscopic observations, conidia were single-celled with hyaline hypea, globular shaped and greenish in colour (Figure 2). These characteristics compared well with the discription of USDA fungal database, reported by Samuels (2006) and Druzhinia et al.(2006).

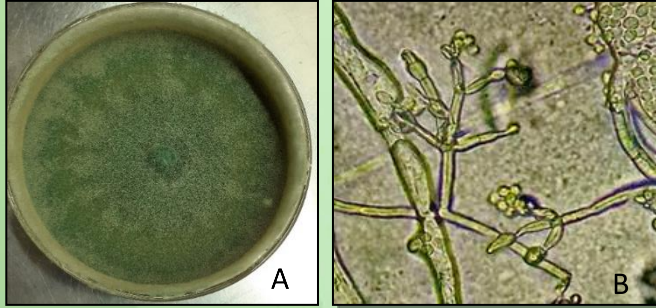

Figure 2: Macroscopic and microscopic features of *Trichoderma* spp. (A) Five-days old culture, (B) Microscopic structures

Molecular confirmation of the isolated *Trichoderma* species

Trichoderma isolate used in this study gave 100 % maximum identity with a query cover of 100 % to the sample (MT256290.1) NCBI gene bank accessions.

Efficiency of isolated antagonistic microorganisms against *Bipolaris oryzae*

Figure 3: Antagonistic ability of *Trichoderma* spp. against *B. Oryzae*

Trichoderma virens had an inhibition ability of 74.6 % (Figure 3). Ng et al. (2015) reported that *Trichoderma* spp. are soil-inhabiting microorganisms with a high degree of antagonism and mycoparasitic potential than other antagonistic agents. For instance, Mukherjee and Maheeshwari (2018) suggest that *Trichoderma* has a higher growth inhibition ability against some rice diseases such as Narrow brown leaf spot. Abdel-Fattah et al. (2007) and Khalili et al. (2011) found that *Trichoderma* spp. inhibit the growth of rice brown spot disease as well. Therefore, *Trichoderma* spp. will be a promising alternative treatment against *B. oryzae*.

Conclusions

The isolated pathogen associated with Rice brown leaf spot disease was morphologically and molecularly confirmed as *Bipolaris oryzae* (accession number MT256290.1). *Trichoderma virens* (accession number MT256290.1) was isolated from the local soil samples and molecularly confirmed. *Trichoderma virens* had an inhibition ability of 74.6 % in dual culture test.

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