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Identification and Parasitism of three fungal diseases of rice by *Trichoderma* spp.

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Abstract
The genus *Trichoderma* is characterized by having pustulate or tufted, repeatedly branched conidiophores. The phialides formed on the conidiophores, are lageniform and hyaline and the conidia are green in colour. This genus is widely used for the management of plant pathogens due to its unique biological properties. Mycoparasitism is one of the important mechanisms used by *Trichoderma* for disease control. In the current study, two isolates of *Trichoderma* were used to screen their mycoparasitic activity against the three pathogens affecting rice. All the three pathogens *viz*, *Pyricularia oryzae*, *Bipolaris oryzae* and *Rhizoctonia solani* were isolated from diseased plant tissues. It was revealed that both *Trichoderma* isolates could grow parallel to the hyphae of all the tested pathogens. The hyphae of *Trichoderma* coil around the mycelium and form appressoria and hook-like structure. Degeneration of pathogen hyphae was also reported by one of the isolates of *Trichoderma*.

Keywords: Mycoparasitism, *Trichoderma*, rice, blast, brown spot, sheath blight

Introduction
Rice (*Oryza sativa* L.) is the world's most widely consumed cereal crop and is a staple food for 50 per cent of the world's population (Pareja *et al*., 2011) [17]. More than 90 per cent of the world's rice is produced and consumed in Asian countries (Karthiba *et al*., 2010) [7]. China is the world's largest producer of rice (214.07 million tons) followed by India (172.58 million tons) (Anonymous, 2019) [3]. It has been reported that rice is affected by more than 70 diseases (Zhang *et al*., 2009) [29] and causes a yield loss of 16 per cent (Mondal *et al*. 2017) [14]. Among the diseases, Rice blast (*Pyricularia oryzae*), sheath blight (*Rhizoctonia solani* AG 1-1A), and brown spot (*Bipolaris oryzae*) are the most common rice diseases in India (Sharma and Thind, 2007) [23].
Generally, the diseases are managed by the use of fungicides however repeated use of chemical fungicides causes the development of resistance in the fungi, environmental contamination and also affects non-target microorganisms (Shabanamol and Jisha, 2014) [21]. The misuse of chemical pesticides may destabilise ecosystems and the infection of people and domestic animals by toxic residues can be harmful. As a result, biocontrol utilising microorganisms is an alternative management strategy. One important mechanism of a biocontrol agent is mycoparasitism. The occurrence of one fungus parasitizing another fungus, when the former is typically not pathogenic to plants, is known as mycoparasitism. This phenomenon offers a method for biological control. The idea of biological control of plant pathogens by mycoparasitism became a reality after it was discovered that *Trichoderma* spp. could parasitize various fungi in culture (Kubicek *et al*., 2011; Poromarto *et al*., 1998) [8, 22]. *Trichoderma* pers. Ex. Fr., a genus under Ascomycota, Sordariomycetes, Hypocreales and Hypocreaceae has gained immense importance in the last few decades due to its biological control ability against several plant pathogens. The researchers are interested in this genus because of its novel biological properties and biotechnological applications. Biocontrol mechanisms are likely to be specific for particular antagonists and plant pathogens and several mechanisms could operate independently or synergistically in any microbial interaction. *Trichoderma harzianum* is one efficient biocontrol agent that is commercially produced to present the development of several soil pathogenic fungi. Different mechanisms have been suggested as being responsible for their bio-control activity, which includes competition for space and nutrients, secretion of chitinolytic enzymes, mycoparasitism and production of

inhibitory compounds. *Trichoderma harzianum* is found to be capable of lysing mycelia of *Rhizoctonia solani*, *Pyricularia oryzae* and *Bipolaris oryzae* (Abdel-Fattah *et al*., 2007; Prabhakaran *et al*., 2015) [1, 18]. In the present study, three important pathogens of rice *viz; Pyricularia oryzae*, *Rhizoctonia solani* and *Bipolaris oryzae* were isolated and characterized morphologically. Thereafter, they were screened for parasitism by two isolates of *Trichoderma* by involving one of the important mechanisms of *Trichoderma*.

Materials and Methods

Isolation of pathogens used for mycoparasitism test

The heavily infected paddy leaves showing typical symptoms of Blast, Brown spot and Sheath blight were used for isolation of the pathogen using the standard technique as per Rajasekar *et al*. (2019) [19]. The leaves were cut into small pieces of three mm size and cut along the edges of the lesions using a sterilized scalpel. These pieces were surface sterilized with 1 per cent sodium hypochlorite solution for 30 seconds and washed 3-5 times repeatedly in sterile distilled water. The sterilized medium (20 ml of PDA) was poured into sterile Petri plates (9mm) and allowed to solidify. The surface-sterilized plant tissue bits were then placed individually at equidistance at the rate of 3 bits per plate. The plates were incubated (25±1 ºC) for 5 days and observed for fungal growth. Identification of cultures was done by microscopic examination based on their unique hyphal and conidial characteristics. The fungal culture was purified by the hyphal tip technique (Narayanasamy, 2010) [15] and maintained on potato dextrose agar at 4±1 ºC through further subculturing.

Pathogenicity test of isolated pathogens

Pathogenicity tests were performed under greenhouse conditions by the methods adopted by Bashar *et al*. (2010) [5]. More than fifteen sprouted seeds of susceptible variety *viz.*, china 1039 and Mushkbudji were sown in earthen pots. Finally, three plants were kept and inoculated with the pathogens. Fifteen-day-old plants were used for inoculation. The conidial suspension was prepared from the 14-day-old culture of *Pyricularia oryzae*, *Bipolaris oryzae* and *Rhizoctonia solani* by adding 10 ml of sterilized water to PDA plates containing the cultures of the pathogens. To remove the conidia, the culture was rubbed with a sterilized hair brush gently and the spore suspension was collected in a sterilized glass beaker. The suspension was adjusted to 10^5 conidia per millilitre of water with the help of a haemocytometer. The prepared conidial suspension was supplemented with 0.15% of Tween 20 and inoculated at the fourth leaf stage of the rice plant by spraying approximately at 0.2 ml plant^{-1}. The un-inoculated plants served as control. The plant pots were kept in the greenhouse. High humidity inside the chamber was maintained by spraying the leaves with sterilized water. The plants were constantly observed for 10 days to rule out any latent infection before inoculation. The symptoms developed were microscopically examined for the presence of conidia. The pathogenicity was confirmed after satisfying Koch's postulate.

Mycoparasitism test

Two isolates of *Trichoderma* sp. which were morphologically and visually dissimilar were obtained from the laboratory of the division of Plant Pathology, Faculty of Agriculture, Wadura and MRCFC Khudwani, SKUAST-K. Under aseptic conditions, PDA was poured into a 90 mm Petri dish up to 3-4 mm thickness which was roughly around the thickness of the glass slide. The plates were then allowed to solidify. On the solidified agar medium a sterilized glass slide was placed and pressed gently to leave an impression of the glass slide. Following that the slide was removed and the agar medium was cut with a sterilized blade according to the impression left by the glass slide that had previously been pressed on it, such that an agar strip was exactly comparable to the dimensions of the glass slide was removed from the Petri plate. A sterile glass slide is inserted into the hollow strip so that all of its sides touch the PDA and the upper surface of the glass slide is uniform with the surface of the PDA. Host fungus was inoculated at various spots on PDA on the borders of glass slides. The plate was incubated for 36 hours, allowing the host fungi to grow on both the medium and the glass slide. After the development of host mycelium growth on the slide margins, the bioagent *Trichoderma* sp was inoculated on the edges of the glass slide in the vicinity where previously host fungi were inoculated, and the Petri plate was incubated for another 24 hours. When the two fungi were visibly engaging, a sharp razor was carefully run all around the glass slide to cut all the mycelium mats between the slide and agar surface without disturbing the interacting fungal growth/mat on the glass slide. The slide has been carefully removed. The underside of the slide was cleaned with bloating paper. The slide was gently stained with cotton blue stain and examined under a microscope for hyphal interaction between the bioagent and host fungi (Bhat, 2017) [6]. Microscopic visualization of co-cultures in slide assay was performed to look for the mycoparasitic ability of *Trichoderma* spp. to degrade, coil and/or develop pegs around pathogens' hyphae. All microscopic observations were realized at 40x, 100x and 400x magnification.

Results

Isolation of associated pathogens

The causal fungi were isolated on potato dextrose agar (PDA) from the infected portion of paddy leaves collected from the experimental field, following the Standard pathological techniques. The white cottony growth appeared after two days on leaf bits after inoculation, which were transferred to PDA slants for further maintenance. The pathogenicity test of the causal pathogen was established on potted rice plants. Observation revealed the initiation of typical symptoms of the disease occurred after 7-10 days of inoculation in all the three diseases studied. In the case of Sheath blight symptom development occurs on the seventh day, while in the case of blast and brown spot, symptoms occurred on the tenth day of inoculation (Plate-1). Re-isolation from these artificially inoculated plants yielded the fungus that resembled initially isolated and inoculated fungus. The pathogenicity of the isolate was proved by satisfying Koch's postulates. The pure culture of the fungus was maintained on potato dextrose agar medium using the single spore technique in the case of *Pyricularia oryzae* and *Bipolaris oryzae* and the hyphal tip method in case of *Rhizoctonia solani*. The pure culture was further maintained by sub-culturing at monthly intervals and stored in a refrigerator for further studies (Plate-2).

Identification

The isolate proving pathogenic on rice plants was used to study its morphological characteristics. The morphological

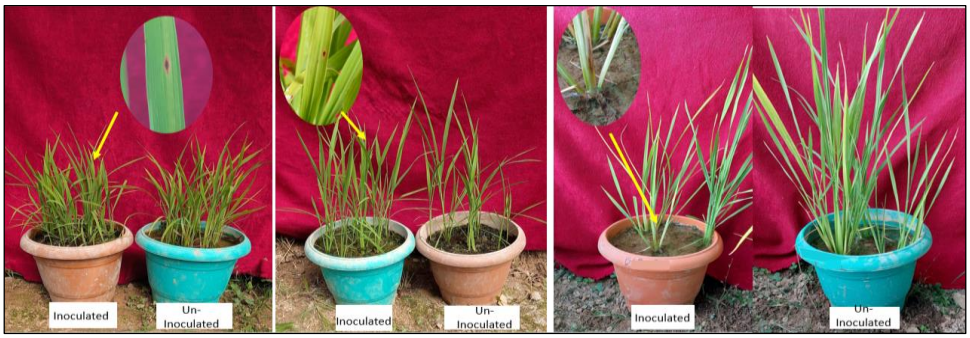
characteristics of the isolated pathogen infecting rice plants were studied from the culture grown on PDA for 5 days in case of *Rhizoctonia solani*, 10 days in case of *Bipolaris oryzae* and 20 days in case of *Pyricularia oryzae* at 25±1 ºC. Observations regarding morphological characteristics of different structures *viz*. mycelium and conidia were noted and are presented in Table 1, Plate 3. The cultural characteristics of the pathogen were studied. The Petri plates inoculated with fungal discs, obtained from a pure culture of the fungus, were critically observed for colony characters and growth behaviour. In case of *Rhizoctonia solani*, the pathogen covers the whole Petri plate of 9 mm within five days. The colony colour was initially white with hyaline mycelium but later on, the colour changed to brown due to the production of brown-coloured red sclerotia. The sclerotia begin to form after 5 days. In case of brown spot, the Petri plate was fully covered after ten days of inoculation. The colony colour was grey and mycelium appears fluffy. The sporulation of the pathogen occurs after seven days. In case of *P. oryzae*, the pathogen produced dark grey coloured colony which has smooth margins and whitish raised mycelium on PDA. The pathogen requires 20 days to cover the whole Petri plate. Sporulation occurs after 15 days of incubation.

Microscopic observations of the isolated fungus revealed that the mycelium was septate, with dark brown coloured hyphae when observed singly and irregularly branched in all the three pathogens studied. However, in case of *R. solani* the mycelium was branched at a right angle measuring 4.32µm-7.92µm in width with an average width of 5.21µm. In case of *B. oryzae* the mycelial width was 3.97-5.65µm, having average width of 4.77µm le as *P. oryzae* has mycelial width of 3.34µm -5.97µm with average width of 4.89µm. In case of *P. oryzae*, the conidia were septate having 2 septa, Pyriform in shape measuring 20.29-26.51×8.24-9.68µm (av. 22.35×9.05µm) and in case of *B. oryzae*, the conidia were septate having 6-12 septa. Conidia were Curved and obclavate in shape measuring 50.11-77×11.19-13.74µm (62.49×12.90µm). In case of *R. solani* the diameter of sclerotia varied from 0.3×0.7mm average diameter of 0.5 mm (Plate-3; Table 1).

The purified culture of the pathogen based on cultural and morphological characteristics and were identified as *Rhizoctonia solani*, *Bipolaris oryzae* and *Pyricularia oryzae* (Ou, 1985) [16].

Table 1: Morphological Characteristics of associated Pathogens

Pathogen	Colony Characteristics	Conidia shape	Spore size(µm)	Mycelial width(µm)
Pyricularia oryzae	Greyish black raised white mycelium	Pyriform, 2 septate	20.29-26.51×8.24-9.68 (av. 22.35×9.05)	3.34-5.97 (av. 4.89)
Bipolaris oryzae	Greyish with flat cottony mycelium	Curved, obclavate,6-12 septate	50.11-77×11.19-13.74 (av.62.49×12.90)	3.97-5.65 (av. 4.77)
Rhizoctonia solani	Initially white later on turn brown	Sclerotia: round	0.4-0.7 mm sclerotia (av.0.5mm)	4.32-7.92 (av. 5.21)



Pyricularia oryzae ***Bipolaris oryzae*** ***Rhizoctonia solani***

Plate 1: Pathogenicity test of *Pyricularia oryzae*, *Bipolaris oryzae* and *Rhizoctonia solani*

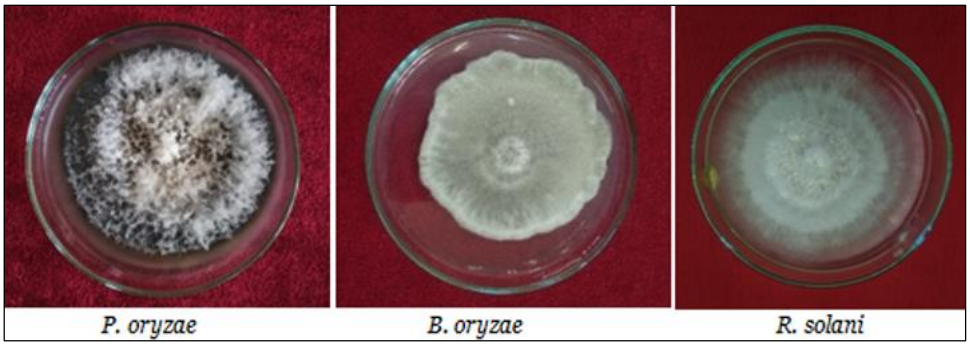


Plate 2: Cultural characteristics of the *Pyricularia oryzae*, *Bipolaris oryzae* and *Rhizoctonia solani* on PDA

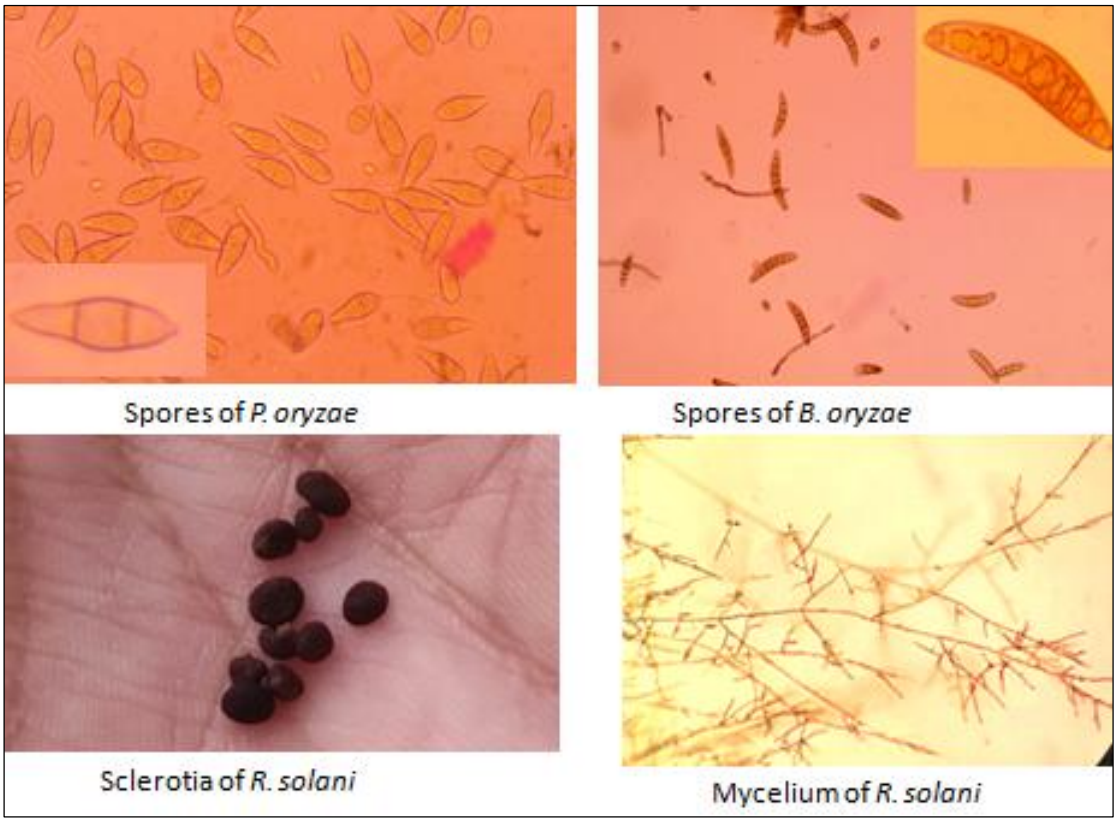


Plate 3: Morphological characteristics of *Pyricularia oryzae*, *Bipolaris oryzae* and *Rhizoctonia solani*

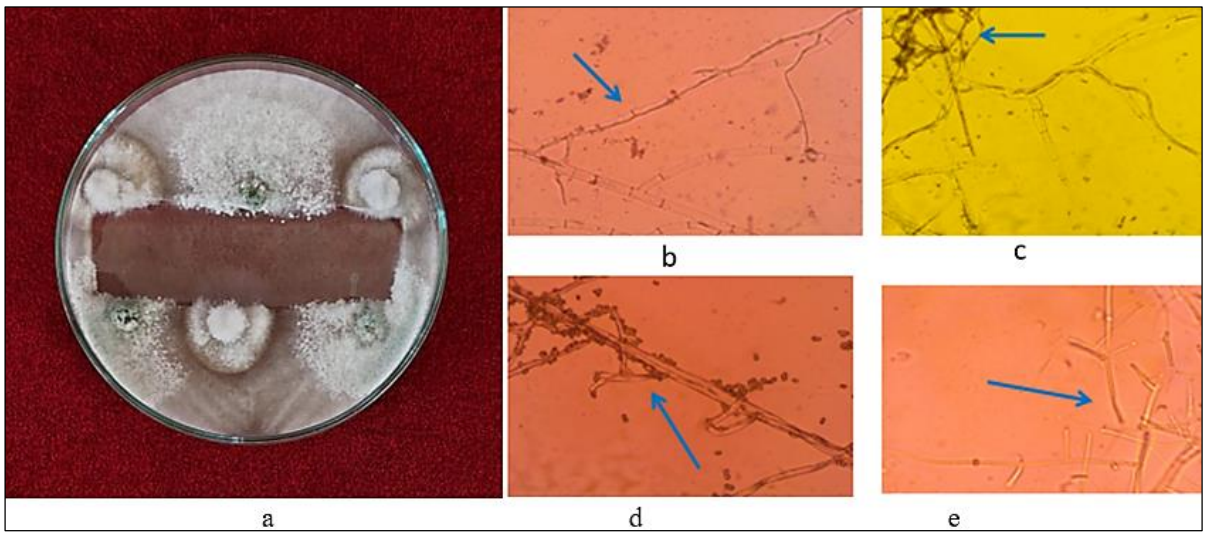
Mycoparasitism

Peg formation and coiling around pathogens hyphae

The slide culture technique was used to screen the *Trichoderma* isolates for Parasitism (Plate 4, a). The slides were gently removed after incubating them for 24 to 36 hours of bioagent inoculation. The slides were stained and directly observed under a microscope both with and without coverslip as well as at low and high power. Microscopic observation was investigated to detect mycoparasitic signs of *Trichoderma* species such as mycoparasitic coils around pathogens' hyphae and degradation of pathogens' mycelium. Both the *Trichoderma* spp. grew in close contact with all the three pathogens studied. Peg formation, coiling and disintegration of pathogen hyphae were also visualized. In case of *R. solani*, the *Trichoderma* spp. grew in close contact with occasional appressoria-like structures seen penetrating *R. solani* (Plate-4b). Further, the coiling by hyphae of both *Trichoderma* sp. was also observed (Plate 4. c, d). Both isolates of *Trichoderma* created tight windings along *R. solani* mycelia as demonstrated in plate 4 c, d and produce a high number of coils around. Similarly, peg formation and tight coiling of the mycelium of *P. oryzae* and *B. oryzae* were also observed by the hyphae of both *Trichoderma* isolates figure (4, f, g, h). It was also visualized that after peg formation the *Trichoderma* hyphae penetrate inside the pathogenic mycelium hence competing for nutrition.

Mycelium degradation

Mycelium degradation was observed only in case of *Trichoderma* 2 isolate for the mycelium of *R. solani* and *B. oryzae* (Plate 4 e, i), however, no mycelium degradation was reported in the case of *P. oryzae*.



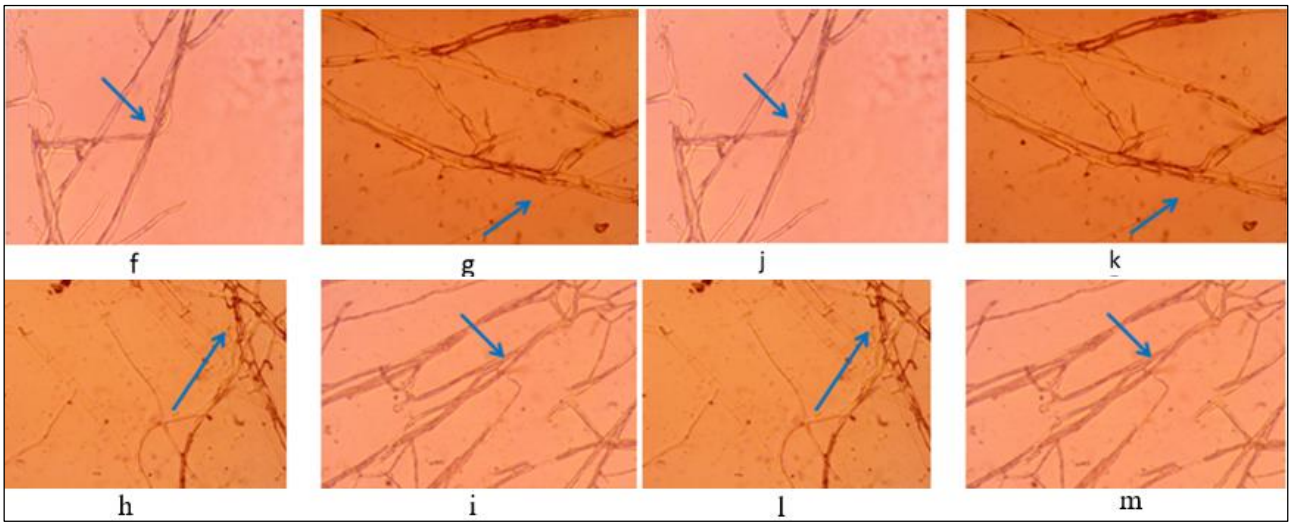


Plate 4: Coiling of mycelium of pathogens by *Trichoderma* hyphae (X400)

a: Slide culture for mycoparasitism of *Trichoderma* against *Pyricularia* mycelium.
b, c: Peg formation and coiling of mycelium of *Rhizoctonia solani* by *Trichoderma* isolate 1.
d: Peg formation and coiling of mycelium of *Rhizoctonia solani* by *Trichoderma* isolate 2.
e: Mycelium degradation of Trichoderma to the mycelium of *Rhizoctonia solani*.
f, g, h: Peg formation and coiling of mycelium of *Bipolaris oryzae* by *Trichoderma* hyphae.
i: Mycelium degradation of Trichoderma to the mycelium of *Bipolaris oryzae*.
j, k, l, m: Peg formation and coiling of mycelium of *Pyricularia oryzae* by *Trichoderma* hyphae.

Discussion and conclusion

In the current study, the infected disease samples of blast, brown spot and sheath blight were collected from fields and the associated pathogens were isolated. The identification of pathogens was done based on morphological characters. In case of *R. solani*, it was found that the mycelium was initially white which later on turn brown. The mycelium was observed under a light microscope and branching occur at a right angle. The morphological characteristics of this species were matched with the key characteristics described by Sneh *et al*. (1991) [26]. The Sclerotia on the field were black in colour. Our results are in agreement with San Aye *et al*. (2008) [20] who reported similar results. In the case of *B. oryzae* conidia were multi septate slightly curved and fusiform in shape. The colony was grey. Similar findings were also reported by Kumar *et al*., (2011) [9], Kumari *et al*., (2015) [10], Valarmathi and Ladhalakshmi (2018) [28] and Manamgoda *et al*., (2014) [11]. In *P. oryzae* the colony was dark grey in colour. The conidia were pyriform, having 1-2 septa. Mycelium was hyaline and branched. Similar results were also reported by Srivastava *et al*. (2014) [27] and Asfaha *et al*. (2015) [4].

Mycoparasitism is a mechanism that implicates mycoparasitic signals such as coiling and degradation. Perceiving signals, compounds and pathways inducing mycoparasitic potential in *Trichoderma* spp. are crucial to understanding how *Trichoderma* antagonists recognize, redirect and attach and consume the pathogen. Microscopic observation of the interaction region between *R. solani*, *B. oryzae* and *P. oryzae* with two isolates of Trichoderma showed that the mycelia of Trichoderma grew on the surface of the pathogens always coiling around their mycelia and later penetrating their cell walls directly without formation of appressorium structures. The pathogen mycelia then disintegrate suggesting enzyme action. Similar results were also reported by Shalini and Kotasthane (2007) [22] who also reported coiling and peg formation by Trichoderma on the mycelium of *R. solani*. Similarly, Mokhtari *et al.* (2018) [13] also reported mycoparasitism by three Trichoderma isolates against *R. solani* by coiling, peg formation and degradation. Ali *et al*., (2014) [2] also reported inhibition by coiling of *Pyricularia oryzae* mycelium by *Trichoderma* sp. Metcalf and Wilson (2001) [12] and Sharon *et al*. (2001) [24] demonstrated the possible role of chitinolytic and/or glucanase enzymes in biocontrol by Trichoderma. These enzymes function by breaking down the polysaccharides, chitin, and glucans that are responsible for the rigidity of fungal cell walls, thereby destroying cell wall integrity and limiting the growth of the pathogen. A mixture of several enzymes might be necessary for efficient cell wall lysis. *T. harzianum* has been reported to apply high β-1,3-glucanase activities (Sivan *et al*., 1984) [25]. Further research should be done to find novel, highly chitinolytic microorganisms which help in developing potential biocontrol agents.

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