MANAGEMENT OF BROWN SPOT DISEASE OF RICE USING FUNGAL ROOT ENDOPHYTE *Piriformospora indica* AND NEW GENERATION FUNGICIDES

by

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THESIS

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DECLARATION

I, hereby declare that this thesis entitled **"Management of brown spot disease of rice using fungal root endophyte *Piriformospora indica* and new generation fungicides"** is a bonafide record of research work done by me during the course of research and the thesis has not previously formed the basis for the award to me of any degree, diploma, associate ship, fellowship or other similar title, of any other University or Society.

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LIST OF ABBREVIATIONS AND SYMBOLS USED

%	Per cent
°C	Degree Celsius
CD	Critical difference
SE (m) ±	Standard error of mean
cm	Centimeter
g	Gram
ha	Hectares
µm	Micrometer
PDA	Potato dextrose agar
PNM	Plant nutrient medium
DTSA	Days taken for symptom appearence
DTCP	Days taken for complete coverage of petridish
Viz.,	Namely
et al.	And other co workers
h	Hour
sp.	Species
mM	Millimolar
CRD	Completely Randomized Design
DAI	Days after inoculation
DAC	Days after co-cultivation
MS medium	Murashige and Skoog medium
M	Molar
PDI	Per cent disease index
mm	Milli meter
PNM	Plant nutrient medium
mL	Milli litre

Introduction

1. INTRODUCTION

Rice (*Oryza sativa L.)*, a cereal grain is the most widely consumed staple food that belongs to genus *Oryza* of family Gramineae. Among the different cultivars of rice *viz.* Asian rice (*Oryza sativa),* African rice (*Oryza glabberima)* and wild rice *(Zizania* sp.), Asian rice is widely known with its subspecies Indica being more popular in India. Owing to its major role as global staple food, rice is grown in more than hundred countries with an approximate annual production of 700 million tons. Even though, efforts have been made for intensifying agricultural production through high yielding varieties and adequate production inputs, there is a sharp decline in cereal yields (Ray *et al.*, 2013) in recent years. The rising demand of rice due to population growth and urbanisation highlight the need for intensifying the production in future.

Pests and diseases are always a challenge for global food security (Flood, 2010; Schut *et al.*, 2014). An evident example for the same is the Great Bengal Famine of 1942-43 caused by *Helminthosporium oryzae* which took away the lives of two million people by starvation (Lenne, 2000). Rice cultivation is drastically affected by many biotic factors such as blast, sheath blight, brown spot, bacterial blight and rice tungro. Among this, brown leaf spot disease is a serious threat of rice cultivating tracts in every growing season worldwide. The diagnostic symptoms of the disease included brownish spots surrounded by yellow halo on various plant parts like coleoptile, leaf blade, leaf sheath, glume and grain. The relative yield loss of the disease ranged between 26-52 per cent (Chakrabarti, 2001) and about 90 per cent loss in grain yield had been reported where the brown spot acquired epiphytotic proportion as in Great Bengal famine of 1943.

H. oryzae (Breda De Haan) (syn. *Bipolaris oryzae / Drechslera oryzae)*, the causal organism of brown leaf spot was described for the first time in 1900. This hemi-biotrophic fungus belongs to the subdivision Pezizomycotina under the division Ascomycota. Ito and Kuribayashi

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(1927) observed the teleomorph of the fungus in culture and was termed initially as *Ophiobolus miyabeanus* which was later included in to *Cochliobolus* genus by Dastur in 1942. The crop suffers pathogen attack from seedling to milky stage and the disease is severe in an environment where water scarcity combines with nutritional imbalance. Being a seed borne disease, it has specific role in post-harvest damage and reduces the quality and market value of the produce. Loss in quantity as well as quality of the produce and the historic background of a leading role in the Great Bengal famine add great significance to the disease.

The management of brown leaf spot is a hot topic for research right from its reporting time till date. The present changing climate scenario and increase in the number of marginal farmers enhance the need for fruitful management strategies for brown leaf spot in Kerala. Though different approaches *viz.,* cultural, chemical, biological and resistant varieties are available variability within the pathogen created a barrier over the successful control of the pathogen. The present study seeks the role of a novel root endophytic fungus *Piriformospora indica* for the management of brown leaf spot disease of rice. *P. indica* has a wide host range of agriculturally important plants. It is a basidiomycetes fungus discovered from the Thar Desert of India and its isolation for the first time was done by Verma *et al.* (1998). Apart from its role in growth promotion and abiotic stress management in crop plants, exploration of its disease management potential has been less attempted. Hence, an effort has been made to evaluate conclusions regarding the efficacy of *P. indica* against *H. oryzae.*

Accounting other ways of disease management, use of fungicide is the easiest and reliable approach to combat diseases in commercial cultivation. Fungicide usage in rice has been recorded to be 8.4 per cent globally (Collines, 2007) and several fungicides have been proved to be effective against various rice fungal pathogens. Mancozeb, iprobenfos,

edifenphos, tetra methyl thiram disulphide, copper hydroxide, captafol, metalaxyl, copper oxy chloride, triadimefon and tridemorph are few among them which showed maximum inhibition of the pathogen growth (Gowda and Gowda, 1985; Vinaykumari *et al.*, 1997; Arshad *et al.*, 2013) under *in vitro* and *in vivo* conditions. However, complete control of the disease using chemicals is still not feasible. The frequent variations in the pathogen pose a disadvantage against the use of conventional fungicides and also for developing resistant varieties. Hence, there is a constant urge for widening the range of fungicides used for the management of fungal diseases as well as for the development and usage of fungicide combinations to prevent the development of resistant strains of the pathogen.

Triazoles and strobilurins are broad spectrum new generation fungicides which show higher efficacy at lower doses. Though literatures are available regarding the usage of azole fungicides for managing brown leaf spot, lesser is known about the utilization of strobilurins and their combination with azoles for the same. A few such recommendations are included in package of practice of KAU (KAU, 2017). It is high time to search for options of triazoles, strobilurins and their combinations for the management of brown leaf spot of rice.

In this context, the present study was undertaken for the management of brown leaf spot disease of rice using fungal root endophyte *P. indica* as well as the new generation fungicides *viz.*, triazoles, strobilurins and their combinations. The major objectives of the research work included,

- Survey and collection of brown leaf spot affected samples of rice in Kerala and the assessment of disease incidence and severity
- Virulence rating of the isolates
- *In vitro* evaluation of *P. indica*-primed rice seedlings against *H. oryzae*.

- *In vitro* evaluation of selected new generation fungicides *viz.*, azole, strobilurin and their combination fungicides against *H. oryzae*.
- *In vivo* evaluation of *P. indica*-primed rice seedlings against *H. oryzae*.
- *In vivo* evaluation of best azole, strobilurin and their combination fungicides against *H. oryzae*

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Review of Literature

2. REVIEW OF LITERATURE

Rice (*Oryza sativa* L.), is a versatile crop grown in both dry and wetland conditions. It is an edible cereal grain on which half of the world population is wholly dependent as staple food. The chief rice producing countries are China, India, Japan, Bangladesh, Indonesia, Thailand and Myanmar. Rice farming holds around 165 million hectares globally with an annual production of 758.8 MT (FAO, 2017) and thus ensures its critical role in the global food security system.

India is the largest producer of rice next to China. The country holds 21.2 per cent of world's total production followed by Indonesia (10.19 %) and Bangladesh (6.93 %) (FAOSTAT, 2018). Annual production of rice in India is around 275.68 MT. In India rice is grown in an area of 43.19 Mha. West Bengal is the largest producer of rice (15.09 MT), followed by Uttar Pradesh and Punjab. There is an increase of 4.5 % in total export value of rice in 2018 whereas export quantity of rice was 6770.83 thousand tonnes in 2017 (DAC&FW, 2017). More than 40,000 cultivated rice varieties exist in the world and India has 6000 varieties.

Rice is a whole grain consumed across the world. Its dietary benefits include reduction in the risk of heart diseases, weight maintenance and decreasing neutral tube defects as per the review of Dietary Guidelines Advisory Committee 2010. According to Marquez *et al.* (2009), rice consumers had less total and saturated fat, more iron, potassium and fiber compared to non-rice consumers. Fulgoni *et al.* (2010) reported that overall diet quality was better for rice consumers in terms of nutrients such as iron, folate and B vitamins. Kennedy *et al.* (2015) opined that rice consumption ensure significantly higher intake of energy and nutrients while less intake of calories and low body mass index.

Rice cultivation is heavily threatened by various abiotic and biotic stress. Diseases caused by fungi, bacteria, virus, insect pests and nematodes hinder large scale production of the crop in every growing season. Economically important diseases that are responsible for loss in quantity and quality of the produce can be

accounted as blast, bacterial blight, brown leaf spot, sheath blight and rice tungro virus diseases. According to Savary *et al.* (2012), sheath blight and brown leaf spot are the highest yield reducers compared to bacterial blight, leaf and neck blast in tropical Asia. Brown leaf spot, also known as orphan disease of rice seeks special attention since its incidence and spread is highly correlated with favourable climate, poor crop management, seed borne primary and secondary air borne inoculum. Epidemic nature of the disease is another trait that brings greater importance for research in brown leaf spot. Being a destructive disease of staple food crop with a back ground of Great Bengal famine that killed millions by starvation; there is a challenge for developing an effective management strategy by means of environmentally safe chemical and biological methods.

2.1 HISTORY, OCCURRENCE AND YIELD LOSS OF BROWN SPOT OF RICE

Brown leaf spot of rice was reported by Hori for the first time in Japan (1901). First report of this fungus in India was done by Sundararaman from Madras in 1922. Description of the causal organism as *Helminthosporium oryzae* was done by Breda de Haan. Later the fungus was transferred to *Drechslera oryzae* by Subramanian and Jain (1966). It was Shoemaker (1959), who observed the germination pattern of conidia from both the ends and referred it as *Bipolaris oryzae* (Baranwal *et al.,* 2013).

Teleomorph of the fungus was earlier termed as *Ophiobolus miyabeanus* (Ito and Kuribayashi, 1927) and its present name is *Cochliobolus miyabeanus* (Ito and Kuribayashi, 1927) Drechsler ex Dastur. *C. miyabeanus* belongs to kingdom-fungi, Division-Ascomycota, Class-Pleosporomycetes, Order-Pleosporales, Family-pleosporomycetaceae (Bockhavan, 2014). Though most investigations revealed that *Helminthosporium oryzae* (Breda De Haan) (syn. *Bipolaris oryzae / Drechslera oryzae)* is the causal organism of brown leaf spot, Motlagh and Kaviani (2008) reported *Bipolaris victoriae* as the pre-dominant species associated with brown spot followed *by B. oryzae, B. bicolor* and *B. indica* in Iran.

The global distribution pattern of brown spot of rice revealed that the disease was prevalent in most of the rice growing tracts including India, Japan, China, Burma, Srilanka, Bangladesh, Iran, Africa, South America, Russia, North America, Philippines, Saudi Arabia, Australia, Malaya and Thailand (Ou, 1985; Khalili *et al.*, 2012). As per Bengyella *et al.* (2017), the spatial and temporal distribution of brown spot of rice pointed out that 99 countries out of 196 countries on the globe had at least a first report of disease and a wide occurrence was reported from Indian sub-continent, China and Pacific countries. According to Gangopadhyay, 1983 and Ou, 1985 the disease is found in all rice growing states of India and it is of particular significance in states where dry/direct seeding is practiced (Sunder *et al.*, 2014).

Harish *et al.* (2007) surveyed 15 different rice cultivation tracts representing nine districts in Tamil Nadu. Fifteen pathogenic isolates were obtained from the survey. Isolate from Ammapetti of Thanjavur district was found to be the most virulent one with a percentage disease index (PDI) of 82.96 followed by Trichy isolate (80.74). Least virulence was shown by Cumbum isolate (20.00). As per Kamal and Mia (2009), 152 isolates of *H. oryzae* were obtained from a survey conducted in Bangladesh on the occurrence of brown spot in rice and were characterized through DNA fingerprinting.

Gupta *et al.* (2013) surveyed prominent rice growing areas of Jammu subtropics in 2011-2012 for disease distribution and severity. Accordingly, area under brown spot progress curve (AUBSPC) ranged between 3150 - 3560 in Jammu, 3025 - 3100 in Kathua, and 2921 - 31050 in Samba districts. Hassanein *et al.* (2018) conducted a survey in El-Dakahlia and El-Qaliubiya of Egypt for the collection of disease specimens during 2014. The highest (29) number of isolates were recorded from El-Qaliubiya governorate. The maximum disease severity was recorded from El-Qaliubiya (43.33) followed by El-Dakahlia (40). Nur *et al.* (2019) observed brown spot symptoms in the leaves during the survey conducted in 23 granary fields throughout Peninsular Malaysia in Kedah, Penang, Perak, and Selangor states in March 2013 to February 2017.The first report of *Bipolaris*

cactivora inciting brown leaf spot of rice was confirmed by this study through morphological and molecular characterisation.

Choudhary *et al.* (2019) conducted a survey in the rice belts of Punjab, Pakistan and gathered the information regarding brown spot incidence of 18 rice producing districts during 2014-2017. The disease was predominantly noticed on rice throughout the observation period. The result of the survey pointed out that maximum disease incidence was reported from Sargod (14.37 %) and minimum disease incidence in Okara (1.13 %). The average incidence of Brown leaf spot disease ranged from 6.50 to 8.75 % for the period of study.

Brown spot is a chronic disease of rice leading to severe yield reduction. Bedi and Gill (1960) found that there was a grain weight loss up to 4.28 – 29.00 per cent due to the disease. Ghose *et al.* (1960) described that the grain yield loss can reach up to 90 per cent when the disease acquires the epiphytotic proportion similar to Great Bengal famine (1942-43). Vidhyasekharan and Ramadoss (1973) reported the reduction in the tiller number; and inhibition of root and shoot elongation due to the disease. Chattopadhyay *et al.* (1975) estimated the yield loss between 26.2 - 51.8 per cent due to heavy grain infection which also depend on cultivars and the stage of infection. Yield loss data of a resistant cultivar, Bhasamanik during heavy infection in West Bengal was 2.2 per cent whereas, higher yield loss up to 11.7 per cent was recorded in Tilakkachery, a susceptible cultivar. It was Ou (1985) described about the disease severity and yield loss due to brown spot of rice. Accordingly severe infection led to the reduction in the number of tillers and grains along with poor grain quality. Yield loss of 30-43 per cent was recorded during severe infection, 12 per cent during moderate infection and negligible during lower infection. Average loss of 9.28 - 24.50 per cent in grain yield was reported from Bangladesh during 1995-1997 by Mia *et al.* (2001).

Savary *et al.* (2006) stated that the disease was responsible for an average yield loss of 10 per cent of the attainable crop yield in the lowlands of tropical and subtropical Asia. Kamal and Mia (2009) observed that reduction in yield due to brown spot of rice ranged from 18.75 to 22.50 per cent in Bangladesh. Mondal *et*

al. (2017) conducted a field experiment during 2015-16 on summer rice cultivar, IET 4786 following system of rice intensification (SRI). The results indicated that the loss in grain yield (15.60 %) and straw yield (12.35 %) was recorded due to brown spot. According to Poudel *et al.* (2019), there was a significant negative correlation between area under disease progress curve (AUDPC) value and grain yield (R^2 = 0.343) and test weight (R^2 = 0.080). The disease severity of brown spot was responsible for approximately 34.3 per cent reduction in the grain yield.

2.2 SYMPTOMATOLOGY OF BROWN SPOT DISEASE OF RICE

Economic importance of the disease is associated with Grain discolouration symptoms. Discolouration symptom starts at heading and continues till maturity, and is favoured by humid environmental conditions. Additionally, glume blight reduces grain quality and weight. At the time of epidemic years, glume blight reduced the grain weight from 29 to 45 per cent and head milling yield from 0 to 14 per cent; and the infected grain resulted in poor seedling emergence (Prabhu *et al.*, 2012).

Disease threatens the crop from seedling stage to harvest. Typical symptom of the disease is cylindrical to oval shaped minute spots of light to dark brown colour. Even though the spots are seen on the coleoptile, leaf blade, sheath, glumes and grains, the conspicuous site for the appearance of spots are leaves. Spots are of similar with sesame seeds hence the disease is often called as sesame leaf spot. Individual spots are often surrounded with a yellow halo (Baranwal *et al.*, 2013).

According to Dallagnol *et al.* (2015) first symptoms of brown leaf spot appeared on leaves within 18 h after inoculation. The brownish lesion development was accompanied with partially collapsed cell wall and intensely damaged cell membranes. Manandhar *et al.* (2016) observed symptoms on rice leaves, panicles, glumes and grains. Spots were small, circular, dark brown to purple-brown and fully developed lesions were circular to oval with a light brown to grey centre, surrounded by a reddish-brown margin which eventually killed the

leaf. Srinivasan *et al.* (2016) observed that brown coloured lesions on leaves reduced nutrient absorption and photosynthetic area which in turn resulted in the decrease of tillering nodes. Along with this, infection on plant parts like coleoptile, leaf sheath, panicle branches, glumes, and spikelet were also noted. Marco *et al.* (2017) observed small round to oval dark brown leaf spots; and larger lesions had dark brown margins with pale greyish centres. Quintana *et al.* (2017) described the symptomatology as seedling blight, leaf and culm infection, and kernel infection in rice cultivar IRGA 424. Leaf spot symptoms appeared throughout the growing season, mostly on leaf blade and sheath. Younger spots were of dark brown to reddish brown shades, circular to oval in shape, while older ones had a light, reddish-brown or grey centre surrounded by a dark to reddish-brown margin.

2.3 MORPHOLOGICAL AND CULTURAL CHARACTERIZATION OF *H. oryzae*

According to Ou, (1985) greyish brown to dark brown inter and intra-cellular mycelial mat of *H. oryzae* was visible in infected tissues whereas in culture, it was grey to olive or black in colour. The sporophores were thick, erect and geniculate. Base of sporophores were dark olivaceous and turned lighter towards tip in clusters of 3-5. The conidia was 5-10 septate and the oldest conidium was observed towards the base. Shapes of the conidia were slightly curved and maximum width was observed at the mid portion. Matured conidia were brownish in colour and showed bipolar germination. Dimension of conidiophores and conidia of isolates had been observed in a range of 68-688 x 7.6-20 µm and 15-132 x 10-26 µm in Japan; 70-175 x 5.6-7 µm and 45-106 x 14-17 µm in India; 99-345 x 7-11 µm and 24-122 x 7-23 µm in China, and 150-600 x 4-8 µm and 35-170 x 11-17 µm in USA.

Sonavane *et al.* (2015) made macroscopic studies on 42 sporulating isolates of *H. oryzae*. Isolates placed under group I were fluffy, cottony and with whitish grey aerial mycelium. Variation in radial growth of the isolates ranged from 2.2 cm to 8.45 cm. The isolates were sub-grouped based on the texture,

margin and form of the cultures as smooth, entire and circular (Sub-group A), smooth, undulate and irregular (Sub-group B), rough, undulate and irregular (Sub-group C). Isolates placed under group II were black in colour and texture was smooth. Radial growth ranged from 8.65 cm to 6.25 cm. The group II was further sub-grouped into smooth, undulate and irregular (Sub-group A) and smooth, entire and circular (Sub-group B). Microscopic studies of eight day-old cultures of the isolates revealed the variations in the conidial shape from fusoid, navicular, oblong, ovoid and curved to straight. Pale brown, olivaceous brown or golden brown conidia with either flat or prominent hilum were observed. The end cells of certain isolates were cut-off by dark septa. They noticed that all the isolates were germinating typically from one or both polar cells there was no germ tube development from intermediate cells.

As per Jaiganesh and Kannan (2019), the isolates of *H. oryzae* showed olivaceous, light brown to black, septate, profuse aerial/submerged and branched mycelium. Conidial colouration was brown to light brown. Shape was slightly curved with a middle bulge and tapering end. Conidial dimensions were noted as 29.3-33.2 µm.x 13.5-14.8 µm.

2.4 Pathogenicity Testing and Screening of Isolates

Variations in virulence and physiological functions are the properties of fungal pathogens which enables them for better survival in various environmental conditions (Narayanaswamy, 2011). Pathogenic variability is assessed by standardised methodologies. Shabana *et al.* (2008) tested the pathogenicity of 13 isolates of *H. oryzae* by spraying 21 day-old rice seedlings with the spore suspension (5×10^5 spore.ml^{-1}) of the pathogen of which the isolate B5 produced disease symptom within 48 h of inoculation. Highest disease severity was recorded with isolate B5 on cultivar Giza 177 hence concluded as the most aggressive isolate of *H. oryzae.*

Bockaven *et al.* (2015) evaluated the pathogenicity of *H. oryzae* isolate on rice cultivar Cm 988. Conidia were suspended in 0.5 per cent gelatine to a final

density of $1x10^4$ conidia ml^{-1}. Inoculation was done on leaves of five week-old plants by spraying conidial suspension (1 ml per plant) using a compressor-powered airbrush gun and kept in a humid and warm infection chamber. Symptom appearance was observed after 24 h of inoculation of pathogen.

Nazari *et al.* (2015) tested pathogenicity of 12 isolates of *H. oryzae* on local rice cultivar 'Tarom'. Thirty day-old plants were sprayed with conidial suspension of the isolates. First symptom in the form of pin head sized spots appeared 24 h after inoculation. 2 days later, the spots developed to oval necrotic lesions and on 7^{th} day, lesions coalesced to develop as leaf blight.

Amorio and Cumagun (2017) evaluated the pathogenicity of *H. oryzae* on one month old rice plants of variety IR-72. The pure culture of *H. oryzae* was maintained on V8-juice agar with alternating UV light (12 h) and dark (12 h) period to ensure faster sporulation. Spore suspension with 2-3 drops of Tween 20 for the adhesion of spores ($1.4x10^4$ conidia ml^{-1}) sprayed to the leaves. The most virulent isolate recorded a mean lesion density, mean lesion size and disease severity (4.22, 1.52 mm and 12.34 % respectively) on 3^{rd} day after inoculation (DAI).

2.5 EVALUATION OF *Piriformospora indica* AGAINST *H. oryzae*

P. indica is a novel basidiomycetous root endophytic fungus belonging to order Sebacinales. The fungus do not have host specificity, thus exhibit a large host range and its colonization is positively correlated with improved plant performances *viz.,* plant growth, abiotic and biotic stress tolerance, increased nutrient uptake, early flowering and enhanced seed production. Discovery of this axenically cultivable root endophytic fungus from the rhizosphere of xerophytic woody shrubs *viz., Prosopis juliflora* and *Zizyphus nummularia* in the Thar Desert of North-Western India (Verma *et al.,* 1998) opened a new era of research for the exploitation of its beneficiary aspects.

2.5.1 Root Colonisation of Rice var. Uma with *P. indica*

Serfling *et al.* (2007) reported that *P. indica* colonized and persisted in roots of wheat plants. Three weeks after colonisation, chlamydospores were observed in the root hair cells and epidermal layers. Mousavi *et al.* (2014) made the first report of root colonisation of *P. indica* on Iranian local rice cultivar 'Tarom'. It was found that the colonised rice roots contained round to pear shaped chlamydospores and was detected by staining. Johnson (2014) studied the cell biology of arabidopsis root colonised with *P. indica* and found that the fungus colonises every portion of root *viz.,* root tip, meristematic, elongation and maturation zones. Inter- and intra- cellur chlamydospores were observed 14 days of co-cultivation. Hajipour *et al.* (2015) reported that there was sufficient root colonisation of *P. indica* in 15 and 35 days old rice plants of local cultivar 'Tarom'. Microscopic analysis of colonised rice roots revealed that, the root colonisation of fungi was primarily through root hairs and later inter cellular growth was noticed.

Narayan *et al.* (2017) observed that *P. indica* enter into the chickpea roots within 5 to 7 days of co-cultivation. Further found that colonization of the roots depends on the size of the inoculum and duration of co-cultivation. In chick pea, minimum 7–9 per cent of colonization by the *P. indica* in the roots of chickpea plants in 7 days and maximum 65 per cent colonization after 30 days of co-cultivation was observed. Nassimi and Taheri (2017) studied the root colonisation of local rice cultivar 'Domsiah' with *P. indica* on 3, 7 and 14 days after inoculation. It was found that on three and seven days of post inoculation, no chlamydospores were detected in rice roots whereas on 14 days after inoculation, there were large number of chlamydospores in the root cells confirming the colonisation of *P. indica.* Root colonisation of *P. indica* in rice plants after 4 days of co-cultivation was reported by Saddique *et al.* (2018).

2.5.2 Dual culture assay of *P. indica* with *H. oryzae*

Dual culture assay is typically used to study the effect of one microorganism on another. Generally the observations vary from antagonism to mutualism. Antagonism is the interaction where the growth of one microbe is inhibited by the other. Mechanisms of antagonism includes antibiosis where, production of antimicrobial compounds for growth inhibition or inhibition zone where lytic enzymes are produced. Parasitic interaction involves coiling around the mycelium and choking, overgrowth and sporulation.

Ghahfarokhi and Goltapeh (2010) carried out a dual culture assay to study the antagonistic interaction between *P. indica* and *Gaeumannomyces graminis* var. *tritici,* the causal agent of take all disease of wheat. Potato dextrose agar, Kafer and their combination media were used for the study. The results indicated that. *P. indica* inhibited the mycelial growth of *G. graminis* var. *tritici* and there was noticeable inhibition zone in the dual culture plate. Microscopic study confirmed the antagonistic action by coiling of *P. indica* around the hyphae of *G. graminis* var. *tritici.*

Johnson *et al.* (2013) evaluated the interaction between *P. indica* and *Alternaria brassicae* through antagonistic assay. It was found that *P. indica* inhibits the growth of *A. brassicae* by the mechanisms of antibiosis and inhibition zone development. In addition to this overgrowth and sporulation were also noticed.

Sun *et al.* (2014) conducted a dual culture assay of *P. indica* and *Verticillium dahlia*. Antagonistic interaction of *P. indica* against *V. dahlia* was evident from the reduced mycelial growth of *V. dahlia* in dual culture plate. Whereas there was sufficient growth of *V. dahlia* in control plates. Apart from this the micro-sclerotia production by *V. dahlia* was lesser in dual culture plates compared to control. A clear cut inhibition zone was absent and there were no evidence for the influence of *V. dahlia* on the growth of *P. indica*. Nassimi and Taheri (2017) conducted an antagonistic assay between *P. indica* and *Rhizactonia*

solani, rice sheath blight pathogen. Five mm mycelial discs of *P. indica* and *R. solani* were placed on the sides of a PDA plate and the interaction was examined. But the study revealed that there is no direct antagonistic interaction between *P. indica* and *R. solani*.

2.5 3. *In vitro* evaluation of *P. indica* primed seedlings against *H. oryzae*

Knecht *et al.* (2010) pointed out the significance of *P. indica* conferred resistance to phytopathogenic fungi namely *Rhizoctonia solani* and *Verticillium longisporum* when inoculated into *Arabidopsis* seedlings. Johnson *et al.* (2013) reported that *P. indica* provides tolerance or resistance to *Alternaria brassicae*, a necrotrophic foliar pathogen infecting *Arabdiopsis thaliana*. *P. indica* colonisation on the epidermis and cortical tissues of roots referred as the priming of roots with the endophyte. The study revealed that priming of roots with *P. indica* and further challenge-inoculation of pathogenic fungi on leaves resulted in remarkable disease suppression in terms of reduced lesion number and size.

Sun *et al.* (2014) conducted an *in vitro* evaluation experiment for *P. indica* and *V. dahlia*. In the investigation, pre-exposure of the seedlings to *P. indica* prior to *V. dahlia* infection was carried out. Seedlings either not exposed to any of the two fungi or to one of them alone kept as control seedlings. On 14th day, seedlings infected by *V. dahlia* expressed intact disease symptoms as paler and browner seedlings, in comparison to the seedlings exposed to *P. indica*. On the 21st day, seedlings exposed to *V. dahlia* shown severe damage along with reduced fresh weights to the extent of no longer measurable. There was an average increase of 30 per cent biomass due to *P. indica* treatment alone. Surprisingly, *P. indica* pre-treated seedlings then exposed to *V. dahlia* had the same fresh weight increase. This was a clear proof for the *P. indica* protection to Arabidopsis seedlings against *V. dahlia* induced wilt.

2.5 4. *In vivo* evaluation of *P. indica*-primed seedlings against *H. oryzae*

Waller *et al.* (2005) identified the potential of *P. indica* to induce resistance to fungal diseases of barley initially under controlled growth conditions

later in field. Infection of *Fusarium culmorum* caused 12-fold decrease in root and shoot fresh weight in 1 month old barley plants. This devastating effect of pathogen was effectively diminished by the root colonization of *P. indica*. Similar results were obtained in case of *Cochliobolus sativus* infection in barley. Additionally, efforts were made to determine the contribution of *P. indica* colonisation to systemic disease resistance to barley plants. The potential of *P. indica* against foliar pathogen *Blumeria graminis* f. sp. *hordei* was evaluated. Results revealed that there was notable reduction in disease severity, by means of reduced powdery mildew colony number. Succeeding microscopic studies gave further insight to the mechanism of disease management by *P. indica* in the form increased frequency of cell death and activated cell wall mediated plant defence in response to the pathogen.

Serfling *et al.* (2007) reported the effect of *P. indica* against various root and foliar diseases of winter wheat. Pathogens in the evaluation included *B. graminis* f. sp. *tritici*, *Pseudocercosporella herpotrichoides* and *F. culmorum*. The study revealed that negative impact of the pathogen was hindered by the root endophyte in a larger extent. The reduction in biomass of the crop due to the pathogenic stress was effectively compensated by root colonization of *P. indica*. It was noted that significant control was lacking over the leaf pathogen (*B. graminis* f. sp. *Tritici*) under the field environment. However, in field *P. herpotrichoides* disease severity was evidently reduced in *P. indica* colonized plants. Role of *P. indica* in induction of systemic resistance was explained by increased numbers of sheath layers and hydrogen peroxide concentrations after challenging with pathogens in colonized plants, suggesting *P. indica* is more suitable for tropical or subtropical farming.

According to Kumar *et al.* (2009) *Fusarium verticilloides* infection in maize roots was effectively reduced by the protective action of *P. indica.* Fakhro *et al.* (2010) investigated the impact of *P. indica* against *V. dahliae* causing verticillium wilt in tomato and Pepino mosaic virus (PepMV). It was concluded that the tomato plants colonized with *P. indica* effectively reduced the stunted

growth, yellowing and wilting symptoms of *V. dahlia* (30 % disease suppression over control). Moreover, there was a visible increase in fresh weight and dry matter content of colonized plants compared to control. Regarding the PepMV tolerance, the viral symptoms was present throughout the study. But the concentration of the virus reduced over time. As claimed by Mousavi *et al.* (2014) that rice blast fungus *Magnaporthe oryzae* was efficiently controlled by the root endophyte *P. indica*. Assessment of the disease severity was done by means of nature of infection, leaf area affected and number of lesions. Results revealed that *P. indica* treated plants had less severe disease symptoms. In addition, expression of defence related genes quantified through Real-time PCR concluded that, beneficiary role of *P. indica* in rice against blast disease was due to boosted expression of defence related genes.

Hajipour *et al.* (2015) reported the pre-inoculation of rice plants with *P. indica* offered effective control over *Fusarium proliferatum*, the causal agent of root rot and crown rot (Bakanae) disease. It was noticed that *F. proliferatum* infected plants expressed the symptoms such as abnormal growth, chlorotic, thin and brownish leaves and reduced seedling health. However, in plants colonized by *P. indica* the symptoms were observed in delay of two weeks compared to non-colonized plant with reduced or no disease and continued healthy during the experimental period. Lakshmipriya *et al.* (2017) explored the potential of *P. indica* against taro leaf blight pathogen *Phytophthora colocasiae*. At different stages of infection a significant reduction of disease severity (50.7, 57.6 and 84.3 % over control) was recorded in *P. indica* colonised taro plants upon infection with *P. colocasiae*.

Narayan *et al.* (2017) reported that the colonisation of *P. indica* in chick pea conferred tolerance against *Botrytis cinera*. It was reported that there were more number of secondary roots and increased crown root length in pre-colonised chick plants at 10 days after inoculation with *B. cinera* in comparison to plants inoculated with *B. cinera* alone. Nassimi and Taheri (2017) illustrated the potential of *P. indica* against *R. solani,* sheath blight pathogen of rice. Infection of

R. solani after 0, 7, and 14 days from pre-inoculation with *P. indica* revealed that best time interval for inoculation of the pathogenic fungus was 14 days. Rice plants pre-inoculated with *P. indica* when challenged with *R. solani* after 14 days, there was a considerable decrease in disease development as compared to plants challenged with pathogen alone. Also there was notable increase in fresh and dry weight of root and shoot in colonized plants.

Athira (2018) evaluated the effect of *P. indica* against *Ralstonia solanacearum* inciting bacterial wilt of tomato. There was around 80 per cent disease suppression of bacterial wilt in *P. indica* treated plants over control. Lin *et al.* (2019) reported the potential of *P. indica* in growth promotion and bacterial wilt tolerance of *Anthurium andraeanum*. *P. indica* promoted the anthurium rooting and shortened the recovery period of anthurium. Panda *et al.* (2019) demonstrated that the pre-colonisation of tomato roots with *P. indica* systemically induced resistance against *Alternaria solani* – the early blight pathogen.

2.6 EVALUATION OF NEW GENERATION FUNGICIDES AGAINST *H. oryzae*

2.6.1 *In vitro* evaluation of new generation fungicides against *H. oryzae*

Fungicide application remains one of the easiest and best means to mitigate the losses due to diseases in commercial cultivation. Though several experiments were carried out to study the inhibitory action of fungicides against brown spot pathogen, literatures on the action of new generation fungicides *viz.* triazoles, strobilurins and their combinations for the same are mentioned here.

Ahmed *et al.* (2002) investigated the effect of different concentrations (50, 100, 250 and 500 ppm) of carbendazim, edifenphos, mancozeb and propiconazole against *H. oryzae* and found that maximum inhibition (95.58 %) of the mycelial growth of the pathogen was shown by propiconazole (Tilt 250 EC) at 500 ppm. Sunder *et al.* (2005) observed that hexaconazole (0.11 ppm) and propiconazole (0.42 ppm) were inhibitory to the mycelial growth of *H. oryzae in vitro*. Hunjan *et al.* (2011) evaluated five fungicides *viz.* trifloxystrobin + tebuconazole,

tebuconazole, propiconazole, pencycuron and thifluzamide *in vitro* against the pathogen causing brown leaf spot in rice and the results revealed that trifloxystrobin + tebuconazole (0.04 %) was the best followed by tebuconazole (0.1 %) and propiconazole (0.1 %) while thifluzamide (0.1 %) was the least effective.

Mahapatra and Hemayathullah (2014) tested the efficacy of difenoconazole, mancozeb, propiconazole, azoxystrobin, chlorothalonil, kasugamycin, carbendazim, mancozeb and hexaconazole + zineb against *H. oryzae* by poisoned food technique. The highest per cent inhibition of mycelial growth (87.49) was shown by propiconazole (0.1 %) and the lowest (34.72 %) by azoxystrobin (0.1 %) among the tested fungicides.

According to Pandey (2015), among the fungicides tested *in vitro* against *H. oryzae viz.* carbendazim, edifenphos, aureofungin, hexaconazole and propineb at 1000 and 1500 ppm, carbendazim (1000 ppm) appeared to be the most effective with lowest (0.5 cm) radial mycelial growth five days after incubation while hexaconazole (1000 ppm) was the least effective (1.6 cm). Mahmud and Hossain (2017) reported that propiconazole (0.1%) seemed to be the best among the tested fungicides for the inhibition of mycelial growth of *H. oryzae*.

In vitro evaluation of thiophanate, myclobutanil, carbendazim, propineb and propiconazole against *H. oryzae* revealed that all the fungicides tested significantly reduced the mycelial growth of the fungus six days after incubation (Kamei and Simon, 2018). The results also indicated that the maximum inhibition (73.04 %) of radial growth (2.73 cm) of mycelium over control was shown by propiconazole followed by propineb (74.26 % and 2.86 cm) and mycobutanil (51.83 % and 5.11 cm).

2.6.2 *In vivo* evaluation of new generation fungicides against *H. oryzae*

According to Thind *et al.* (2004) among the eight fungicides namely propiconazole, carbendazim, bitertanol, hexaconazole, zineb, iprobenphos, carbendazim + Mancozeb and validamycin; propiconazole 25 EC @ 0.1 per cent

was found to be the most effective fungicide against brown spot, with a lowest mean disease severity (9.0 %) compared to untreated check (48.5 %) whereas, hexaconazole 5 EC @ 0.1 per cent was not much effective for the brown spot (33.20 %). Celmer *et al.* (2007) evaluated the effect of thiophanate methyl, tebuconazole, azoxystrobin and trifloxystrobin + propiconazole, against brown spot pathogen. Lore *et al.* 2007 reported that propiconazole as the most effective fungicide against brown spot with 75.3 per cent disease control followed by hexaconazole.

Sunder *et al.* (2010) evaluated fungicides *viz.,* propiconazole, hexaconazole, azoxystrobin, iprabenphos, mancozeb and kasugamycin; and concluded that all the six fungicides significantly reduced leaf spot and stalk rot phases of the infection. Among the fungicides, propiconazole (0.1 %) and hexaconazole (0.2 %) significantly reduced leaf spot severity to 76.8 and 64.2; and stalk rot incidence to 49.1 and 41.2 respectively over control. Kumar and Kumar (2012) evaluated the effect of Captan 70% + Hexaconazole 5% WP (0.2%) and found to be effective for managing brown leaf spot of rice.

Akter *et al.* (2013) conducted a field study to evaluate the efficacy of the selected fungicides *viz.*, iprodione, tebuconazole and hexaconazole against brown spot disease in rice. The Percentage of unfilled grain varied from 4.4 to 14.1 per cent. The lowest unfilled grain percentage was found in plants treated with hexaconazole (13.6 %) followed by tebuconazole (14.2 %). Application of tebuconazole reduced the percentage of spotted grain (15.8 %) compared to control (23.6 %). Divya (2015) investigated the effectiveness of different fungicides against brown leaf spot severity. Propiconazole 0.1 per cent (18.51), hexaconazole 0.2 per cent (20.48) and tricyclazole 0.06 per cent (22.99) significantly reduced brown spot severity compared to unsprayed check. The same trend was observed for the reduction of grain discolouration symptom. Number of grains per panicle and grain yield did not differ significantly with fungicidal treatments, as the fungicidal treatments were carried out only after

panicle initiation. However, highest grain yield (39.58 q ha -1) was observed in hexaconazole treated plots.

Kamei and Simon (2018) analysed fungicides *viz.,* thiophanate, myclobutanil, carbendazim, propineb and propiconazole consecutively for two crop seasons (2014-15 and 2015-16) and the results revealed that all selected fungicides led to significant reduction on disease incidence. However, among the treatments highest per cent reduction over control was recorded in propiconazole (72.39 and 72.12) followed by propineb (69.40 and 70.83). Qudsia *et al.* (2017) conducted field trials for the evaluation of different fungicides *viz.*, azoxystrobin + difenconazonle, tebuconazole + trifloxystrobin, difenoconazole, difenoconazole + propiconazole, sulfur and copper hydroxide during kharif season of 2016. The results revealed that all the six fungicides had efficacy on the brown leaf spot compared to control (61.33 %). Among the fungicides, sulfur showed the best result with minimum disease incidence (10 %) followed by fungicides azoxystrobin (14.66 %), tebuconazole + trifloxystrobin (15.66 %) and difenoconazole (17.66 %). However, copper hydroxide was least effective to control the brown leaf spot and had 22.33 per cent disease incidence.

Poudel *et al.* (2019) carried out a field experiment to evaluate the efficacy of different chemical fungicides *viz.,* propiconazole, hexaconazole, carbendazim, Mancozeb, carbendazim + mancozeb, azoxystrobin + tebuconazole against brown leaf spot of rice in completely Randomized Block Design. The area under disease progressive curve (AUDPC) value was calculated on the basis of the disease severity recorded 85, 92, 99, 106 and 113 days after treatment. Significantly lowest AUDPC value was observed in the field treated with the propicanazole (415.7) which was statistically on par with azoxystrobin + tebuconazole. (464.3) followed by carbendazim + mancozeb (464.3). The control plants expressed maximum AUDPC value (723.40). Plants treated with propiconazole recorded highest grain yield (4.28 t ha^{-1}) followed by azoxystrobin + tebuconazole (4.12 t ha^{-1}).

Materials and Methods

3. MATERIALS AND METHODS

The location of current study entitled 'Management of brown spot disease of rice using fungal root endophyte *Piriformospora indica* and new generation fungicides' was Department of Plant Pathology, College of Agriculture, Vellayani during the period 2017-19.

3.1. SURVEY ON INCIDENCE AND SEVERITY DUE TO BROWN SPOT OF RICE

A survey was conducted at six different locations of Kerala with an aim to collect *Helminthosporium oryzae* isolates. Disease incidence and severity of brown spot of rice during season of 2018-19 was also collected. Different research stations of KAU such as College of Agriculture (COA) Vellayani, IFSRS Karamana, RARS, Kumarakom, RRS Moncombu, RRS Vytilla and RARS, Pattambi were the survey locations. From the respective locations, diseased fields were identified and from each field random selection of 100 plants were done for calculating disease incidence. Disease incidence was recorded on the basis of number of healthy and diseased leaves. The prevalence of the disease in an area was expressed in terms of per cent infected plants in the host population. Incidence of brown spot of rice was calculated using the formula by (Wheeler, 1969).

$$\text{Per cent disease incidence} = \frac{\text{Number of infected leaves}}{\text{Total number of leaves observed}} \times 100$$

The disease severity was calculated by assessing 20 infected plants and 25 leaves from each plant. The calculation of per cent disease index (PDI) based on a standard score chart was followed to determine the diseases severity. According to the percentage leaf area infected different grades were allocated, where the whole

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leaf area was considered as 100 per cent. A zero to nine disease scale developed by IRRI (2002) was used as standard score chart (Table 1).

PDI calculation was based on the formula (Mc Kinney, 1923).

$$PDI = \frac{\text{Sum of all disease ratings}}{\text{Total no. of leaves observed} \times \text{Maximum disease grade}} \times 100$$

Table 1. Score chart for assessing the severity of brown spot of rice caused by *H. oryzae*

Grade	Description
0	No incidence
1	Less than 1% leaf area affected
2	1-3 % leaf area affected
3	4- 5 % leaf area affected
4	6-10 % leaf area affected
5	11-15% leaf area affected
6	16-25% leaf area affected
7	26-50% leaf area affected
8	51-75% leaf area affected
9	76-100% leaf area affected

3.2. ISOLATION OF THE PATHOGEN

Collection and labelling of diseased specimens from the surveyed locations (Thiruvananthapuram, Moncombu, Vytilla and Pattambi) was carried

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out for the isolation of the pathogen. For the isolation of pathogen, a leaf bit with fresh infection along with a portion of healthy tissue was chosen. Surface sterilization of the leaf bit using 0.1 per cent mercuric chloride for 30 seconds followed by two subsequent washing with sterile water was carried out next to that. Further the excess moisture from the tissue bits were removed with sterile tissue paper. The tissue bits were then placed on sterilized potato dextrose agar medium (PDA) in sterile petri plates. The fresh mycelial strands, from the plates after 3 days of incubation at room temperature (28±2°C) were transferred to PDA slants and also to another set of petri plates as per the procedure of Biswas *et al.*, (2010). Same procedure was repeated to get pure culture of *H. oryzae.*

3.2.1. Maintenance of Culture

Monthly sub culturing of isolates to the sterile PDA slants were done in order to maintain the viability. The cultured slants allowed attain the growth under room temperature for seven days. Later it was stored at 4°C in refrigerator (Vidhyasekaran *et al*, 1992).Periodic inoculation of the fungus to host plant and subsequent re-isolation was performed to maintain the culture virulence.

3.3. PATHOGENICITY TEST

Leaf inoculation method was performed to test the pathogenicity of all the five obtained isolates. Five week old plants of a susceptible variety 'Uma' grown under greenhouse condition were inoculated with *H.oryzae*. Prior to making the injury, proper washing of the leaves with sterile water and air drying of washed area was done. Pinpricking was followed for making injury to the leaves using sterile needle. *H. oryzae* mycelial disc of dimension 5 mm was kept over the injured area. A thin layer of moistened cotton kept over the inoculated area. Pinpricked leaves with plain agar disc without served as the control. The inoculated plants were labeled and kept inside polypropylene bags, to maintain humidity (Vidhyasekaran *et al.,* 1990). The inoculated plants were placed for symptom development and periodic observations were taken. After the characteristic symptom establishment, re isolation of the pathogen from the

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artificially inoculated leaves was performed. Comparison of the obtained cultures with original cultures was done to prove Koch's postulates. Appropriate maintenance of all the isolates were done for further studies.

3.4. SCREENING OF VIRULENT ISOLATE OF *H. oryzae*

Detached leaf assay was performed in 5 week old leaves of variety 'Uma' for the experiment. Sterile conical flasks with wide mouth were taken and the bases of the flasks were provided with layers of wet cotton. Detached leaves were washed and pat dried, and then the leaves were injured with a sterile needle. Over the injured area disc culture of *H. oryzae* was placed next to this covering of the inoculated area with a thin layer of wet cotton was also performed. After the successful inoculation the entire system was placed under poly propylene bag to ensure adequate humidity. Plants are observed for symptom development and after the symptom appearance; observations on lesion size and PDI were recorded on 3rd, 5th, 7th, 10th and 15th day of inoculation. PDI was calculated based on a score chart developed by IRRI (2002).

3.5. CHARACTERIZATION OF PATHOGEN CAUSING BROWN SPOT OF RICE

The cultural and conidial characteristics of individual isolates were performed to draw the conclusions regarding the morphological variation among the isolates. For the same colour and appearance of colony, colour and size of mycelium, conidial size and shape of all the five isolates were studied.

3.5.1. Cultural Characterization

Sterile petri plates containing PDA media were used for growing the fresh culture of all the five isolates. 5mm mycelial disc of 7 days old culture of individual isolates were kept at the centre of petri plates and incubated at (28 ± 2°C) temperature for 7 days. There were three replications for each isolate. Observation on mycelial growth was noted on 3rd, 5th, 7th, 10th and 15th day. Growth rate was calculated as average of mean daily growth in cm per day for

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seven days. Observations on appearance of mycelial growth, colour, zonation and sporulation of the isolates were recorded.

For the microscopic studies, slide culture technique was followed. A petri plate with blotter paper at the base was provided with glass rods, glass slide and coverslips. After sterilizing the unit, the blotter paper was moistened with sterile water and glass slides kept over the glass rods under aseptic condition. 2 per cent agar blocks were taken and kept on the sides of glass slide on which inoculation of fungi was carried out (Cai *et al.*, 2009). After the inoculation, careful placement of coverslip over the agar bit was done. Observations were taken within 48-72 h to study the morphology.

3.5.2. Conidial Characteristics

H. oryzae isolates shown poor or no sporulation under laboratory conditions. Sporulation can be induced by using a specific medium and alternate exposure of the culture to near UV light and dark for 12 hour. Rabbit food agar media was plated in sterile petri plates and 5mm mycelial disc of individual isolates kept at the center of the plate. After 5 days of incubation at room temperature, the plates were alternately exposed to near UV light and dark for 12hour for a week. Conidial features such as shape, size, and septation were studied by performing slide culture experiment using these sporulation induced cultures. Coverslips from the slide culture unit were stained with lacto phenol cotton blue and observed under a ZEN camera associated compound microscope. The measurement of size (length and width) and shape of conidia were recorded.

3.6. EVALUATION OF *PIRIFORMOSPORA INDICA* AGAINST *H. oryzae*

3.6.1. Co-cultivation and root colonization efficiency of *P. indica* with rice seedlings

Co cultivation of rice seedlings with *P. indica* is done as described by Johnson *et al.*, (2013). Medium used for the study was modified PNM media

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which supports the growth of both the organism. Before conducting the co cultivation experiment, two separate steps were done.

Initially, 60 ml modified PNM media filled in glass jar bottles and kept for sterilization. A 5 mm mycelial disc of 9 days old *P. indica* culture was placed in the centre of jar bottle and allowed for incubation under room temperature. Simultaneously sterile petri plates were plated with sterile half M S media on to which surface sterilized rice seeds of variety 'Uma' were placed and kept for germination. Surface sterilization of rice seeds involved dipping of de husked seeds in 0.1% mercuric chloride for 3 minutes and 3 subsequent washing in sterile water. Cold treatment of 4^0C for 48 h was performed to ensure uniform germination. Then plates are incubated for about 5 days at 22^0C under continuous illumination. After 5 days of inoculation, sufficient emergence of root and shoot were observed. The uniformly germinated rice seeds then transferred to the modified PNM media with fungal lawn. Jar bottles were closed tightly and sealed with para film and kept for incubation under room temperature.

Colonisation efficiency of *P. indica* on rice roots was determined on 3rd, 5th, 7th, 10th and 14th day of co-cultivation. It was monitored by randomly collecting ten root bits from the co cultivated rice seedlings. The collected roots were dipped in 10% KOH for overnight, followed by five consecutive washing with water. Later the washed root bits were treated with 1% HCl for about 4 minutes. Then the treated roots were mounted on a sterile glass slide and the stain used was lacto phenol cotton blue (Vahabi *et al.*, 2011). Detailed examination of the colonized roots was then taken under the microscope.

3.6.2. Dual culture assay of *P. indica* against *H. oryzae*

Sterile petri plates plated with PDA medium was used for the assay. A mycelial disc (5 mm) of *P. indica* was placed at one end of petri plate and *H. oryzae* on the other end. Fungal plugs were placed at 1cm apart from the rim of petri plate. Plates inoculated with *H. oryzae* alone served as the control. After the inoculation plates were sealed and kept for incubation under room temperature.

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Periodically mycelial growth of *H. oryzae* in dual culture and control plates were measured and percentage growth inhibition of *H. oryzae* by *P. indica* was calculated as per the formula given by Vincent (1927)

$$PI = \frac{C - T}{C} \times 100$$ where,

PI = Per cent Inhibition

C = Growth of the pathogen in control plates (cm)

T = Growth of the pathogen in dual culture (cm)

Similarly sterile petri plates plated with PDA medium on which sterile cellophane membranes were placed was used for evaluating the antagonistic mechanism of *P. indica* against *H. oryzae*. Mycelial disc (5 mm) of *P. indica* and *H. oryzae* were placed at ends of petri plate. Fungal plugs were placed at 1cm apart from the rim of petri plate. Plates inoculated with *H. oryzae* alone served as the control. After the inoculation plates were sealed and kept for incubation under room temperature. Periodically observations on antagonistic actions of *P. indica* against *H. oryzae viz.*, lysis zone/antibiosis/inhibition zone and mycelial growth inhibition were recorded.

3.7. *IN VITRO* EVALUATION OF *P. indica*-PRIMED RICE SEEDLINGS AGAINST *H. oryzae*

Prior to the evaluation procedure a few preparatory steps were followed. Initially *P. indica* mycelial plugs of 5 mm were inoculated to the sterile 250 ml flasks containing potting mixture. The potting mixture comprised of sterile vermiculate-perlite in 2:1 proportion. The inoculated flasks were then kept for incubation under room temperature for about 25 days. After sufficient mycelial growth, potting mixture transferred into sterile beakers under aseptic condition. Beakers containing potting mixture alone served as the control treatment (Kumar *et al.*, 2009).

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Later rice seeds of variety 'Uma' surface sterilized with 0.1% mercuric chloride followed by repeated washing with sterile water for 4-5 times. Treated seeds then placed to the petri plates containing sterile MS media for germination. After 3-4 days of incubation, uniformly germinated seedlings were selected and aseptically transferred to the beakers containing potting mixture with *P.indica* and to the control beakers. Beakers with rice Seedlings were placed under an isolated chamber and required fertilizers (N, P, K) were applied using a micro pipette to the root zone. After 15 days, artificial inoculation of *H. oryzae* was performed. A fine spray of crushed mycelial mass of pathogen was given to the leaves. The inoculated plants were covered with poly propylene bags to ensure sufficient humidity for successful infection. Observations were taken for days of symptom development, lesion size, PDI and plant biomass.

3.8. *IN VIVO* EVALUATION OF *P. indica*-PRIMED RICE SEEDLINGS AGAINST *H. oryzae*

3.8.1. Preparation of fungal lawn in potting mixture

100 g sterile potting mixture of composition, 2:1 vermiculate-perlite was weighed and filled 250 ml sterile conical flasks. Fungal plugs (5 mm) from 1 week old *P. indica* culture plates were inoculated to the flasks and kept for incubation about 2 weeks under room temperature. After observing the sufficient mycelial growth, the potting mixture was taken for growing rice seedlings.

3.8.2. Germination of rice seeds

Seeds of susceptible variety 'Uma' were used for the experiment. Required amount of seeds were soaked overnight. Then these seeds were tied tightly in clean wet muslin cloth and kept in a petri plate. After 3 days of incubation germinated rice seeds are collected and a wet seed treatment using the root endophyte *P. indica* was also performed.

3.8.3. Preparation of pathogen inoculum and inoculation

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Culture disc of 7 days old *H. oryzae* was inoculated into sterile potato dextrose broth and allowed for incubation under room temperature. After 2 weeks, complete coverage of mycelial mat was observed. The mycelial mat was collected by straining through sterile muslin cloth. The strained mat then crushed with pestle and mortar and a fine suspension was made in sterile water. Using a hand sprayer mycelial suspension was sprayed to the leaves of 25 days old rice variety 'Uma'.

3.8.4. Pot culture experiment

Pot culture experiment was performed at Department of Plant Pathology, College of Agriculture, Vellayani during 2018-19. Objective of the study was the evaluation of *P. indica* against brown spot disease of rice. The experimental design followed was completely randomized design (CRD) with 4 treatments and five replications per treatment. 14 days old rice seedlings grown on portray, transplanted to the pots. Crop management in terms of cultural operations and fertilization was followed as per the package of practices of Kerala Agricultural University (KAU, 2017). Ten days after establishment of seedlings evaluation of four treatments were carried out. Treatments were,

T1: *P. indica* alone

T2: *H. oryzae* alone

T3: P. *indica*-primed seedlings + *H. oryzae*

T4: control

To check the effectiveness against pathogen a fine mycelial spray to the foliage was followed and periodic observations for symptom development, lesion size, PDI and root and shoot biomass were taken.

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3.9. *IN VITRO* EVALUATION OF FUNGICIDES AGAINST *H. oryzae*

3.9.1. Poisoned Food Technique

New generation fungicides such as azoles, strobilurins and their combinations given in table 5 were evaluated against *H. oryzae* by poisoned food technique under *In vitro* condition (Nene and Thapliyal, 1993).

Table 2. New generation fungicides and concentrations used for *in vitro* evaluation by poisoned food technique against *H. oryzae*

Sl. No.	Chemical	Formulation	Concentrations of fungicides tested
1	Tebuconazole	25.9 EC	10, 50, 100 & 250 ppm
2	Difenoconazole	25 EC	10, 50, 100 & 250 ppm
3	Azoxystrobin	23 SC	10, 50, 100 & 250 ppm
4	Pyraclostrobin	20 WG	10, 50, 100 & 250 ppm
5	Pyraclostrobin + Tebuconazole (Physical mix)	20 WG + 25.9 EC	10, 50, 100 & 250 ppm
6	Azoxystrobin (11%) + Tebuconazole (18.3%)	11%+18.3% SC	10, 50, 100 & 250 ppm

In poisoned food technique, preparation and sterilization of 50 ml of distilled water and 50 ml of double strength PDA medium were done separately. Desired concentration of poisoned media was prepared by mixing the sterile distilled water with dissolved fungicide and 50 ml molten double strength PDA. Then the medium was plated in sterilized petri plates and kept for solidification. These steps were repeated for all the fungicides. At the centre of each plate mycelial disc of 7-day old *H. oryzae* culture was placed and allowed for

incubation at 28±2°C. Plates with non-poisoned media were maintained as control. Periodic observations were taken for radial growth of the pathogen. Calculation of per centage inhibition of growth over control was based on the formula.

$$I = (C-T)/ C \times 100$$

Where, I = Per centage inhibition.

C = Growth of *H. oryzae* in PDA medium

T = Growth of *H. oryzae* in poisoned PDA medium

3.10. *IN VIVO* MANAGEMENT OF THE PATHOGEN

3.10.1. Preparation of Pathogen Inoculum and Inoculation

Culture disc of 7 days old *H. oryzae* was inoculated into sterile potato dextrose broth and allowed for incubation under room temperature. After 2 weeks, complete coverage of mycelial mat was observed. The mycelial mat was collected by straining through sterile muslin cloth. The strained mat then crushed with pastel and motor and a fine suspension was made in sterile water. Using a hand sprayer mycelial suspension was sprayed to the leaves of 35 days old rice variety 'Uma'.

3.10.2. Pot Culture Experiment

Location of pot culture experiment was Department of Plant Pathology, College of Agriculture, Vellayani. During 2018-19, the study was conducted with an aim of developing an effective management practice for brown spot disease of rice. The followed experimental design was completely randomized design (CRD) with 10 treatments and four replications per treatment. The rice variety Uma was raised as an irrigated crop during April – May. Crop management in terms of cultural operations and fertilization was followed as per the package of practices of Kerala Agricultural University.

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As per the *in vitro* evaluation results, the best triazole, strobilurin fungicides along with two combination fungicides were selected for the study. Tebuconazole 25.9 EC at 0.05 % and 0.1 %, pyraclostrobin 20 WG at 0.05 % and 0.1 %, pyraclostrobin 20 WG + tebuconazole 25.9 EC at 0.05 % and azoxystrobin11 % + tebuconazole18.3 % SC at 0.1 % were used. Performance of these fungicides were compared with the recommended fungicide Trifloxystrobin (25%) + Tebuconazole (50%) at 0.05%. After a week of inoculation, the typical symptoms of the disease appeared then the fungicide spraying was given. The treatments were as follows,

T1: Best azole fungicide 0.05%

T2: Best azole fungicide 0.1%

T3: Best strobilurin fungicide 0.05%

T4: Best strobilurin fungicide 0.1%

T5: Pyraclostrobin (20%) +Tebuconazole (18.3%) - 0.05 %

T6: Azoxystrobin (11%) +Tebuconazole (18.3%)
SC - 0.1%

T7: Trifloxystrobin (25%) +Tebuconazole (50%) (0.05% - treated control)

T8: *H. oryzae* (treated control)

T9: Untreated control

Prior to the application of treatments, calculation of disease severity based on a 0-9 scale was done and the PDI was also recorded. Application of treatments in the form of foliar spray was given at 10^{th} day of inoculation. Observation on PDI was taken regularly on 3^{rd}, 5^{th}, 7^{th}, 10^{th} and 15^{th} day after treatment, on reference to the formula given by Mc Kinney (1923).

Disease suppression over control was calculated using the formula given by Rai and Mamatha (2005).For this PDI observation on the 15^{th} day was taken.

$$\text{Disease Suppression (\%)} = \frac{\text{(PDI in control - PDI in treatment)}}{\text{PDI in control}} \times 100$$

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Results

4. RESULTS

The current study entitled 'Management of brown spot disease of rice using fungal root endophyte *Piriformospora indica* and new generation fungicides' was conducted during 2017 – 2019 at the Department of Plant Pathology, College of Agriculture (COA), Vellayani, Thiruvananthapuram, Kerala. The objectives of the study included evaluation of the effect of the root endophyte *P. indica* against brown spot disease of rice caused by *Helminthosporium oryzae* (Breda De Haan) (syn. *Bipolaris oryzae / Drechslera oryzae)* and new generation fungicides *viz.,* strobilurins, azoles and their combinations for the management of brown spot disease. The results obtained detailed below.

4.1 SURVEY AND COLLECTION OF BROWN SPOT AFFECTED LEAF SAMPLES FROM DIFFERENT RESEARCH STATIONS OF KAU, ISOLATION OF PATHOGEN, PROVING KOCH'S POSTULATE AND MAINTENANCE OF THE CULTURE.

4.1.1. Survey and collection of brown spot affected samples

A survey was conducted during June 2018 – January 2019, in six different research stations of Kerala Agricultural University *viz.*, College of Agriculture, Vellayani; IFSRS Karamana; RRS Moncombu; RARS, Kumarakom; RRS Vyttila and RARS, Pattambi; to collect brown spot affected plant samples and to assess the incidence and severity of brown spot disease of rice (Plate 1). Disease incidence and severity of brown spot disease of rice in the surveyed locations were recorded (Table 3). Percentage of diseased plants out of the total number of plants observed was recorded as the percentage disease incidence and the disease severity was assessed in terms of percentage disease index (PDI) based on standard score chart (Plate 2).

The brown spot disease incidence in the various surveyed locations varied from 24 to76 per cent (Plate 3). The highest disease incidence was recorded from the rice field of IFSRS Karamana (76.0 %), followed by RRS, Moncombu (72.0 %). The lowest disease incidence of brown spot was recorded from RRS, Vyttila (24.0 %). The disease severity of brown spot was calculated as PDI and the highest PDI was recorded from the rice fields of IFSRS Karamana (64.6 %)

Table 3: Brown spot disease incidence and severity in different locations

Sl. No.	Location	Variety	DI (%)	PDI (%)
1	COA, Vellayani	Uma	52	51.8
2	RRS, Vyttila	Vyttila 6	24	39.8
3	RRS, Moncombu	Uma	72	60.2
4	RARS, Pattambi	Jyothi	44	34.6
5	IFSRS, Karamana	Uma	76	64.6
6	RARS, Kumarakom	Sreyas	0	0

DI : Disease incidence

PDI : Percentage disease index

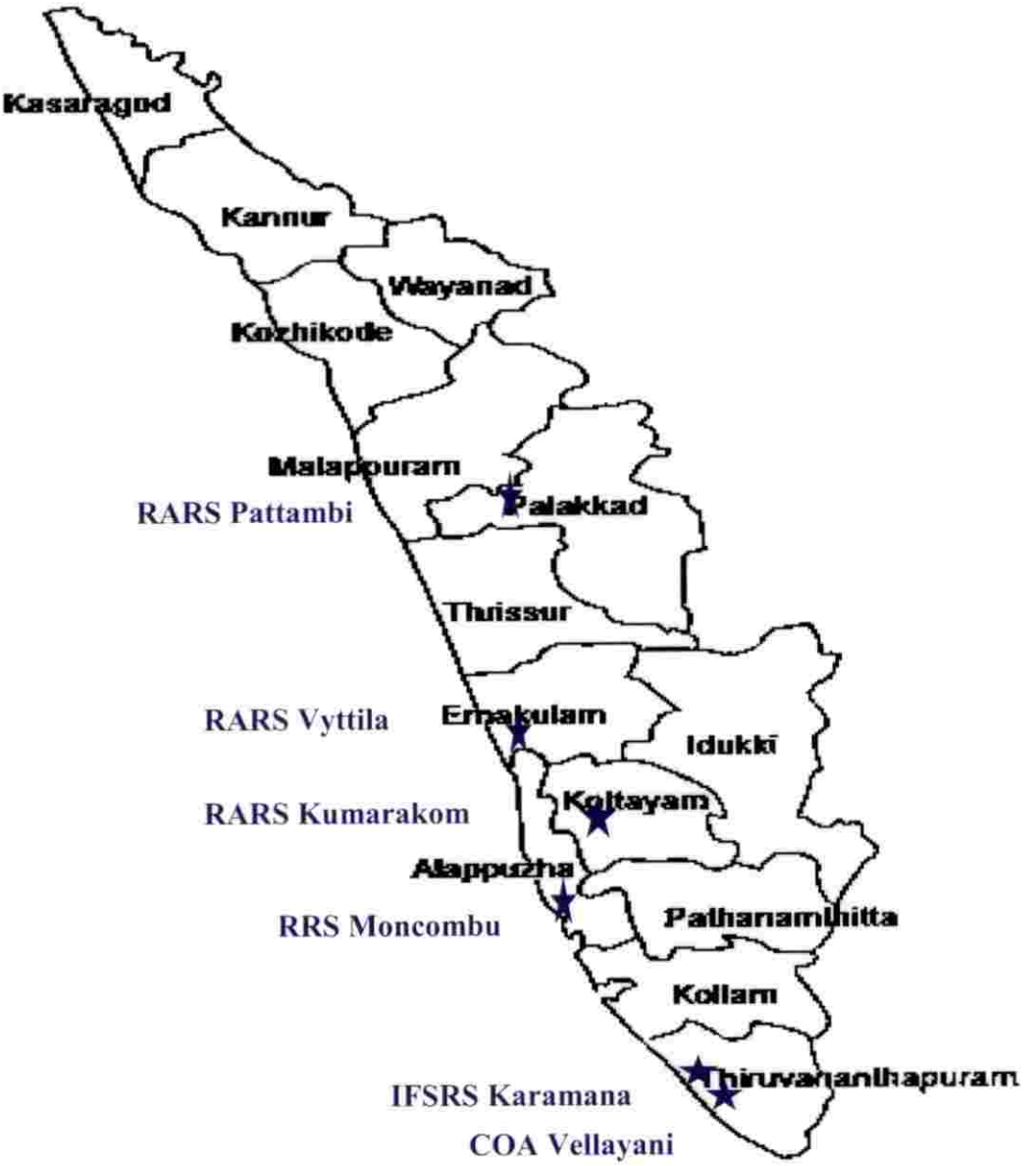


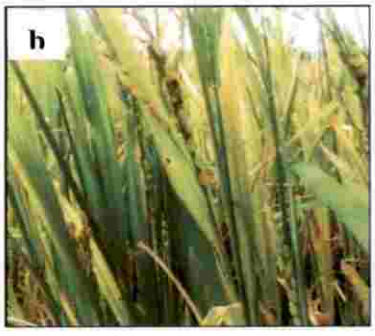
Plate 1. Different locations surveyed in Kerala for the collection of brown spot disease of rice caused by *H. oryzae*

Plate 2: Score chart for assessing the disease severity brown spot disease of rice

Leaf spot

Leaf spot

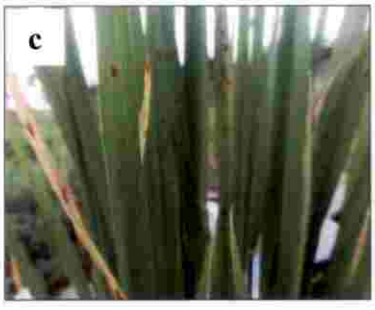
Field view



Leaf spot

Leaf spot

Field view



Leaf spot

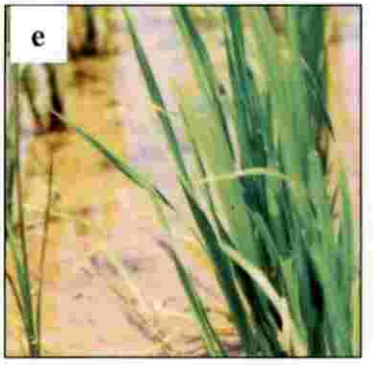
Grain discolouration

Leaf spot

Leaf spot

Leaf blight

Grain discoloration



Leaf spot

Leaf spot

Field view

Plate 3. Symptoms of brown spot of rice caused by *H. oryzae* in locations (a) leaf spot in Vellayani (b) leaf spot and grain discoloration in Vyttila (c) leaf spot and grain discoloration in Mancombu (d) Leaf spot, leaf blight and grain discoloration in Karamana (e) Leaf spot in Pattambi

followed by those from RRS Moncombu (60.2%). The lowest PDI was recorded from RARS, Pattambi with 34.6 per cent disease severity. Thus, from the survey, a total of five isolates of *H. oryzae* were collected.

4.1.2. Symptomatology of the disease under natural condition

Characteristic symptoms of the disease were seen on leaves as oval to round shaped leaf spots with brown coloration. The lesions which later coalesced to form blight symptoms on the leaves. The spotted and blighted leaf area was surrounded by yellow halo with leaf withering. Panicle blight and discoloration of developing grains were other characteristic symptoms of the disease. The brown spot symptoms was appeared at all stages of crop, but higher susceptibility is observed at crop maturity. During the early stages of infection, seedling blight and young brown spots on the leaves were observed. On later stages of infection, severe blighting of the leaf and dark brown to black spots over the grains were observed. Grain infection resulted in seedling blight and damping off. Infected seeds had dark brown to black coloured seed surface and acted as the source of primary inoculum for the pathogen (Plate 4).

4.1.3. Isolation of rice brown spot pathogen

Leaves with characteristic brown spot symptoms were collected during the survey in various locations. The fungus was isolated in potato dextrose agar (PDA) medium from each sample using standard procedure of isolation. Within 24 h of inoculation, mycelial growth of the fungus was observed in the media from leaf bits. A total of five isolates of *H. oryzae* were obtained. The isolates collected from Vellayani, Vyttila, Moncombu, Pattambi and Karamana were named as Isolate 1, Isolate 2, Isolate 3, Isolate 4 and Isolate 5 respectively (Plate 5).

4.1.4. Proving Koch's postulates for the isolates of *H. oryzae*

The pathogenicity of different *H. oryzae* isolates were proven by artificial leaf inoculation. Each isolate was inoculated to the healthy leaves of 35 day-old of rice seedlings of the variety 'Uma' separately. The inoculated leaves were injured

Plate 4. Common symptoms of brown spot of rice caused by *H. oryzae*
(a) Leaf spot (b) leaf blight (c) panicle blight (d) grain discoloration

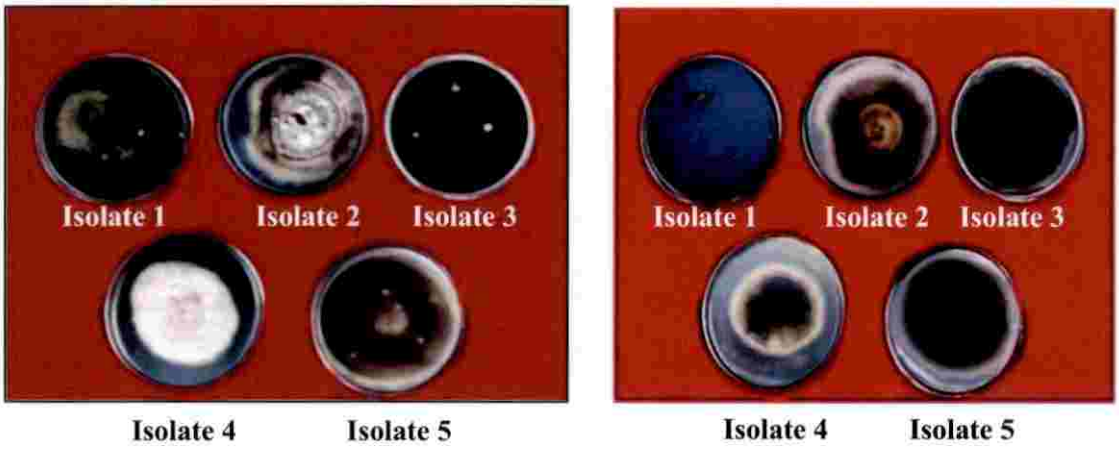


Plate 5: Pure culture of five isolates of *H. oryzae* in potato dextrose agar medium on 7th day (a) upper side view and (b) rear side view of culture plates. Isolate 1 - Vellayani, Isolate 2 - Vyttila, Isolate 3 - Moncombu, and Isolate 4 - Pattambi and Isolate 5 – Karamana.

through pinpricking method. Among the different isolates of *H. oryzae*, inoculation of isolate 1 and isolate 5 produced leaf spot and blight symptoms within 24 h of inoculation. On the fifth day after leaf inoculation, isolate 5 exhibited 46.91 per cent disease severity with a mean lesion size of 0.36 cm followed by isolate 1 where the recorded disease severity was 40.73 per cent with a lesion size of 0.32 cm (Table 4). On further incubation, the lesions got enlarged and hence confirming their pathogenicity (Plate 6). The pathogen was re-isolated from the artificially inoculated leaves onto PDA medium. On comparison, the re-isolated cultures confirmed similarity in its morphology with the original isolates and thus the Koch's postulates were proved.

4.2. MORPHOLOGICAL AND CONIDIAL CHARACTERS OF *H. oryzae*

4.2.1. Morphological Characters

All the five isolates of *H. oryzae* were grown on PDA medium and their morphological characters were recorded by observing the colony growth. Each *H. oryzae* isolate exhibited different mycelial colour and nature of growth. The mycelial colour of the isolates varied from white, grey and black. Mycelium of isolate 1 was grey to black in colour on its upper side and black in reverse side with a fluffy growth. The margin of the growth was regular. The isolate 2 exhibited whitish colour with greyish colour on their upper side, brownish black in the reverse side; and it has an irregular margin with a fluffy nature of growth. Isolate 3 and isolate 5 isolates had grey to black mycelial growth and black in the reverse side, isolate 3 had smooth mycelial growth with irregular margin whereas isolate 5 had fluffy mycelial growth with regular margin. Mycelium of isolate 4 exhibited white colour on upper side and brown to black in the reverse side; and it got a cottony growth with regular margin (Table 5; Plate 7).

4.2.2. Mycelial Characters of *H. oryzae*

Microscopic characters of different isolates were studied through slide culture technique. Slides were observed under microscope (100x). The mycelium of the fungus was slender and septate. The mycelial width of the isolates varied

Table 4: Pathogenicity of *H. oryzae* causing brown spot disease in rice var. Uma

Isolates	DTSA*	Lesion size (cm) 5 DAI**	* PDI (%)
Isolate 1	1	0.327 ± 0.018^{b}	40.73 (39.65)[a]
Isolate 2	2	0.231 ± 0.041^{cd}	29.62 (32.94)[b]
Isolate 3	1	0.264 ± 0.025^{c}	33.32 (35.23)[b]
Isolate 4	3	0.139 ± 0.010^{d}	18.51 (25.41)[c]
Isolate 5	1	0.362 ± 0.031^{a}	46.91 (43.22)[a]
SEm±		0.027	1.27
CD(0.05)		0.087	4.05

Values are mean of three replications ± standard deviation

Values in parenthesis are angular transformed values

DSTA: Days taken for symptom appearance

DAI: Days after inoculation

PDI: Percent disease index

Table 5: Morphological characters of different isolates of *H. oryzae* causing brown spot of rice

Isolates	Colour of mycelia and appearance		Margin
	Upper side	Reverse side	
Isolate 1	Grey to black fluffy growth	Greyish black	Regular
Isolate 2	White with grey fluffy growth	Brownish black	Irregular
Isolate 3	Grey to black smooth growth	Black	Irregular
Isolate 4	White cottony growth	Brown to black	Regular
Isolate 5	Grey to black fluffy growth	Black	Regular

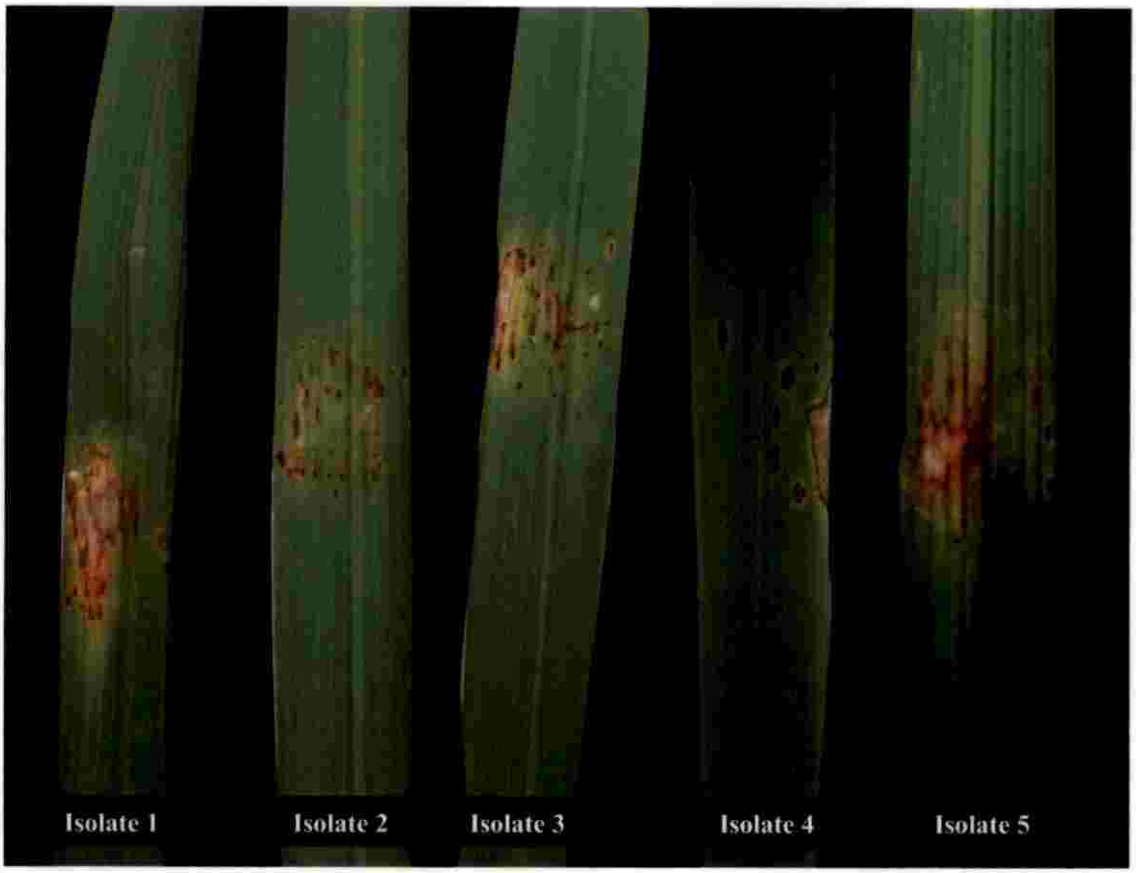
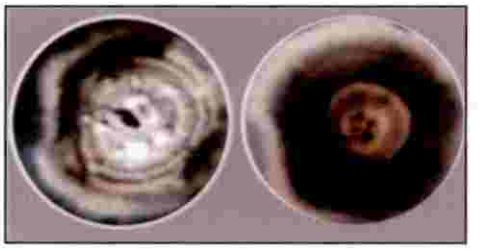
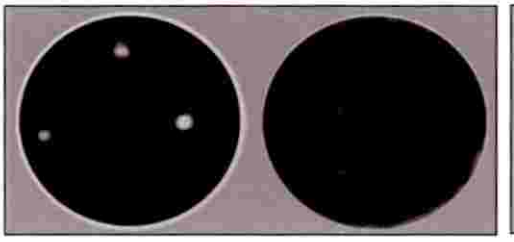


Plate 6. Symptom appearance on artificial inoculation of different isolates of *H. oryzae* on rice var. Uma at 5th day after inoculation.

Isolate 1

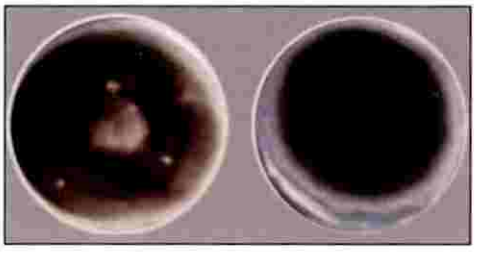


Isolate 2



Isolate 3

Isolate 4



Isolate 5

Plate 7. Upper and rear side views of different isolates of *H. oryzae* in PDA medium on 7th day of growth under laboratory condition.

from 1.49 µm to 2.81 µm (Table 6; Plate 8). The fungus exhibited poor sporulation under lab conditions. Sporulation was observed in isolates 1 and 5, and both of them produced brown coloured slightly curved conidia with bulged centre. The conidial dimensions were higher for isolate 5 (length 50 µm and width 5.5 µm) followed by isolate 1 (length 46.5 µm and width 6.6 µm) (Table 6; Plate 9).

4.2.3 Radial mycelial growth of the isolates

The radial mycelial growth of all the five isolates was compared and it was concluded that the maximum growth of 8.93 cm was recorded by isolate 1, which was on par with isolate 5 (8.58 cm). The days taken for completing the mycelial growth in petridish (9 cm) was maximum (11 days) for isolate 4 and minimum (7 days) for isolate 1 and isolate 5 (Table 7).

4.3. SCREENING OF VIRULENT ISOLATE OF *H. oryzae*

An *in vitro* screening experiment by detached leaf assay was conducted to find the most virulent isolate among the different isolates of *H. oryzae* collected during the survey. On the basis of days taken for symptom appearance, lesion size on the leaves and disease severity on leaves of rice variety 'Uma', the most virulent isolate of *H. oryzae* was identified. All the five isolates developed lesions of varying sizes on artificial inoculation.

4.3.1. Leaf Inoculation

Detached leaf assay by inoculation of *H. oryzae* to the leaves of rice variety 'Uma' produced leaf spots, leaf blight and yellowing symptoms within 24 to 72 h of inoculation which later spreads to the whole leaf area (Plate 10).

Among the different isolates screened, isolate 5 exhibited maximum disease severity of 63.7 per cent with a mean lesion size of 2.94 cm on fifth day of leaf inoculation. On the third day of inoculation, the isolate 3 produced maximum lesion size of 0.97 cm. After 5 days of incubation, complete rotting of inoculated leaf was observed (Table 8 and Table 9). Thus the isolate 3 was confirmed to be as the most virulent isolate of *H. oryzae*.

Table 6: Microscopic characters of mycelia and conidia of different isolates of *H. oryzae* causing brown spot disease of rice

Isolates	Width of mycelia (µm)*	Characteristics of conidia (at 400X)		
		Shape	Length (µm)	Width (µm)
Isolate 1	1.49 ± 0.21	Slightly curved widest at the middle	46.5	6.6
Isolate 2	2.81 ± 0.22	No sporulation	-	-
Isolate 3	1.85 ± 0.05	No sporulation	-	-
Isolate 4	2.64 ± 0.03	No sporulation	-	-
Isolate 5	2.29 ± .040	Slightly curved widest at the middle	50	5.5

Values are mean of five replications ± standard deviation

Table 7: Radial mycelial growth of different isolates of *H. oryzae* causing rice brown spot in PDA medium

Isolates	Radial growth (cm)* at different days of incubation					DTCP
	3th day	5th day	7th day	10th day	15th day	
Isolate 1	5 ± 0.087[a]	6.9 ± 0.06[a]	8.93 ± 0.07[a]	9	9	7
Isolate 2	4.13± 0.02[c]	6.3 ± 0.04[c]	7.3 ± 0.06[d]	9	9	10
Isolate 3	4.6 ± 0.04[a]	7.08 ± 0.11[a]	8.03 ± 0.09[c]	9	9	9
Isolate 4	3.6 ± 0.06[a]	4.41 ± 0.06[a]	5.6 ± 0.06[e]	7.2	9	11
Isolate 5	4.95 ± 0.07[b]	7.12 ± 0.05[b]	8.89 ± 0.06[a]	9	9	7
SEm ±	0.119	0.098	0.067	--	--	--
CD(0.05)	0.379	0.222	0.213	--	--	--

DTCP- Days taken for complete coverage of petridish; Mean ± SD of three replication Values followed by superscripts are not significantly different at 5% level

Table 8: Days taken for symptom appearance and lesion size at different days after inoculation of different isolates of *H. oryzae* causing brown spot disease in rice var. Uma

Isolates	DTSA	Lesion size (cm)* at different days after inoculation				
		3	5	7	10	15
Isolate 1	1	0.75 ± 0.05^{b}	2.61 ± 0.14^{b}	Complete rotting of leaf		
Isolate 2	2	0.37 ± 0.04^{d}	0.86 ± 0.09^{d}	1.34 ± 0.24	Complete rotting of leaf	
Isolate 3	2	0.54 ± 0.03^{c}	1.26 ± 0.10^{c}	2.06 ± 0.07	Complete rotting of leaf	
Isolate 4	3	0.28 ± 0.03^{d}	0.604 ± 0.06^{e}	0.99 ± 0.06	Complete rotting of leaf	
Isolate 5	1	0.97 ± 0.06^{a}	2.94 ± 0.09^{a}	Complete rotting of leaf		
SEm±		0.045	0.101			
CD(0.05)		0.134	0.255			

Values are mean of five replications ± standard deviation .Values followed by similar superscripts are not significantly different at 5% level

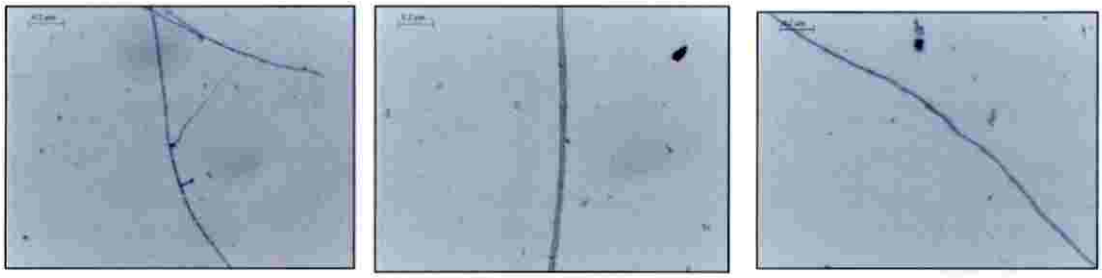
DTSA : Days taken for symptom appearance

Table 9: Nature of symptoms developed and severity of brown spot by different isolates of *H. oryzae* in rice var. Uma (detached leaf assay)

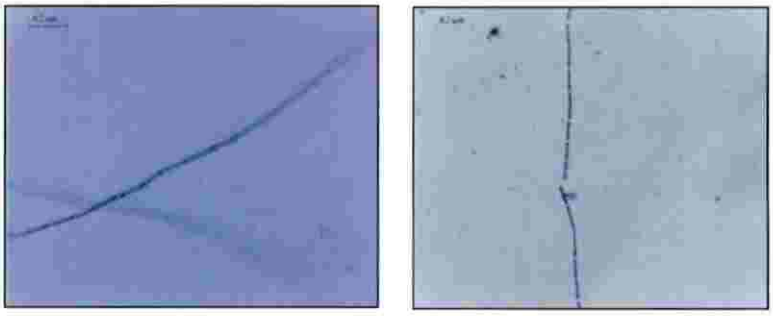
Isolates	Nature of symptoms developed	PDI (%)				
		3rd day	5th day	7th day	10th day	15th day
Isolate 1	Leaf blighting with yellowing	22.96 (28.59)^{b}	54.07 (47.34)^{b}	100	100	100
Isolate 2	Minute spots later turns to lesions	12.58 (20.56)^{d}	34.81 (36.12)^{c}	55.38	100	100
Isolate 3	Leaf spots later turns to lesions	17.77 (24.87)^{c}	43.70 (41.36)^{b}	74.61	100	100
Isolate 4	Minute lesions	7.40 (15.58)^{e}	28.14 (31.97)^{d}	47.54	100	100
Isolate 5	Leaf blighting with yellowing	28.91 (32.50)^{a}	63.7 (52.96)^{a}	100	100	100
SEm±		1.3	0.101			
CD(0.05)		3.5	3.53			

Values are mean of five Replications; Values followed by similar superscripts are not significantly different at 5% level;

Values in parenthesis are arc sine transformed values

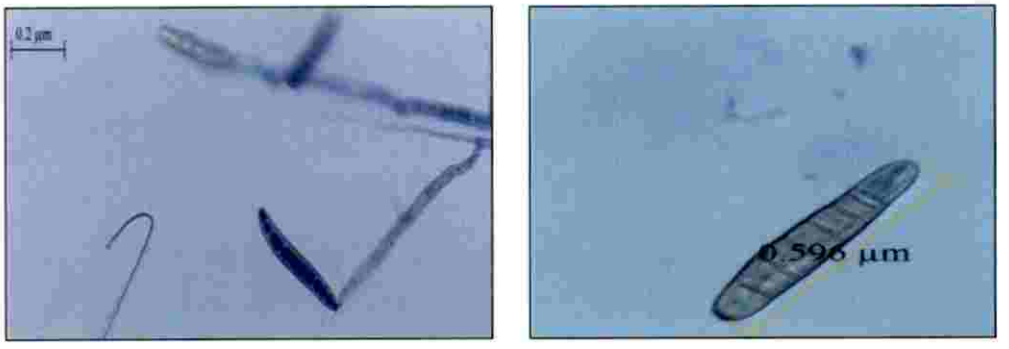


Isolate 1 **Isolate 2** **Isolate 3**



Isolate 4 **Isolate 5**

Plate 8. Microscopic view of mycelia of different isolates of *H. oryzae* (100X)



Isolate 1 **Isolate 5**

Plate 9. Microscopic observation of conidia of different isolates of *H. oryzae* (400x). (a) Isolate 1 (b) Isolate 5.

Plate 10. Screening of the most virulent isolate of *H. oryzae* by detached leaf assay in rice var. Uma on 5th day of leaf inoculation

4.4. EVALUATION OF *P. indica* AGAINST *H. oryzae*

4.4.1 Root Colonization of Rice var. Uma with *P. indica*

Colonization efficiency of *P. indica* on rice variety Uma was standardized as per the protocol of Johnson *et al.*, (2013). Maximum root colonization of *P. indica* (100 %) was recorded at 10 days after co-cultivation (DAC) (Table 10). Mature chlamydospores were noticed inside the rice root cell from 5 DAC and on 15 DAC, large number of chlamydospores covering the whole root cells were noticed (Plate 11).

4.4.2 Dual Culture of *P. indica* with *H. oryzae*

Dual culture assay of *P. indica* with *H. oryzae* revealed that the endophyte exihibits multiple antagonistic actions against the pathogen which includes lysis / inhibition zone (0.65 cm) and antibiosis (0.34 cm) observed at 9th and 15th days after incubation of dual culture plate respectively (Table 11). Microscopic observations on the antagonistic action of *P. indica* against *H. oryzae* indicated that the endophyte induced thickening and lysis of the pathogen mycelia and secreted certain secondary metabolites to inhibit the pathogen (antibiosis) (Plate 12).

Mycelial inhibition of *H. oryzae* by *P. indica* in dual culture plate was recorded during different intervals of incubation. It was found that there was a reduced and suppressed nature of mycelial growth of *H. oryzae* in dual culture in comparison with the control plate was noticed. On 5th day after incubation a percentage growth inhibition of 54.44 was recorded in *H. oryzae* growth and it was 62.3 per cent on 10th day (Table 12; Plate 13).

4.4.3 *In vitro* Evaluation of *P. indica*-Primed Rice Seedlings (var. Uma) against *H. oryzae*

In vitro evaluation of *P. indica*-primed rice seedlings against *H. oryzae* revealed that *P. indica*-primed rice seedlings significantly delayed the symptom development of brown spot disease by a day with a minimum lesion size of 1.43 cm on 7th day after inoculation (DAI) of *H. oryzae* (Table 13). Similarly the *P.*

Table 10: Root colonisation efficiency of *P. indica* in rice var. Uma

Treatment	Days after co-cultivation of *P. indica* in rice roots				
	3rd day	5th day	7th day	10th day	15th day
Colonisation percentage (%)	-	40	70	100	100

Values are mean ten replications

Table 11: Antagonistic properties of *P. indica* against *H. oryzae* causing brown spot of rice in dual culture grown on PDA medium

Nature of interaction	Days taken for appearance	Measurement (cm)
Lysis / Inhibition zone	9	0.65
Antibiosis	15	0.37
Over growth	-	-

Values are mean of three replications

Table 12: Mycelial inhibition of *H. oryzae* causing rice brown spot by *P. indica* in dual culture grown on PDA medium

Treatments	Mycelial growth (cm)					Nature of mycelial growth
	3rd day	5th day	7th day	10th day	15th day	
P. indica (dual culture)	4.03	5.13	5.76	5.76	5.76	Normal
H. oryzae (dual culture)	2.66 (18.4)	3.20 (36.4)	3.24 (54.4)	3.24 (62.3)	3.24 (54.4)	Suppressed
P. indica (control)	4.06	5.70	7.10	7.90	9.00	Normal
H. oryzae (control)	3.26	5.03	7.1	8.60	9.00	Normal

Values are mean of three replications

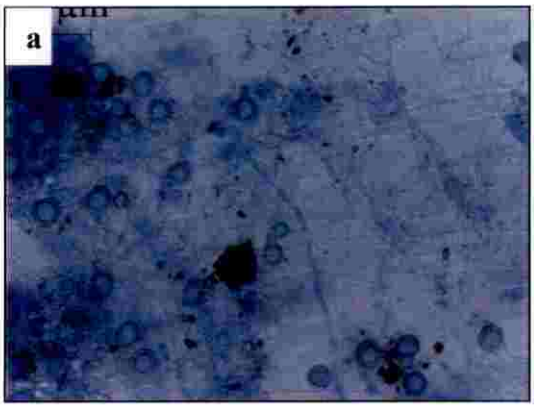
Values in parenthesis represent the percentage growth inhibition of *H. oryzae*

Table 13: Days taken for symptom appearance and lesion size of brown spot caused by *H. oryzae* in *P. indica*-primed rice seedlings var. Uma

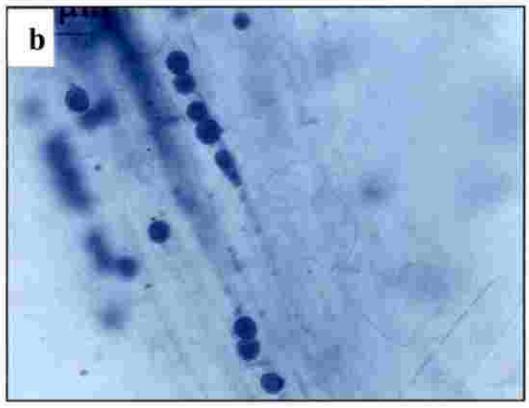
Treatments	DTSA	Lesion size (cm)				
		3rd day	5th day	7th day	10th day	15th day
P. indica alone	0	0	0	0	0	0
H. oryzae alone	2	1.69 ± 1.11	3.9 ± 0.95	4.45 ± 0.59	Complete leaf blight	Complete leaf blight
P. indica-primed seedlings + *H. oryzae*	3	0.52 ± 0.24	1.08 ± 0.23	1.43 ± 0.18	2.08 ±0.3	2.6± 0.40
Control	0	0	0	0	0	0

Values are mean of five replications ± standard deviation

DTSA: Days taken for symptom appearance

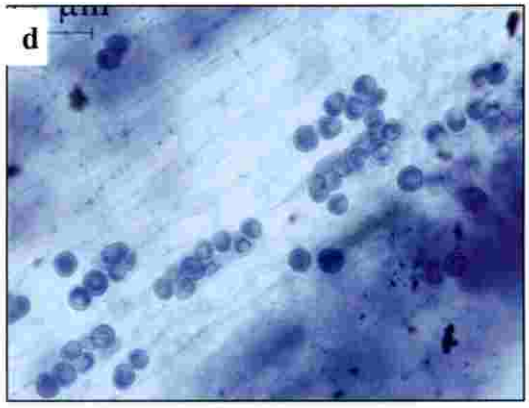


3rd DAC

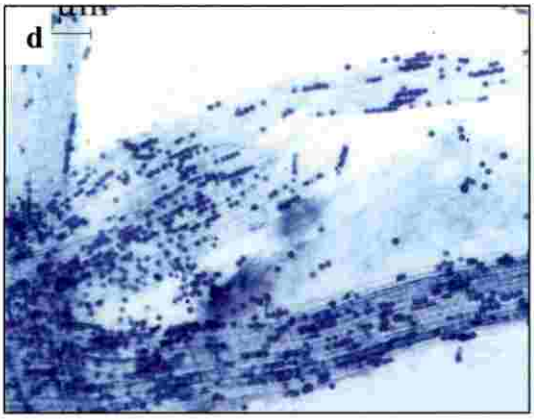


5th DAC

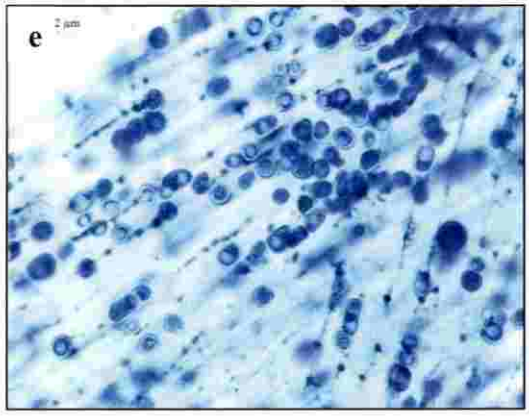
7th DAC (100x)



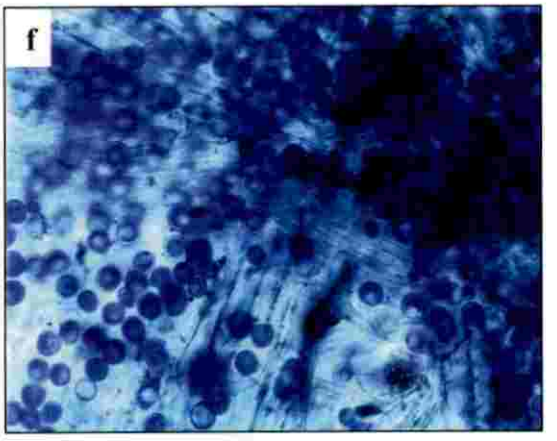
7th DAC (400x)



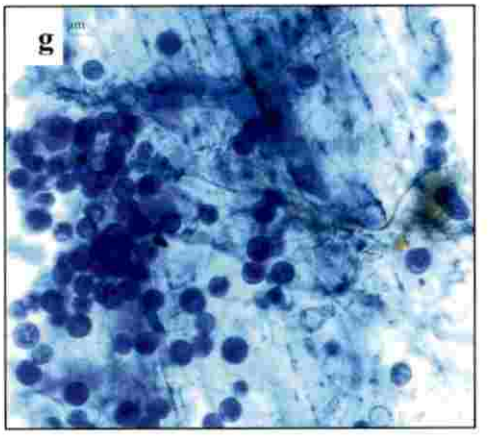
10th DAC (100x)



10th DAC (400x)

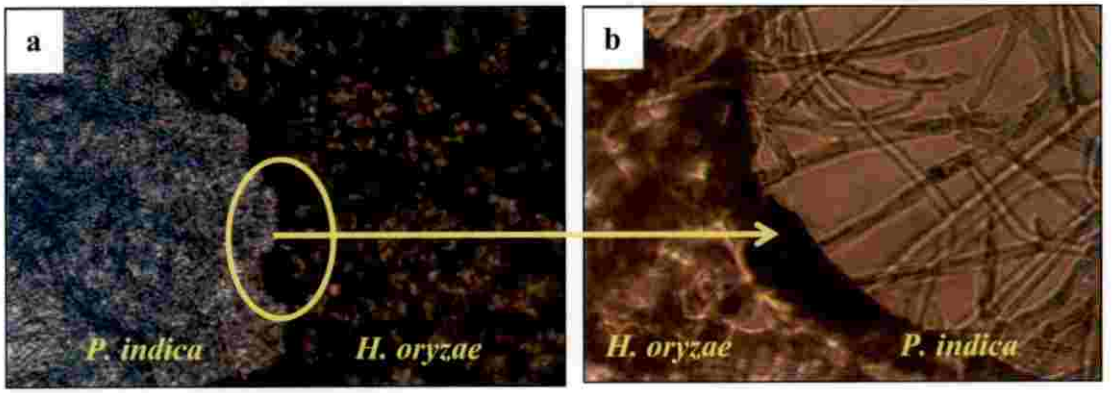


15th DAC (100x)

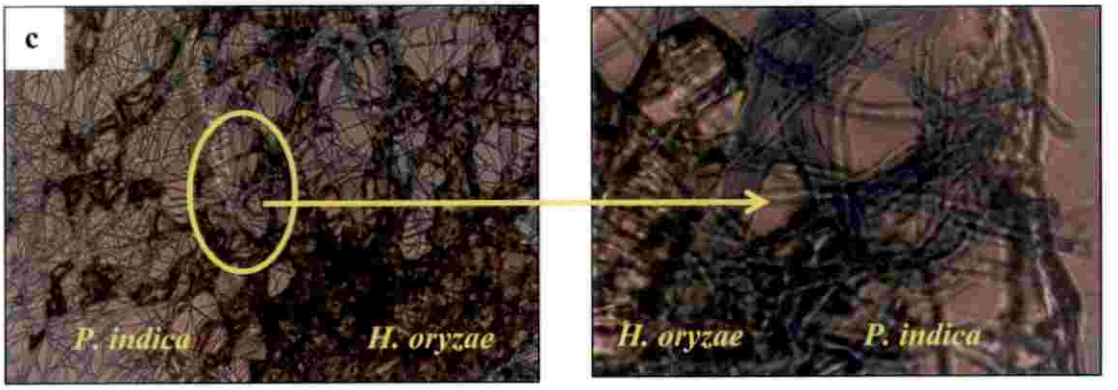


15th DAC (100x)

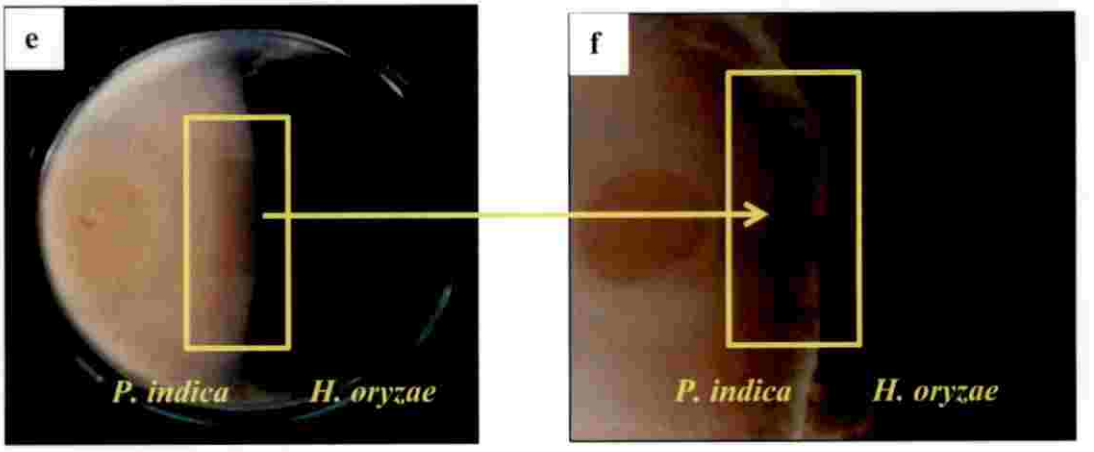
Plate 11: Root colonisation of *P. indica* in rice var. Uma after 3, 5 ,7, 10 and 15 days of co-cultivation on PNM medium. (a) hyphae and young chlamydospores on 3rd day after co-cultivation (DAC); (b) mature chlamydospores inside the root cells on 5th DAC; (c) mature chlamydospores inside the root cells on 7th DAC (100x); (d) mature chlamydospores inside the root cells on 7th DAC (400x); (e) mature chlamydospores inside the root cells on 10th DAC (100x); (f) mature chlamydospores inside the root cells on 10th DAC (400x) (g-h) mature chlamydospores completely covered the entire root.



Thickening of mycelia (100x) Thickening of mycelia (1000x)



Lysis of mycelia (100x) Lysis of mycelia (1000x)



Antibiosis Antibiosis - enlarged view

Plate 12. Multiple antagonstic action of *P. indica* against *H. oryzae* in dual culture (a) Mycelial thickening of *H. oryzae* in response to the antagonism of *P. indica* (100x); (b) Enlarged of the same (1000x); (c) Lysis of *H. oryzae* mycelia (100x); (d) Enlarged view (1000x); (e) antibiosis of *P. indica* against *H. oryzae*; (f) enlarged view.

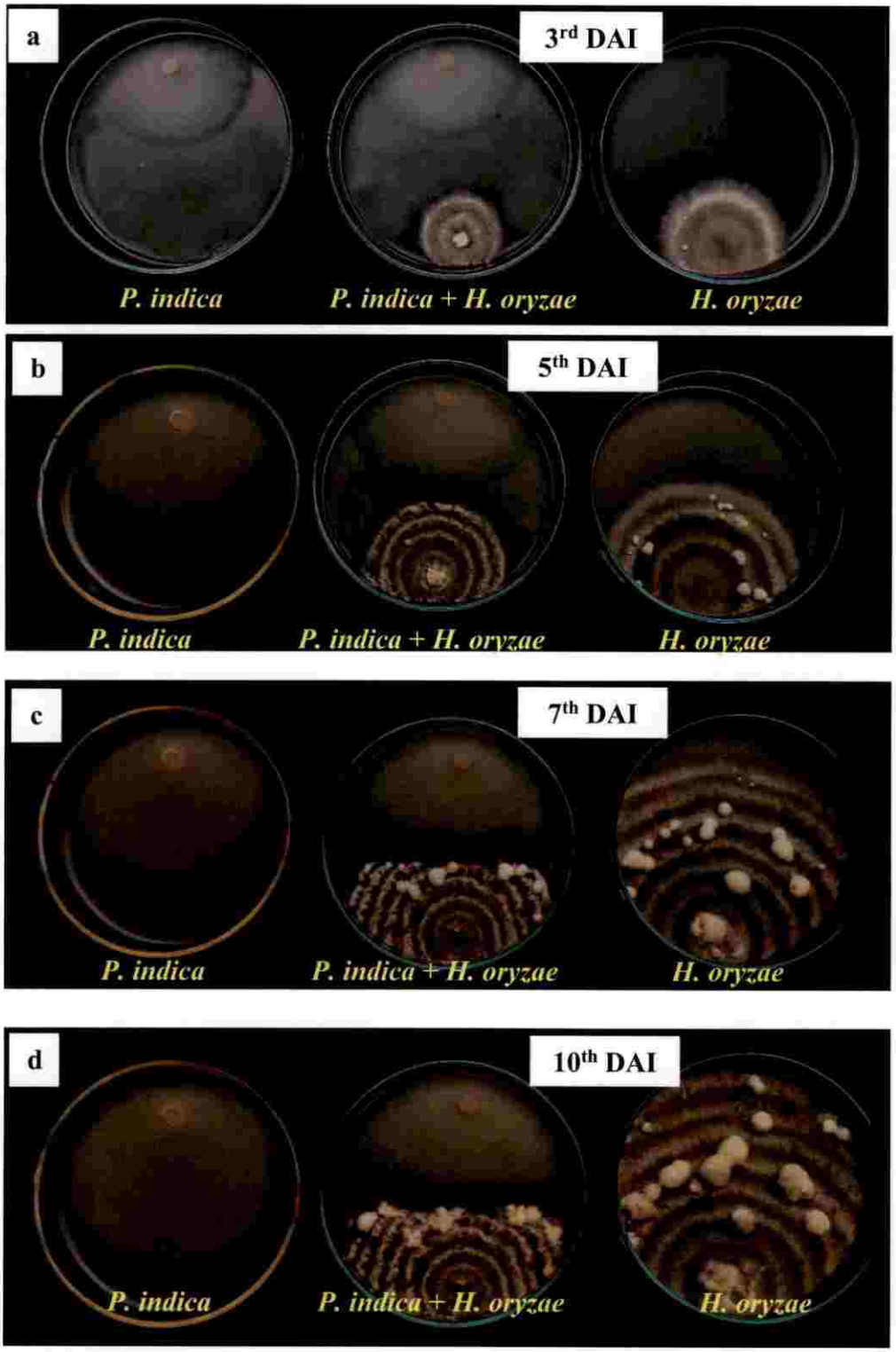


Plate 13. Mycelial growth and inhibition of *H. oryzae* by *P. indica* in dual culture on PDA medium at different intervals of incubation (a) on 3rd day (b) 5th day (c) 7th day and (d) 10th day.

indica-primed rice seedlings exhibited minimum brown spot disease severity (12.4 %) in comparison to the non-primed plants (100 %) on 15 DAI of *H. oryzae* (Table 14; Plate 14).

Enhanced biomass production in terms of fresh and dry weight of shoot and root was observed in rice seedlings primed with *P. indica,* and the seedlings primed with *P. indica* and inoculated with *H. oryzae*, compared to the control and seedlings inoculated with *H. oryzae* alone. The highest shoot fresh and dry weight were recorded in plants primed with *P. indica* and inoculated with *H. oryzae* (0.32 g and 0.05 g respectively) followed by plants primed with *P. indica* alone (0.32 and 0.04 respectively). The lowest shoot fresh and dry weight were recorded in plants inoculated with *H. oryzae* alone (0.07 and 0.02 respectively) followed by control plants (0.11 and 0.03 respectively). Similarly, root fresh and dry weight was higher in plants primed with *P. indica* and inoculated with *H. oryzae* (0.13 and 0.07 respectively) followed by plants primed with *P. indica* alone (0.12 and 0.06 respectively). The plants inoculated with *H. oryzae* alone recorded the lowest root fresh and dry weight (0.05 and 0.03 respectively) followed by control plants (0.08 and 0.02 respectively) (Table 15).

Root colonisation of *P. indica* conferred brown spot disease tolerance and growth promotion to the rice seedlings. The *P. indica*-primed rice seedlings produced more number of tillers and leaves with increased height and size. The primed rice seedlings also exhibited more secondary and tertiary roots with profuse root hairs. *P. indica*-priming in rice seedlings thus ensured a synergism in growth promotion and brown spot disease tolerance (Plate 15).

4.4.4 *In vivo* Evaluation of *P. indica*-Primed Rice Seedlings (Var. Uma) against *H. oryzae*

Pot culture experiment designed for evaluating the effect of *P. indica*-primed rice plants against *H. oryzae* (Plate 16). *In vivo* evaluation of *P. indica*-primed rice plants against *H. oryzae* revealed that *P. indica*- primed rice plants significantly delayed the symptom development of brown spot disease up to 8 day with a minimum lesion size of 1.02 cm on 15th DAI of *H. oryzae* (Table 16).

Table 14: Disease severity of brown spot caused by *H. oryzae* in *P. indica*-primed rice seedlings var. Uma

Treatments	Percentage disease index (PDI)				
	3rd day	5th day	7th day	10th day	15th day
P. indica alone	0	0	0	0	0
H. oryzae alone	27.99 ± 3.3	59.73 ± 3.12	89.7 ± 4.6	98.6±1.9	100
P. indica-primed seedlings + *H. oryzae*	6.66 ± 9.1	9.3 ± 12.80	10.2 ± 14.0	11.1 ± 13.6	12.4 ± 15.2
Control	0	0	0	0	0

Values are mean of five replications ± standard deviation

Table 15: Root and shoot biomass of *P. indica*-colonised rice seedlings var. Uma

Treatments	Fresh weight (g plant^{-1})		Dry weight (g plant^{-1})	
	Shoot	Root	Shoot	Root
P. indica alone	0.32 ± 0.02	0.12 ± 0.01	0.04 ± 0.01	0.06 ± 0.01
H. oryzae alone	0.07 ± 0.02	0.05 ± 0.01	0.02 ± 0.01	0.03 ± 0.01
P. indica + *H. oryzae*	0.32 ± 0.01	0.13 ± 0.02	0.05 ± 0.01	0.07 ± 0.01
Control	0.11 ± 0.02	0.08 ± 0.01	0.03 ± 0.01	0.02 ± 0.01

Values are mean of five replications ± standard deviation

Table 16: Days taken for symptom appearance and lesion size of brown spot caused by *H, oryzae* in *P. indica*-primed rice plants var. Uma

Treatments	DTSA	Lesion size (cm)				
		3rd day	5th day	7th day	10th day	15th day
P. indica alone	0	0	0	0	0	0
H. oryzae alone	2	0.55 ± 0.16	1.02 ± 0.17	1.46 ± 0.07	1.9 ± 0.2	2.8 ± 0.3
P. indica + *H. oryzae*	8	0	0	0	0.48 ± 0.07	1.02 ± 0.1
Control	0	0	0	0	0	0

Values are mean of five replications ± standard deviation

DTSA: Days taken for symptom appearance

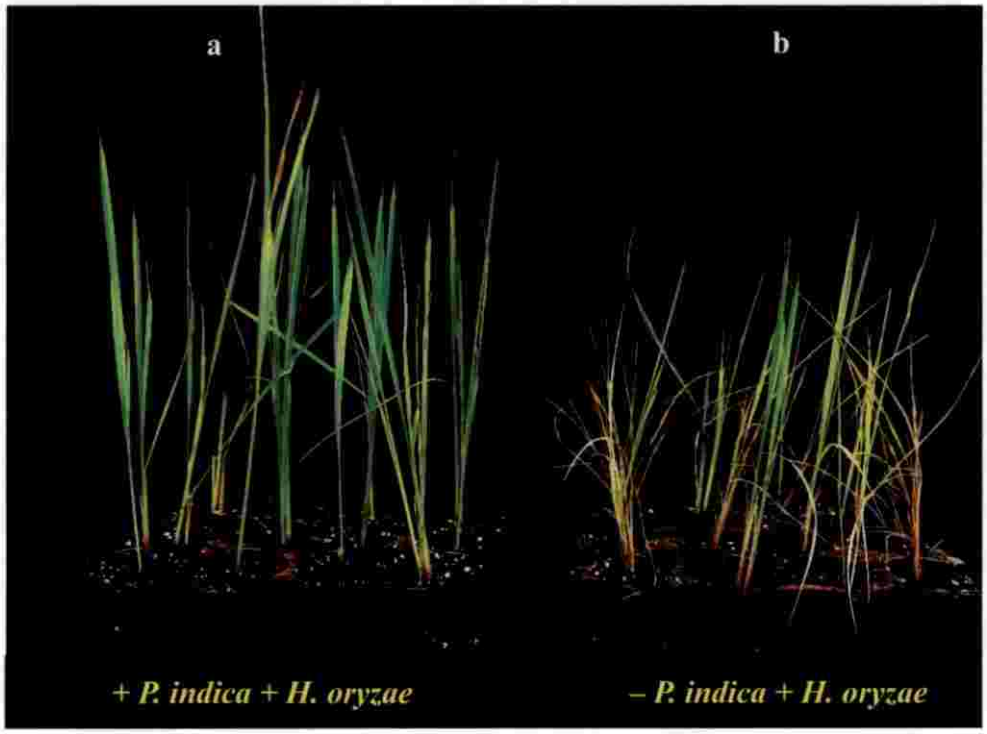


Plate 14. Less severity of brown spot disease caused by *H. oryzae* in *P. indica*-primed rice seedlings var. Uma under *in vitro*. Brown spot lesions and seedling blight on rice leaves treated with *P. indica* followed by *H. oryzae* (a) and *H. oryzae* alone (b), at 7th day after inoculation.

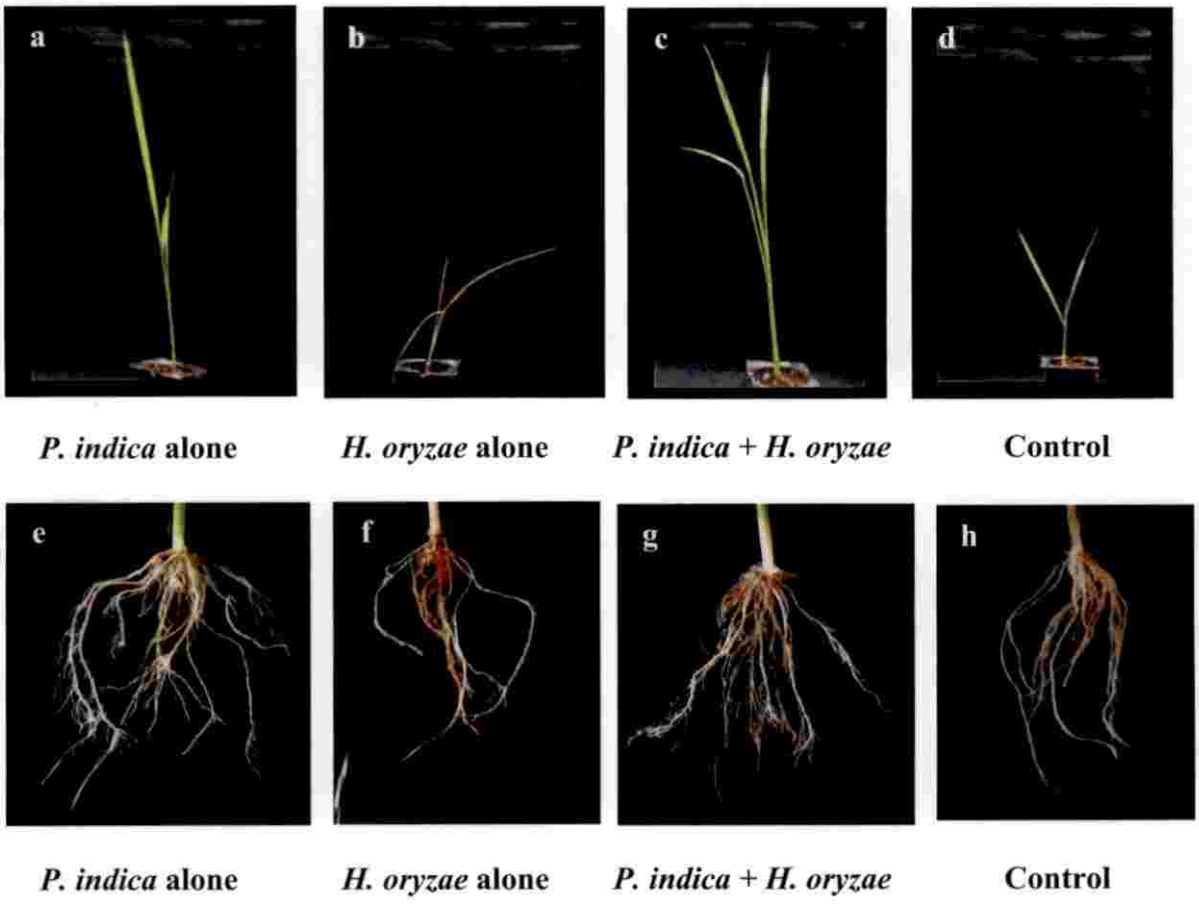


Plate 15. ***In vitro*** **evaluation of** ***P. indica*****-primed rice seedlings var. Uma for growth promotion (a-d) Shoot growth of** ***P. indica*** **alone,** ***H. oryzae*** **alone,** ***P. indica*** **+** ***H. oryzae,*** **and control. (e-h) Root growth of** ***P. indica*** **alone,** ***H. oryzae*** **alone,** ***P. indica*** **+** ***H. oryzae,*** **and control**

Plate 16. General view of pot culture experiment of *P. indica*-primed rice seedlings var. Uma against brown spot pathogen, *H. oryzae*

Similarly, the *P. indica*-primed rice plants recorded minimum brown spot disease severity (13.7 %) in comparison to the non-primed plants (69.7 %) on 15 DAI of *H. oryzae* (Table 17; Plate 17).

Enhanced biomass in terms of fresh and dry weight of shoot and root was also observed in rice plants primed with *P. indica* and plants primed with *P. indica* and inoculated with *H. oryzae* compared to control and plants inoculated with *H. oryzae* alone. The highest shoot fresh and dry weight were recorded in plants primed with *P. indica* alone (10.84 g and 4.22 g respectively) followed by plants primed with *P. indica* and inoculated with *H. oryzae* (9.10 g and 3.52 g respectively). The lowest shoot fresh and dry weight were recorded in plants inoculated with *H. oryzae* alone (4.54 g and 1.60 g respectively) followed by control plants (5.36 g and 1.36 g respectively). Similarly, root fresh and dry weight was higher in in plants primed with *P. indica* and inoculated with *H. oryzae* (6.58 g and 2.82 g respectively) followed by plants primed with *P. indica* alone (6.20 g and 2.04 g respectively). The plants inoculated with *H. oryzae* alone recorded the lowest root fresh and dry weight (2.64 g and 1.18 g respectively) followed by control plants (2.80 g and 1.48 g respectively) (Table 18).

Root colonisation of *P. indica* conferred brown spot disease tolerance and growth promotion to the rice plants. The *P. indica*-primed rice plants exhibited more number of tillers and leaves with increased number and size. The primed rice plants also exhibited more secondary and tertiary roots with profuse root hairs. The *P. indica*-priming thus ensured a synergism in growth promotion and brown spot disease tolerance in rice plants (Plate 18).

4.5. EVALUATION OF SELECTED NEW GENERATION FUNGICIDES *viz.*, STROBILURINS, AZOLES AND THEIR COMBINATION FUNGICIDES AGAINST *H. oryzae*

Evaluation of new generation fungicides *viz.*, triazoles, strobilurins and their combinations were performed against *H. oryzae*. *In vitro* evaluation was performed by poisoned food technique. Further, the evaluation of best triazole,

Table 17: Disease severity of brown spot caused by *H. oryzae* in *P. indica*-primed rice plants var. Uma

Treatments	Percentage disease index (PDI)				
	3rd day	5th day	7th day	10th day	15th day
P. indica alone	0	0	0	0	0
H. oryzae alone	14.2 ± 2.3	27.5 ± 2.9	43.9 ± 1.8	57.7 ± 2.7	69.7 ± 1.8
P. indica + *H. oryzae*	0	0	0	9.62 ± 1.9	13.7 ± 1.7
Control	0	0	0	0	0

Values are mean of five replications ± standard deviation

Table 18: Root and shoot biomass of *P. indica*-colonised rice plants var. Uma

Treatments	Fresh weight (g plant^{-1})		Dry weight (g plant^{-1})	
	Shoot	Root	Shoot	Root
P. indica alone	10.84^{a}	6.20^{a}	4.22^{a}	2.04^{b}
H. oryzae alone	4.54^{c}	2.64^{b}	1.60^{c}	1.18^{c}
P. indica + *H. oryzae*	9.10^{b}	6.58^{a}	3.52^{b}	2.82^{a}
Control	5.36^{c}	2.80^{b}	1.36^{c}	1.48^{c}
SEm±	0.44	0.26	0.12	0.25
CD (0.05)	1.31	0.77	0.412	0.38

Values are mean of five replications

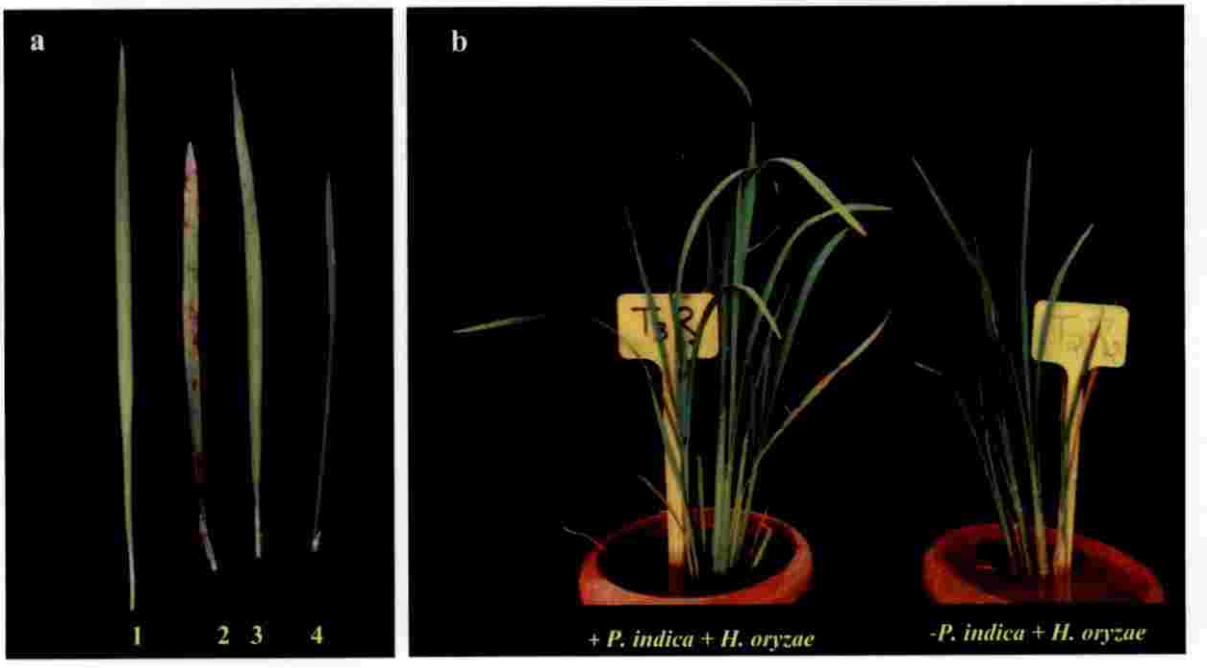
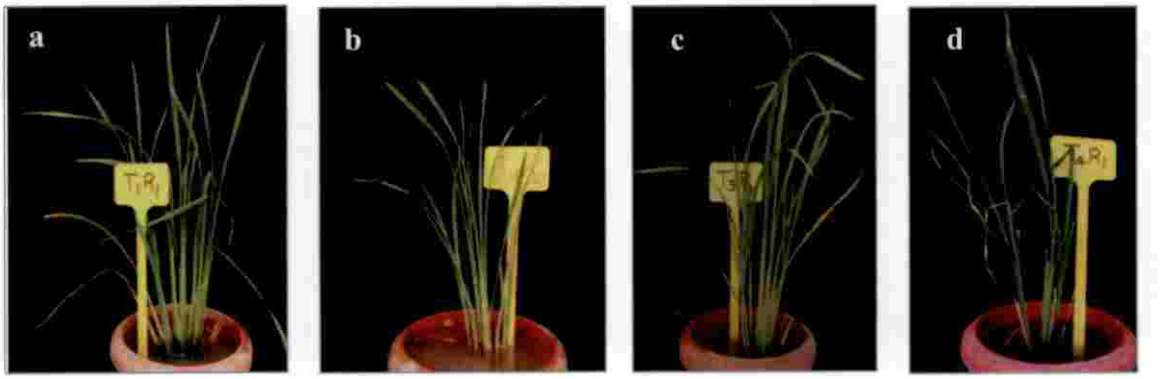
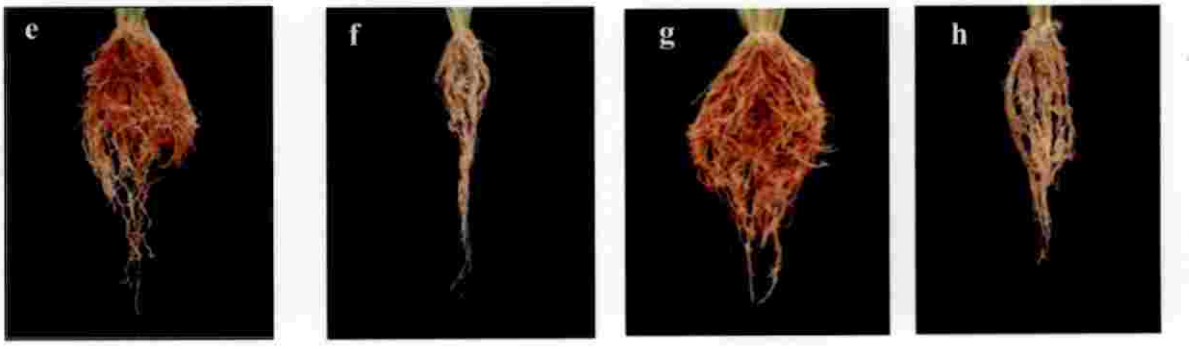


Plate 17. Severity of brown spot disease caused by *H. oryzae* in *P. indica*-primed rice seedlings var. Uma under *in vivo.* (a.1) *P. indica* alone, (a.2) *H. oryzae* alone , (a.3) *P. indica* followed by *H. oryzae*, and (a.4) control (b) disease severity of brown spot in *P. indica*-primed and control rice plants at 15th day after inoculation of *H. oryzae*.



P. indica alone *H. oryzae* alone *P. indica* + *H. oryzae* Control



P. indica alone *H. oryzae* alone *P. indica* + *H. oryzae* Control

Plate 18. ***In vivo*** **evaluation of** ***P. indica*****-primed rice plants var. Uma for growth promotion (a-d) Shoot growth of** ***P. indica*** **alone,** ***H. oryzae*** **alone,** ***P. indica*** **+** ***H. oryzae,*** **and control. (e-h) Root growth of** ***P. indica*** **alone,** ***H. oryzae*** **alone,** ***P. indica*** **+** ***H. oryzae,*** **and control.**

best strobilurin and their combination fungicides from *in vitro* were performed in *in vivo* (pot culture) experiment.

4.5.1. *In vitro* evaluation of fungicides against *H. oryzae*

Poisoned Food Technique

The efficacy of 6 different fungicides *viz.*, tebuconazole 25.9 % EC, difenoconazole 25 % EC, azoxystrobin 23 % SC, pyraclostrobin 20 % WG, pyraclostrobin 20 % WG + tebuconazole 25.9 % EC, and azoxystrobin 11% + tebuconazole 18.3 % SC at 10, 50, 100 and 250 ppm concentrations against the mycelial growth of *H. oryzae* was evaluated by poisoned food technique.

Among the six fungicides evaluated, at 10 ppm concentration on different intervals of incubation (3rd, 5th, 10th and 15th day), the lowest mycelial growth of *H. oryzae* on 10th day of incubation was recorded in azoxystrobin + tebuconazole combination fungicide (4.10 cm) followed by pyraclostrobin + tebuconazole combination (4.23 cm) (Table 19). At 50 ppm concentration the minimum mycelial growth was recorded in pyraclostrobin + tebuconazole combination (2.80 cm) followed by azoxystrobin + tebuconazole (3.60 cm) on 10th day of incubation (Table 20). In higher concentrations *viz.*, 100 and 250 ppm, the complete mycelial growth inhibition was recorded in pyraclostrobin + tebuconazole combination followed by azoxystrobin + tebuconazole (1.77 cm and 1.27 cm respectively) on 10th day of incubation (Table 21 and Table 22).

In vitro evaluation results revealed that among the tested azole fungicides, maximum inhibition to the mycelial growth of *H. oryzae* was recorded in tebuconazole at 250 ppm (Table 22 and Plate 19). Among the strobilurin fungicides, the highest inhibition was noticed in pyraclostrobin at 250 ppm (Table 22 and Plate 22). Both the tested combination fungicides significantly inhibited the mycelail growth of *H. oryzae* at 250 ppm. However the complete inhibition was recorded in pyraclostrobin + tebuconazole combination (Table 22 and plate 23).

Regarding the percentage growth inhibition of *H. oryzae* by different fungicides, maximum percentage growth inhibition at lower concentration (10

96

Table 19: Evaluation of new generation fungicides at 10 ppm on mycelial growth of *H. oryzae* causing brown spot of rice in PDA medium (Poisoned food technique)

Treatments (Fungicides)		Radial mycelial growth at 10 ppm (cm)				Nature of mycelial growth
		5th day	7th day	10th day	15th day	
T1	Tebuconazole 25.9% EC	4.37 ± 0.19	5.40 ± 0.11	6.83 ± 0.09	7.50 ± 0.12	Fluffy growth
T2	Difenoconazole 25 % EC	5.03 ± 0.07	6.27 ± 0.18	6.97 ± 0.03	8.10 ± 0.06	Cottony growth
T3	Azoxystrobin 23 % SC	5.63 ± 0.09	7.17 ± 0.09	8.10 ± 0.06	9.00 ± 0.00	Sparse growth
T4	Pyraclostrobin 20 % WG	3.90 ± 0.06	5.13 ± 0.09	6.68 ± 0.10	8.23 ± 0.09	Cottony growth
T5	Pyraclostrobin 20%+ Tebuconazole 25.9% EC	2.40 ± 0.06	3.43 ± 0.03	4.23 ± 0.03	5.73 ± 0.09	Cottony growth
T6	Azoxystrobin 11 % + Tebuconazole 18.3 % SC	2.10 ± 0.06	3.00 ± 0.06	4.10 ± 0.06	5.40 ± 0.06	Cottony growth
T7	Control	7.12± 0.05	8.89 ± 0.06	9 ± 0.00	9 ± 0.00	Fluffy growth
	SEm±	0.092	0.096	0.062	0.071	
	CD (0.05)	0.281	0.294	0.190	0.218	

Values are mean of three replications ± standard deviation

Table 20: Evaluation of new generation fungicides at 50 ppm on mycelial growth of *H. oryzae* causing brown spot of rice in PDA medium (Poisoned food technique)

Treatments (Fungicides)		Radial mycelial growth at 50 ppm (cm)				Nature of mycelial growth
		5th day	7th day	10th day	15th day	
T1	Tebuconazole 25.9% EC	3.37 (2.09 ± 0.008)	4.13 (2.27 ± 0.02)	5.33 (2.57 ± 0.007)	6.23 (2.69 ± 0.02)	Fluffy growth
T2	Difenoconazole 25 % EC	4.35 (2.35 ± 0.014)	5.33 (2.52 ± 0.03)	5.80 (2.61 ± 0.01)	6.63 (2.76 ± 0.02)	Cottony growth
T3	Azoxystrobin 23 % SC	5.03 (2.46 ± 0.030)	6.47 (2.73 ± 0.04)	7.73 (2.95 ± 0.02)	9.0 (3.16 ± 0.00)	Sparse growth
T4	Pyraclostrobin 20 % WG	3.33 (2.08 ± 0.008)	4.10 (2.26 ± 0.01)	5.70 (2.59 ± 0.01)	7.30 (2.88 ± 0.03)	Cottony growth
T5	Pyraclostrobin 20%+ Tebuconazole 25.9% EC	0.60 (1.264 ± 0.02)	1.13 (1.46 ± 0.03)	2.80 (1.95 ± 0.044)	2.87 (1.97 ± 0.03)	Cottony growth
T6	Azoxystrobin 11 % + Tebuconazole 18.3 % SC	1.67 (1.63 ± 0.027)	2.40 (1.84 ± 0.03)	3.60 (2.14 ± 0.013)	4.90 (2.43 ± 0.01)	Cottony growth
T7	Control	7.12 (2.85 ± 0.008)	8.89 (3.1 ± 0.001)	9 (3.16 ± 0.00)	9 (3.16 ± 0.00)	Fluffy growth
	SEm±	0.019	0.027	0.020	0.019	
	CD (0.05)	0.058	0.082	0.062	0.058	

Values are mean of three replications ± standard deviation; Values in parenthesis are square root transformed values

Table 21: Evaluation of new generation fungicides at 100 ppm on mycelial growth of *H. oryzae* causing brown spot of rice in PDA medium (Poisoned food technique)

Treatments (Fungicides)		Mycelial growth in 100 ppm (cm)				Nature of mycelial growth
		5th day	7th day	10th day	15th day	
T1	Tebuconazole 25.9% EC	2.57 (1.9 ± 0.009)	3.03 (2.01 ± 0.008)	3.57 (2.1 ± 0.008)	4.27 (2.3 ± 0.007)	Fluffy growth
T2	Difenoconazole 25 % EC	1.3 (1.5 ± 0.038)	2.56 (1.8 ± 0.039)	2.83 (1.96 ± 0.02)	3.30 (2.07 ± 0.01)	Cottony growth
T3	Azoxystrobin 23 % SC	4.93 (2.4 ± 0.007)	6.10 (2.6 ± 0.039)	7.03 (2.83 ± 0.02)	8.70 (3.1 ± 0.009)	Sparse growth
T4	Pyraclostrobin 20 % WG	3.33 (2.08 ± 0.01)	4.23 (2.3 ± 0.026)	5.23 (2.45 ± 0.03)	7.13 (2.85 ± 0.01)	Cottony growth
T5	Pyraclostrobin 20% + Tebuconazole 25.9% EC	0.00 (1.0 ± 0.00)	0.00 (1.0 ± 0.00)	0.00 (1.0 ± 0.00)	0.00 (1.0 ± 0.00)	No growth
T6	Azoxystrobin 11 % + Tebuconazole 18.3 % SC	1.57 (1.6 ± 0.04)	1.67 (1.63 ± 0.027)	1.77 (1.6 ± 0.036)	2.07 (1.75 ± 0.01)	Cottony growth
T7	Control	7.12 (2.85 ± 0.008)	8.89 (3.1 ± 0.001)	9 (3.16 ± 0.00)	9 (3.16 ± 0.00)	Fluffy growth
	SEm±	0.019	0.027	0.020	0.01	
	CD (0.05)	0.059	0.083	0.063	0.03	

Values are mean of three replications ± standard deviation; Values in parenthesis are square root transformed values

Table 22: Evaluation of new generation fungicides at 250 ppm on mycelial growth of *H. oryzae* causing brown spot of rice in PDA medium (Poisoned food technique)

Treatments (Fungicides)		Mycelial growth in 250 ppm (cm)				Nature of mycelial growth
		5th day	7th day	10th day	15th day	
T1	Tebuconazole 25.9% EC	0.53 (1.2 ± 0.013)	1.03 (1.43 ± 0.01)	1.20 (1.48 ± 0.02)	1.43 (1.56 ± 0.02)	Fluffy growth
T2	Difenoconazole 25 % EC	1.00 (1.4 ± 0.020)	1.83 (1.68 ± 0.04)	2.06 (1.75 ± 0.02)	2.13 (1.77 ± 0.009)	Cottony growth
T3	Azoxystrobin 23 % SC	4.27 (2.3 ± 0.026)	5.17 (2.48 ± 0.03)	6.10 (2.66 ± 0.03)	7.10 (2.85 ± 0.02)	Sparse growth
T4	Pyraclostrobin 20 % WG	3.23 (2.06 ± 0.042)	4.00 (2.24 ± 0.01)	5.00 (2.45 ± 0.01)	6.10 (2.66 ± 0.011)	Cottony growth
T5	Pyraclostrobin 20%+ Tebuconazole 25.9% EC	0.00 (1.0 ± 0.00)	0.00 (1.0 ± 0.00)	0.00 (1.0±0.00)	0.00 (1.0 ± 0.00)	No growth
T6	Azoxystrobin 11 % + Tebuconazole 18.3 % SC	0.57 (1.25 ± 0.026)	1.10 (1.45 ± 0.02)	1.27 (1.5 ± 0.011)	1.47 (1.57 ± 0.01)	Cottony growth
T7	Control	7.12 (2.85 ± 0.008)	8.89 (3.1 ± 0.001)	9 (3.16 ± 0.00)	9 (3.16 ± 0.00)	Fluffy growth
	SEm±	0.024	0.023	0.016	0.012	
	CD (0.05)	0.074	0.070	0.049	0.038	

Values are mean of three replications ± standard deviation; Values in parenthesis are square root transformed values

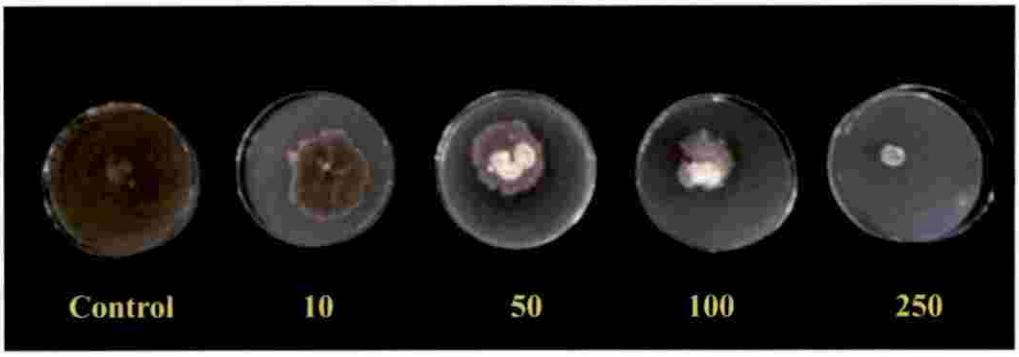


Plate 19. Effect of different concentrations of tebuconazole (ppm) on mycelial growth of *H. oryzae*.

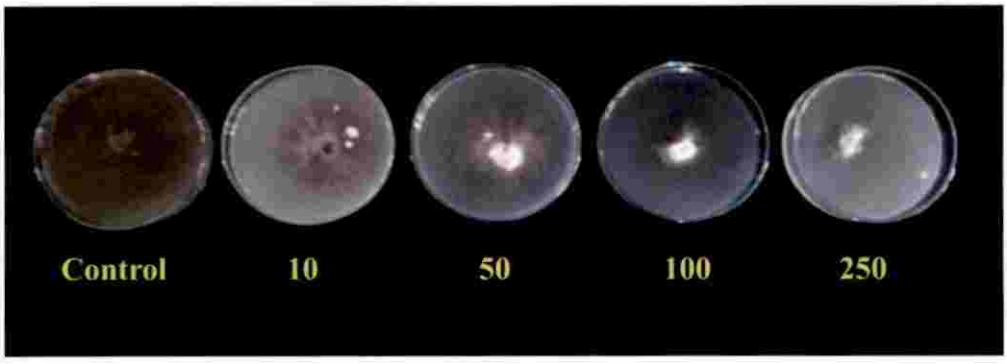


Plate 20. Effect of different concentrations of difenoconazole (ppm) on mycelial growth of *H. oryzae*.

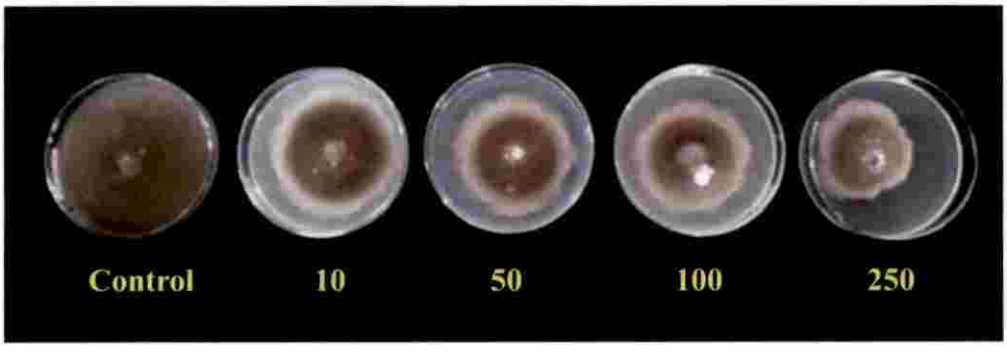


Plate 21. Effect of different concentrations of azoxystrobin (ppm) on mycelial growth of *H. oryzae*.

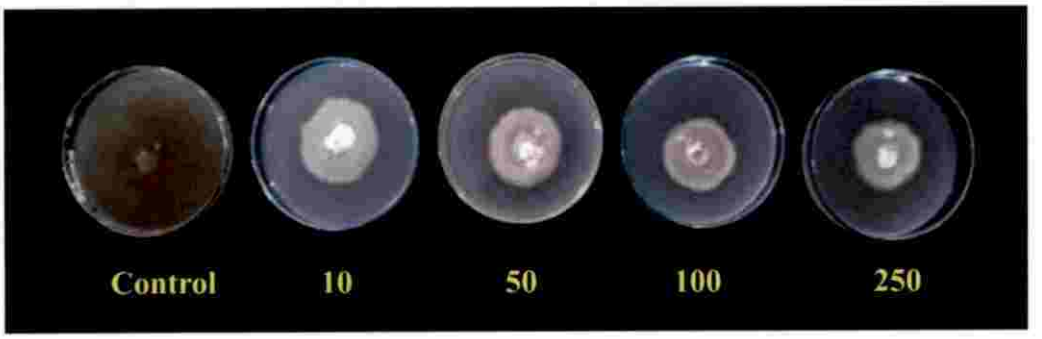


Plate 22. Effect of different concentrations of pyraclostrobin (ppm) on mycelial growth of *H. oryzae.*

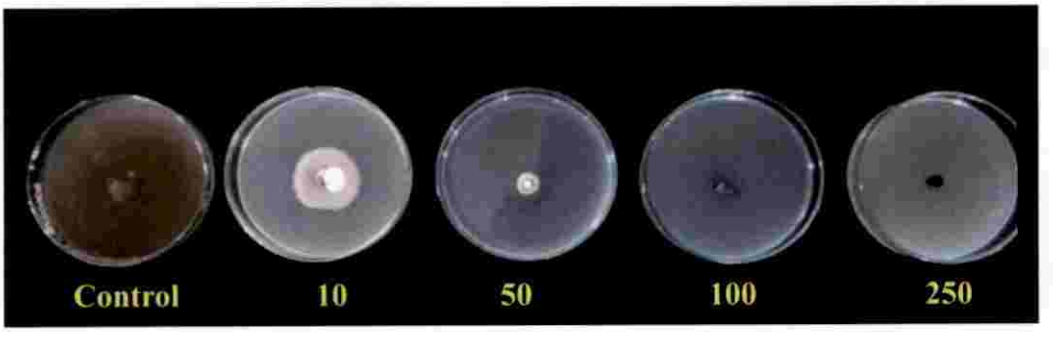


Plate 23. Effect of different concentrations of pyraclostrobin + tebuconazole (ppm) on mycelial growth of *H. oryzae.*

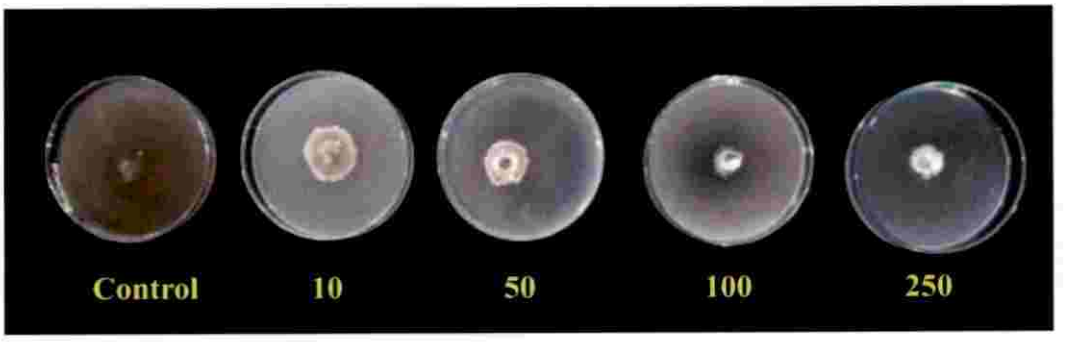


Plate 24. Effect of different concentrations of azoxystrobin + tebuconazole (ppm) on mycelial growth of *H. oryzae.*

ppm) was exhibited by azoxystrobin + tebuconazole (66.60) followed by pyraclostrobin + tebuconazole (61.8). However, at higher concentration (50 ppm) the maximum growth inhibition was recorded with pyraclostrobin + tebuconazole (87.3) followed by azoxystrobin + tebuconazole (73.30). At 100 and 250 ppm concentrations, the highest growth inhibition was recorded with pyraclostrobin + tebuconazole followed by azoxystrobin + tebuconazole (82.56 and 87.70 respectively) (Table 23).

4.5.2. *In vivo* evaluation of fungicides against *H. oryzae*

A pot culture experiment was conducted at Dept. of Plant Pathology, College of Agriculture, Vellayani during June-July 2019 to find the effect of the best azole, strobilurin and combination fungicides at recommended and higher doses from *in vitro* evaluation of fungicides. The experiment was designed in CRD with 4 replications and the variety used was 'Uma' (Plate 25).

Thirty five-day old rice plants were inoculated with mycelial suspension of *H. oryzae* and sufficient leaf spot symptoms developed after 5 days of inoculation. The treatments imposed for the infected plants included, tebuconazole 25.9 % EC (0.05% and 0.1%), pyraclostrobin 20 % WG (0.05% and 0.1%), pyraclostrobin 20 % WG + tebuconazole 25.9 % EC (0.05%), azoxystrobin 11% + tebuconazole 18.3 % SC (0.1%) and trifloxystrobin + tebuconazole (0.05%) - positive control along with *H. oryzae* treated control and untreated control. Lesion size of brown spot of rice was recorded for each treatment at different days after spraying (DAS). Among the treatments the lowest lesion size was recorded with trifloxystrobin + tebuconazole (0.65 cm) followed by azoxystrobin + tebuconazole (0.84 cm) and pyraclostrobin + tebuconazole (1.17 cm) on 15 DAS (Table 24).

Regarding the disease severity, PDI was calculated before imposing the treatments and it ranged from 28.76 to 33.70 per cent. Further, variation in PDI due to the effect of treated fungicides was recorded subsequently on 3, 5, 7, 10 and 15 days after spraying (Table 25). On 3rd day after treatment, an increase in disease severity was recorded in all treatments. But the increase in disease severity was less in plants treated with higher doses of fungicides compared to lower

Table 23: Percentage growth inhibition of *H. oryzae* causing brown spot of rice in PDA medium (Poisoned food technique)

Treatments (Fungicides)		Percentage growth inhibition at			
		10 ppm	50 ppm	100 ppm	250 ppm
T1	Tebuconazole 25.9% EC	39.97(39.19)^{d}	53.86(47.19)^{c}	66.96(54.89)^{d}	88.43(70.09)^{b}
T2	Difenoconazole 25 % EC	30.33(33.39)^{e}	40.7(39.62)^{e}	71.43(57.69)^{c}	79.96(63.44)^{c}
T3	Azoxystrobin 23 % SC	20.7(27.04)^{f}	28.1(31.95)^{f}	32.16(34.51)^{f}	42.53(40.68)^{e}
T4	Pyraclostrobin 20 % WG	42.93(40.92)^{c}	52.93(46.66)^{d}	52.2(46.24)^{e}	54.76(47.72)^{d}
T5	Pyraclostrobin 20%+ Tebuconazole 25.9% EC	61.8(51.82)^{b}	87.3(61.15)^{a}	100(90.00)^{a}	100(90.00)^{a}
T6	Azoxystrobin 11 % + Tebuconazole 18.3 % SC	66.60(54.68)^{a}	73.30(58.87)^{b}	82.56(65.32)^{b}	87.7(69.45)^{b}
	SEm±	0.624	0.88	0.783	0.740
	CD (0.05)	1.97	2.56	2.27	2.18

Mean ± SD of three replications; Values followed by similar Values in parenthesis are arc sine transformed values

Table 24: Lesion size of brown spot of rice caused by *H. oryzae* in rice var. Uma under *in vivo* evaluation of new generation fungicides

Treatments		Lesion size of brown spot (cm) at different intervals				
		3rd day	5th day	7th day	10th day	15th day
T1	Tebuconazole 25.9% EC	1.70 ± 0.039	2.91 ± 0.028	3.14 ± 0.031	2.99 ± 0.043	2.24 ± 0.031
T2	Difenoconazole 25 % EC	1.73 ± 0.028	2.53 ± 0.048	3.05 ± 0.037	2.56 ± 0.031	1.69 ± 0.027
T3	Azoxystrobin 23 % SC	1.86 ± 0.042	3.24 ± 0.040	3.75 ± 0.034	3.69 ± 0.028	2.99 ± 0.048
T4	Pyraclostrobin 20 % WG	1.80 ± 0.058	2.94 ± 0.022	3.49 ± 0.050	3.19 ± 0.041	2.40 ± 0.039
T5	Pyraclostrobin 20% + Tebuconazole 25.9% EC	1.97 ± 0.051	2.69 ± 0.038	3.11 ± 0.028	2.96 ± 0.040	1.17 ± 0.073
T6	Azoxystrobin 11 % + Tebuconazole 18.3 % SC	1.89 ± 0.040	2.35 ± 0.034	2.62 ± 0.071	2.40 ± 0.037	0.84 ± 0.071
T7	Trifloxystrobin (25%) + Tebuconazole (50%)	1.88 ± 0.053	2.21 ± 0.038	2.42 ± 0.036	2.13 ± 0.040	0.65 ± 0.053
T8	*H. oryzae* (treated control)	2.05 ± 0.048	3.61 ± 0.035	3.88 ± 0.038	4.01 ± 0.082	4.09 ± 0.089
T9	Untreated control	0.99 ± 0.091	1.75 ± 0.031	3.09 ± 0.035	3.32 ± 0.037	3.92 ± 0.033
SE(m)±		0.054	0.035	0.065	0.048	0.055
CD(0.05)		0.151	0.099	0.129	0.136	0.155

Mean ± SD of ten replications

Table 25: Severity of brown spot of rice caused by *H. oryzae* in rice var. Uma under *in vivo* evaluation of new generation fungicides

Treatments		Severity of brown spot at different intervals					
		Before treatment	3 DAT	5 DAT	7 DAT	10 DAT	15 DAT
T1	Tebuconazole 25.9% EC	29.46 (32.87)^{e}	30.49 (33.51)^{e}	46.25 (42.84)^{c}	52.46 (46.41)^{d}	49.08 (44.47)^{d}	37.97 (38.03)^{e}
T2	Difenoconazole 25 % EC	31.26 (33.99)^{d}	31.94 (34.40)^{de}	42.77 (40.84)^{d}	48.17 (43.95)^{e}	44.60 (41.89)f	31.77 (34.30)^{f}
T3	Azoxystrobin 23 % SC	28.76 (32.43)^{e}	33.86 (35.57)^{cd}	53.9 (47.25)^{b}	61.47 (51.63)^{b}	58.17 (49.70)^{b}	46.90 (43.22)^{c}
T4	Pyraclostrobin 20 % WG	31.92 (34.39)^{cd}	33.39 (35.30)^{cd}	47.30 (43.45)^{c}	56.87 (48.95)^{c}	53.87 (47.22)^{c}	41.50 (40.14)^{d}
T5	Pyraclostrobin 20% + Tebuconazole 25.9% EC	33.70 (35.48)^{a}	36.48 (37.15)^{ab}	45.66 (42.51)^{c}	53.53 (47.02)^{d}	46.61 (43.05)^{e}	21.01 (27.26)^{g}
T6	Azoxystrobin 11 % + Tebuconazole 18.3 % SC	29.06 (32.62)^{e}	33.64 (35.45)^{cd}	40.56 (39.56)^{e}	46.05 (42.73)^{f}	41.17 (40.26)^{g}	17.46 (24.69)^{h}
T7	Trifloxystrobin (25%) + Tebuconazole (50%)	32.17 (34.55)^{bcd}	34.28 (35.84)^{bc}	37.93 (38.01)^{f}	41.97 (40.38)^{g}	36.62 (37.24)^{h}	10.81 (19.81)^{i}
T8	*H. oryzae* (treated control)	33.48 (35.35)^{ab}	37.61 (37.82)^{a}	57.62 (49.38)^{a}	63.48 (52.82)^{a}	73.57 (59.07)^{a}	93.40 (75.34)^{a}
T9	Untreated control	10.81 (19.18)^{f}	21.01 (27.25)^{f}	31.95 (34.41)^{g}	49.81 (44.89)^{e}	54.37 (47.51)^{c}	73.21 (58.83)^{b}
SE(m)±		1.120	1.409	0.986	2.198	0.34	0.57
CD(0.05)		1.24	1.41	1.12	1.09	0.968	1.3

Mean of four replications.Values followed by similar superscripts are not significantly different at 5% level. Values in parenthesis are arcsine transformed values DAT: Days after treatment

Plate 25. General view of pot culture experiment for the evaluation of selected new generation fungicides against *H. oryzae*

doses. On 3 DAS, the lowest disease severity was recorded in plants treated with tebuconazole 0.05 per cent and 0.1 per cent (30.49 % and 31.94 % respectively). On 5th day the lowest disease severity was recorded in plants treated with trifloxystrobin + tebuconazole (37.93 %) followed by azoxystrobin + tebuconazole (40.56 %) and pyraclostrobin + tebuconazole (45.66 %). Similarly on 7th day after spraying, an increase in disease severity in treated plants were noticed. However the plants treated with trifloxystrobin + tebuconazole (41.97 %) followed by azoxystrobin + tebuconazole (46.05%) and pyraclostrobin + tebuconazole (53.53%) exhibited a decreased rise in disease severity. Decrease in disease severity after imposing treatments was noticed on 10th day; the minimum disease severity was recorded in plants treated with trifloxystrobin + tebuconazole (36.62 %) followed by azoxystrobin + tebuconazole (41.17 %) and pyraclostrobin + tebuconazole (46.61 %). Disease severity of brown spot on 15 days after fungicide treatment indicated that there was a remarkable decrease in PDI due to different treatments. The lowest PDI was recorded in plants treated with trifloxystrobin + tebuconazole (10.81 %) followed by azoxystrobin + tebuconazole (17.46 %) and pyraclostrobin + tebuconazole (21.01 %) (Table 25, Plate 26).

On the basis of PDI value at 15 days after treatment, 'disease suppression over control' was calculated. The result indicated that at recommended dosage, highest disease suppression was recorded by positive control; i.e. trifloxystrobin + tebuconazole (88.42 %) followed by azoxystrobin + tebuconazole (81.30 %) and pyraclostrobin + tebuconazole (77.52 %) (Table 26, Plate 27).

New generation fungicides *viz.*, triazoles, strobilurins and their combinations effectively managed brown spot disease of rice by reducing the disease incidence and severity to result in a better disease suppression. The results clearly indicated the curative action new generation fungicides on the infected plants. There was an effective hindering of disease spread from affected plant parts to the non-affected area; but the opposite trend was clearly visible in control plants which exhibited the typical leaf spot, leaf blight and leaf withering symptoms with increased incidence and severity of the disease.

Table 26: Brown spot disease suppression over control in rice var. Uma due to different new generation fungicides at recommended and double the recommended dose

Treatments	Fungicides and dosage	Disease suppression over control on 15th day (%)
T1	Tebuconazole 25.9 EC - 0.05 %	59.34
T2	Tebuconazole 25.9 EC - 0.1 %	66.05
T3	Pyraclostrobin 20 WG - 0.05 %	49.79
T4	Pyraclostrobin 20 WG - 0.1 %	55.56
T5	Pyraclostrobin 20 WG + Tebuconazole 25.9 EC - 0.05 %	77.52
T6	Azoxystrobin 11% + Tebuconazole 18.3% SC - 0.1 %	81.30
T7	Trifloxystrobin 25% + Tebuconazole 50% SC - 0.05 %	88.42
T8	*H. oryzae* treated control	0
T9	*H. oryzae* untreated control	21.62

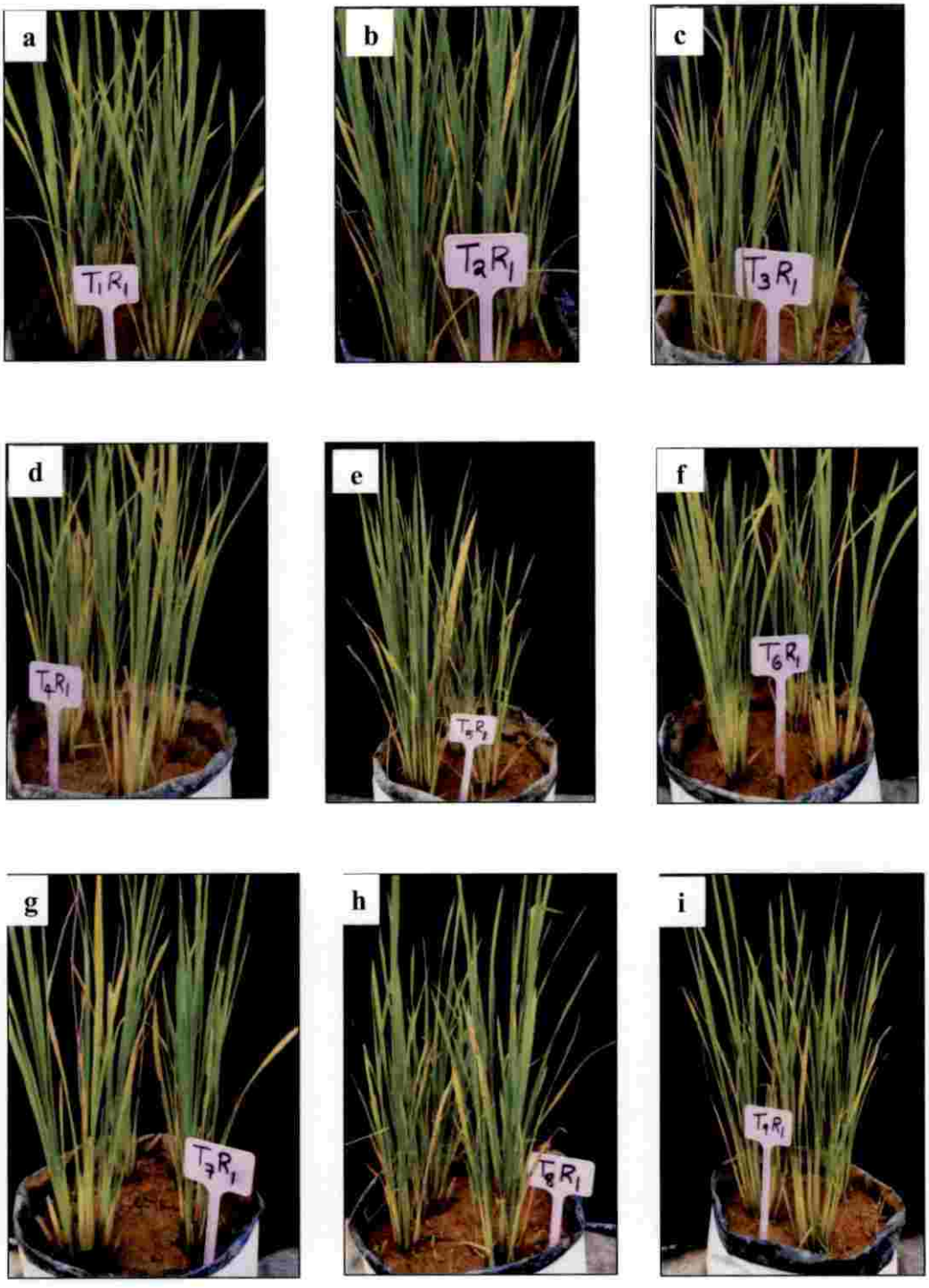


Plate 26. ***In vivo*** **evaluation of different new generation fungicides against brown spot of rice caused by** ***H. oryzae;*** **rice plants before treatments (a) Tebuconazole 0.05 %; (b) Tebuconazole 0.1 %; (c) Pyraclostrobin 0.05 %; (d) Pyraclostrobin 0.1 %; (e) Pyraclostrobin + Tebuconazole 0.05 %; (f) Azoxystrobin + Tebuconazole 0.1 %; (g) Trifloxystrobin +Tebuconazole 0.05 % - positive control; (h)** ***H. oryzae*** **(treated control) (i) Untreated control**

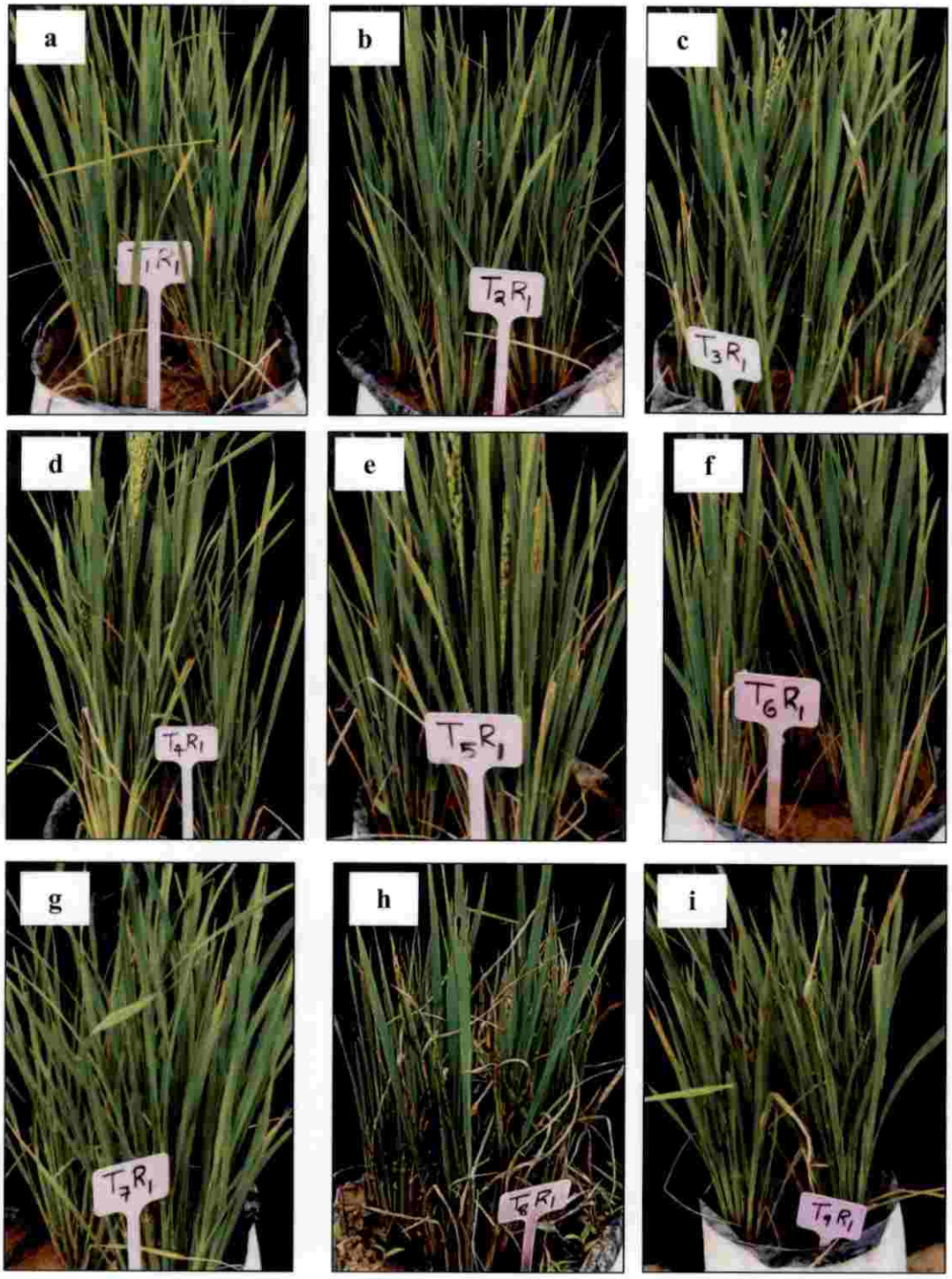


Plate 27. ***In vivo*** **evaluation of different fungicides against brown spot of rice caused by** ***H. oryzae,*** **rice plants after treatments (a) Tebuconazole 0.05% (b) Tebuconazole 0.1% (c) Pyraclostrobin 0.05% (d) Pyraclostrobin 0.1% (e) Pyraclostrobin+ Tebuconazole 0.05% (f) Azoxystrobin+Tebuconazole 0.1% (g) Trifloxystrobin+Tebuconazole 0.05% -positive control (h)** ***H. oryzae*** **(treated control) (i) Untreated control**

Discussion

5. DISCUSSION

Rice is a principle food grain that feeds more than half of the world population and appreciably meets the caloric requirement. The global rice production system is facing various threats and among them, the yield reduction attributed by rice diseases is of particular significance. Major rice diseases includes blast, sheath blight, brown spot, bacterial leaf blight and rice tungro. Brown spot of rice incited by *Helminthosporium oryzae* is an important disease responsible for the reduction in quantity and quality of rice grains. Since the conventional chemical management strategies of brown spot are not safe to the environment, it is necessary to find eco-friendly alternatives for the disease management.

Piriformospora indica is a multifunctional axenically culturable fungal root endophyte belonging to Sebacinales of Basidiomycota (Weib *et al.*, 2011). Root colonization with the endophyte resulted in enhanced growth and increased stress tolerance to the host plants. Since the fungus lacks host specificity there is a wide host range for *P. indica*. Apart from the abiotic stress tolerance *viz.,* drought and salinity, the fungus conferred biotic stress tolerance *viz.,* tolerance to root and foliar fungal, bacterial, viral and nematode diseases (Waller *et al.*, 2005; Serfling *et al.*, 2007; Oelmuller *et al.*, 2009; Fakhro *et al.*, 2010; Athira , 2018; Varkey *et al.*, 2018).

Currently farmers rely on various conventional fungicides to manage the brown spot disease. Taking into account of resistance development of pathogen against many of fungicides, it is high time to look for alternative strategies and safe chemicals to manage the disease. New generation fungicides are the new molecules with very specific action and possess higher efficacy even at lower concentration against a wide range of pathogenic fungi. In this context, the present study entitled 'Management of brown spot disease of rice using fungal root endophyte *Piriformospora indica* and new generation fungicides' was under taken with the objectives of evaluation of the effect of a beneficial root endophyte

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P. indica and new generation fungicides (triazoles and strobilurins) alone and in combination against *H. oryzae* for the management of brown spot of rice. Discussion of the obtained results are included in this chapter.

5. SURVEY AND COLLECTION OF BROWN SPOT AFFECTED LEAF SAMPLES FROM DIFFERENT RESEARCH STATIONS OF KAU, ISOLATION OF PATHOGEN, PROVING KOCH'S POSTULATE AND MAINTENANCE OF THE CULTURE.

5.1.1. Survey and Collection of Brown Spot Affected Samples

A survey was conducted in the rice fields of 6 locations of Kerala *viz.,* Vellayani, Karamana, Moncombu, Kumarakom, Vyttila and Pattambi and brown spot disease was observed as an important disease of rice incited by *H. oryzae.* Association of *H. oryzae* with brown spot disease of rice in India was first reported by Sundararaman from Madras in 1922 (Baranwal *et al.*, 2013). In accordance with the observations of Ou (1985); Chakrabarthi (2001); Harish *et al.* (2007); and Kumar *et al.* (2016) the present study also described the causal fungus of brown spot of rice as *H. oryzae.*

The disease was found to be positively correlated with favourable weather conditions (Padmanabhan, 1973). Further Choudhary *et al.* (2019) reported that the disease incidence of brown spot was not effectively related with the temperature fluctuation, whereas, the relative humidity was highly correlated with the disease incidence. Accordingly, in the present study also higher disease prevalence was recorded in locations with higher relative humidity. Pannu *et al.* (2005) described that there was a remarkable reduction in disease severity in years of high rainfall in comparison with lower rainfall year in Punjab. In contradictory to the above study, it was found in the current study that season followed by heavy rain fall resulted in higher disease severity. Alawa (2017) recorded positive correlation of high incidence and severity of the brown spot disease with high rainfall which also supports the present investigation.

The survey revealed that the brown spot disease incidence and severity varied from location to location (24 – 76 % and 34.6 to 64.6 % respectively (Figure 1 and 2). The variation in disease incidence and severity was due to the variation climatic condition, diversity of rice varieties grown and the pathogenicity of *H. oryzae* isolates present at different survey locations. The obtained results were in agreement with the observations of survey conducted in 15 different rice cultivation areas of Tamil Nadu by Harish *et al.* (2007). It was reported that the disease severity varied from 20 - 80.74 per cent. An extensive survey conducted in various geographical locations of India representing major rice growing regions by Kumar *et al.* (2016) where a disease incidence of 8.11 to 42.37 per cent was recorded.

5.1.2 Symptomatology

H. oryzae infection on the rice plants from young seedling to mature grains resulted in various symptoms such as seedling blight, leaf spot, leaf blight, panicle blight and grain discoloration. Leaf spots appeared as round to oval shaped brown spots with a yellow halo which later progressed as leaf lesion. On mycelial inoculation, detached leaves developed brown to black lesions with yellow halo that eventually led to the complete leaf destruction within a shorter period. Brown to dark brown lesions appeared on panicle at the joining point of flag leaf to stalk which later extended as wet rotting of sheath resulting in chaffy grains. The most damaging form of the disease was grain discoloration in which dark brown to black spots appeared on the developing grains and in severe cases, the entire grain surface was covered with dark brown conidiophore and conidia of pathogen which resulted the velvety fungal mass on the grain surface. Such severe symptoms were observed under higher humidity. Chaffy and discoloured grains were observed in severely infected plants.

The observed symptoms were similar to the descriptions given by Sunder *et al.* (2014) whom described about symptoms from seedling blight to grain discoloration. They also pointed out that the lesion dimensions developed by the pathogen could be more than 1 cm on a susceptible cultivar. Sunder *et al.* (2005)

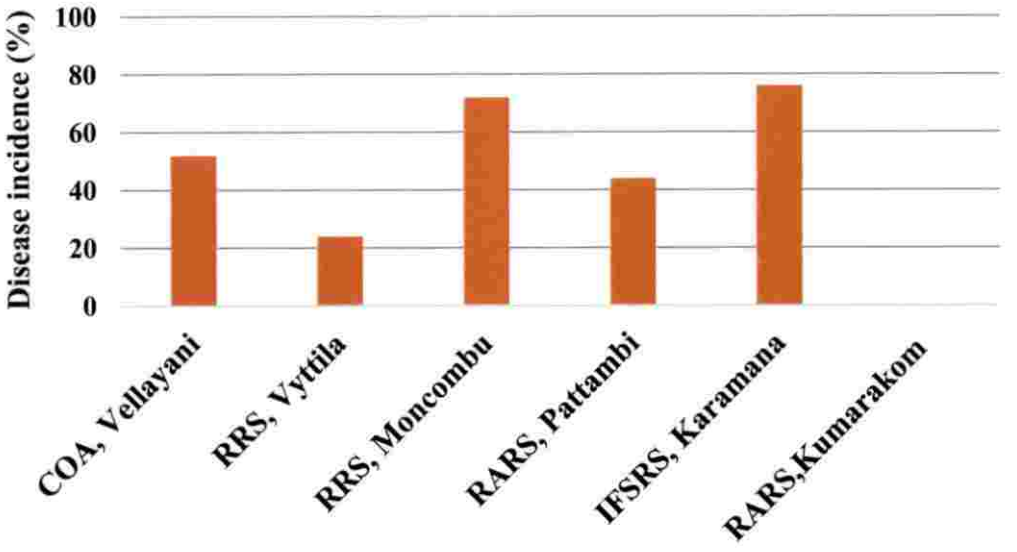

Figure 1: Brown spot disease incidence in different survey locations

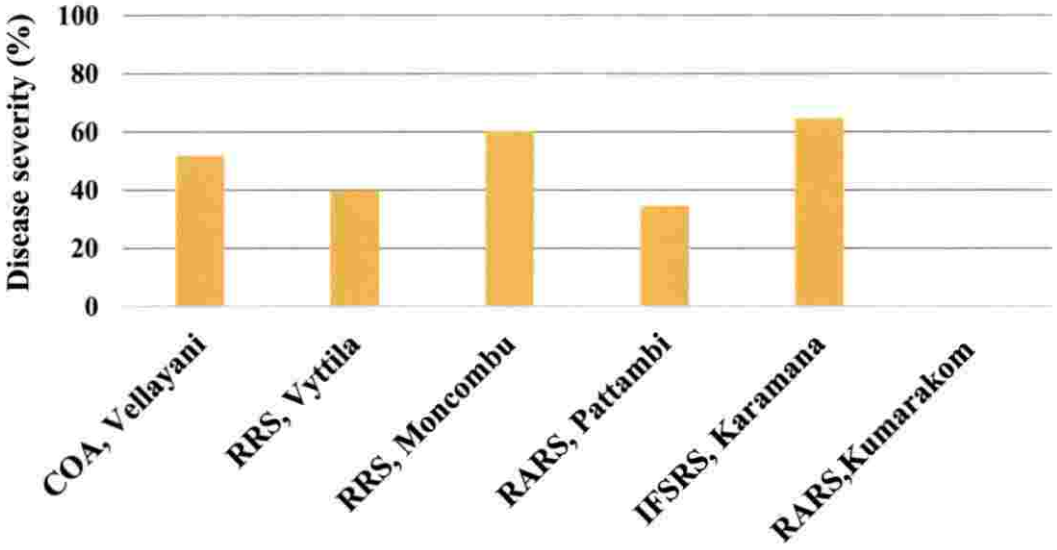

Figure 2: Brown spot disease severity in different survey locations

also reported that panicle blight symptoms appeared as dark brown lesion at the point of flag leaf and stalk which led to wet rotting of sheath. Quintana *et al.* (2017) described the disease symptoms as seedling blight, leaf and culm infection, and kernel infection. They also noticed that the leaf spot symptoms appeared throughout the growing season, mostly on leaf blade, which were in similarity with the current study.

Symptomatological studies of the present investigation indicated about development of the symptoms from seedling to crop maturity. Prominent symptoms such as leaf spots, blight to partial or complete grain discoloration and chaffy grains that could even cause complete damage to the crop. Utilization of infected grains could result in poor germination and seedling damping off. Hence the use of healthy seeds could be an effective strategy of the disease management. The identification of symptoms at early stages of pathogen infection could be helpful in better management of any disease.

5.2. MORPHOLOGICAL AND CONIDIAL CHARACTERS OF *H. oryzae*

All the five isolates of *H. oryzae* varied in morphological characters. The mycelial colour of isolates varied from white, grey and black. Mycelium of isolate 1 was grey to black in colour in upper side and black in reverse side with a fluffy growth and regular margin of growth. The isolate 2 was white with grey in upper side, brownish black in the reverse side and it has an irregular margin with a fluffy nature of growth. Isolate 3 and isolate 5 isolates had grey to black mycelial growth and black in the reverse side, isolate 1 had smooth mycelial growth with irregular margin whereas isolate 5 had fluffy mycelial growth with regular margin. Mycelium of isolate 4 was in the upper side and brown to black in the reverse side and it got a cottony growth with regular margin.

Sonavane *et al.* (2015) also observed the variation in mycelial growth of different *H. oryzae* isolates as fluffy with cottony aerial mycelium. Variation in radial growth of the isolates ranged from 2.2 cm to 8.45 cm. The variation in texture, margin and form of the cultures, the observed characters in the isolates of

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the pathogen were smooth or rough, regular or irregular and entire or undulate. Mycelial colour of the *H. oryzae* isolates varied from gery and black.

Regarding the radial mycelial growth of five isolates, the maximum diameter of growth of 8.93 cm was recorded by isolate 1, which was on par with isolate 5 (8.89 cm). The days taken for complete coverage of 9 cm petridish was maximum (11 days) for isolate 4 and minimum (7 days) for isolate 1 and isolate 5 (Figure 3).

Microscopic characters of different isolates through slide culture technique revealed that the mycelium of the fungus was slender and septate. The mycelial width of isolates varied from 1.49 µm to 2.81 µm. The fungus exhibited poor sporulation under lab conditions. Sporulation was observed in isolate 1 and isolate 5, where both produced brown coloured slightly curved conidia with wide centre. Conidial dimensions were higher for isolate 5 (length 50 µm and width 5.5 µm) followed by isolate 1 (length 46.5 µm and width 6.6 µm).

The mycelium of *H. oryzae* was inter and intra-cellular and greyish to olive or black in colour. The conidia had 5-10 septae and the oldest conidium arranged towards base of the conidiophore. The conidia were slightly curved with widest middle portion. Fully aged conidia were fuliginous or brownish with a dimension of 99 - 345 µm x 7-11 µm (Ou, 1985).

Alawa (2017) found that conidial shape of *H. oryzae* isolates varied from fusoid, navicular, oblong, ovoid and curved to straight. Results of the present study were in accordance with the observation of Valarmathi and Ladhalakshmi (2018) who described *H. oryzae* isolates with black fluffy growth, grey fluffy growth, white spot growth and grey cottony growth. Conidia of isolates varied in shape *viz.,* curved, fusoid or obclavate with mild to golden brown colour and 5-6 septae. Length and bredth of conidia varied from 56.89-113.32 µm x 13.75-27.41 µm. Jaiganesh and Kannan (2019) reported that all the five isolates of *H. oryzae* had septate, branched, olivaceous, light brown to black, profuse aerial / submerged mycelia. Conidial colouration was light brown to brown. Shape was

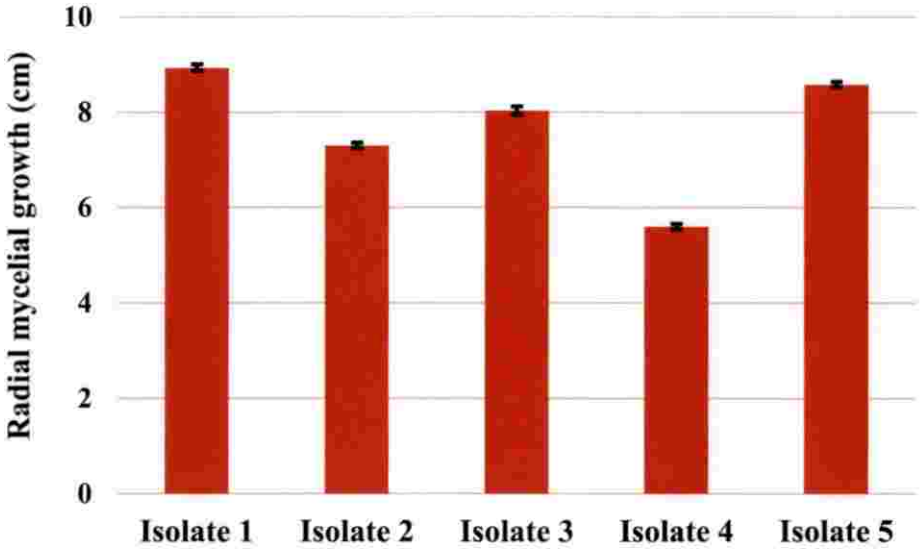

Figure 3: Radial mycelial growth of different isolates of *H. oryzae* in PDA medium on 7th day after inoculation

slightly curved with a middle bulge and tapering end. Conidial dimensions were 29.3-33.2 µm x 13.5-14.8 µm. The above observations were in tandem with the present study.

Absence of sporulation in *H. oryzae* on artificial and natural media under different environmental as well as nutritional conditions has been reported in different studies (Leach, 1961; Hau and Rush, 1980; Kulkarni *et al.,* 1980; Valarmathi and Ladhalakshmi 2018). Morphological studies on appearance, colour and rate of growth of mycelia and microscopic studies on conidial characteristics showed a significant difference among the isolates suggesting the variability among *H. oryzae* isolates of Kerala.

5.3. PATHOGENICITY TESTING AND SCREENING OF ISOLATES

The studies on pathogenicity and comparative virulence of the isolates of *H. oryzae* were done following Koch's postulates. On artificial inoculation on to the rice leaves, the fungus produced the characteristic symptoms as brown spots and lesions. The fungus re-isolated from the artificially inoculated leaves was similar to the original isolates in its morphological and cultural characters. Dallagnol *et al.* (2015) also proved Koch's postulates of *H. oryzae* by artificial inoculation. Symptom development was observed within 18 h after inoculation as brown coloured leaf spot on leaves. Manandhar *et al.* (2016) observed symptoms on rice leaves, as small, circular, and dark brown to purple-brown spots which later developed as lesions of circular to oval shape with a light brown to grey center on artificial inoculation; thus proved the pathogenicity.

Comparative study on virulence of *H. oryzae* isolates revealed that under similar conditions of incubation, the isolates produced lesions of varying size on leaves of rice confirming the pathogenic variability. Among the five isolates, isolate 5 was identified as the most virulent. Five days after leaf inoculation, isolate 5 exhibited maximum disease severity of 63.7 per cent with a mean lesion size of 2.94 cm. After 5 days of incubation, complete rotting of inoculated leaf was observed (Figure 4). Higher virulence of isolate 5 could be substantiated by

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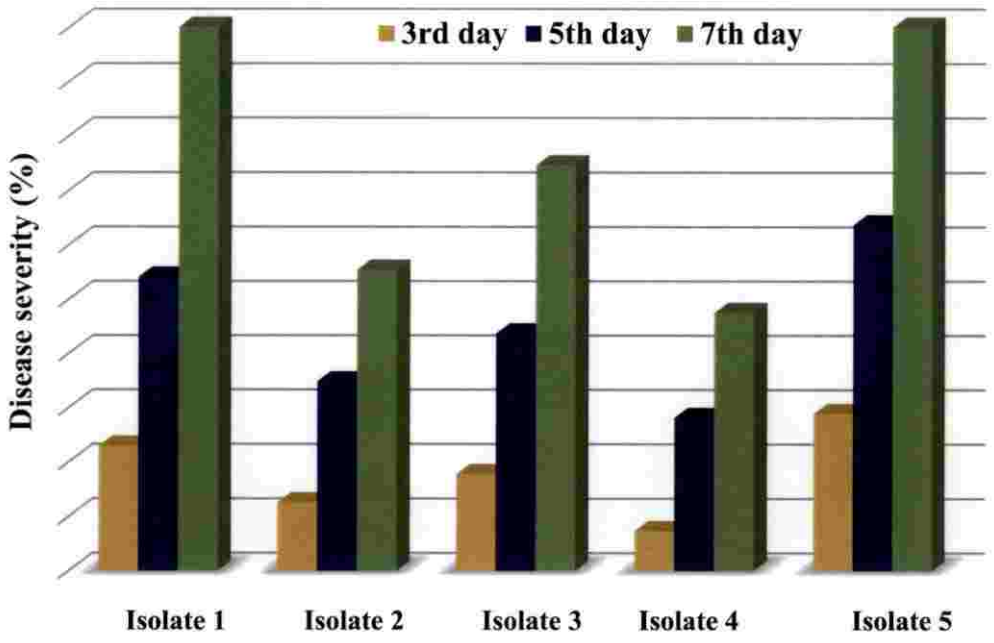

Figure 4: Virulence of different isolates of *H. oryzae* in rice var. Uma at different intervals in detached leaf assay

its faster growth (8.89 cm on 7th day of incubation in PDA medium) and sporulation with larger conidial dimensions (50 µm x 5.5 µm) compared to other isolates. Nazari *et al.* (2015) screened the pathogenicity of 12 isolates of *H. oryzae* on rice cultivar 'Tarom'. First symptom in the form of pin head spots appeared 24 h after inoculation. Later, the spots developed into oval necrotic lesions and on 7th day, coalesced lesions developed as leaf blight. These results were in accordance with the results of the current study. Similarly, Amorio and Cumagun (2017) observed that the most virulent isolate of *H. oryzae* on one-month old rice plants of variety IR-72, recorded a mean lesion density, mean lesion size and disease severity of 4.22, 1.52 mm and 12.34 per cent respectively on 3rd DAI.

The pathogenic variability among isolates could be an indication of presence of different pathotypes or races of *H. oryzae* in different regions of Kerala. The results of present study showed the prevalence of variation within *H. oryzae* populations from different locations of Kerala. The five isolates of *H. oryzae* differed in cultural, morphological and pathogenic characteristics. Understanding of pathogen population will help in successful execution of disease management programme (Masoodi *et al.*, 2013).

5.4 EVALUATION OF *P. indica* AGAINST *H. oryzae*

5.4.1 Root Colonization of Rice var. Uma with *P. indica*

Colonization efficiency of *P. indica* on rice variety Uma revealed that, maximum root colonization of *P. indica* (100 %) was recorded at 10 days after co-cultivation (DAC). Mature chlamydospores were noticed inside the rice root cell from 5 DAC and on 15 DAC large number of chlamydospores covering the whole root cells were noticed. These results were in accordance with the observations of Saddique *et al.* (2018) who reported the root colonisation of *P. indica* in rice plants after 4 days of co-cultivation. Whereas, Nassimi and Taheri (2017) reported that on three and seven days of post inoculation, no chlamydospores were detected in rice roots and only on 14 days after inoculation, there were large numbers of chlamydospores on local rice cultivar 'Domsiah'. Root colonisation of

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P. indica on rice was also reported by Mousavi *et al.* (2012) and Hajipour *et al.* (2015) on rice cultivar 'Tarom'. Data obtained from the present study were in accordance with the observations of Kumar *et al.* (2009), who reported that *P. indica* colonization in plants is time-dependent.

In the present study root colonisation was observed in all parts of the root including root hairs. The results were in agreement with Johnson (2014) who noticed that in *Arabidopsis*, the fungus colonizes every parts of the root, *viz.,* root tip and meristematic, elongation and maturation zones, and its colonization is restricted to epidermis and cortex. The fungal entry to the living root cells by direct penetration, colonization in the root epidermal and cortical tissues, and inter- and intra-cellular growth by forming pear- shaped chlamydospores were observed in the present study.

5.4.2 Dual Culture of *P. indica* with *H. oryzae*

Dual culture assay of *P. indica* with *H. oryzae* revealed that the endophyte exhibited multiple antagonistic actions against the pathogen which included lysis / inhibion zone (0.65 cm) and antibiosis (0.34 cm) observed at 9 and 15 days after incubation of dual culture plate respectively. It was found that there was reduced and suppressed mycelial growth of *H. oryzae* in dual culture in comparison with the control plate. On 5th and 10th day of incubation, percentage growth inhibition of 54.44 and 62.3 respectively was recorded in *H. oryzae* growth (Figure 5).

Varma *et al.* (2001) also reported that the *P.indica* started to invade into the growth area of *Gaeumannomyces graminis* var. *tritici* causing take-all disease of wheat, and caused lysis of the pathogen hyphae in dual culture. Similarly, antagonism of *P. indica* against *G. graminis* var. *tritici*, reported by Ghahfarokhi and Goltapeh (2010) indicated that. *P. indica* could inhibit the mycelial growth of *G. graminis* var. *tritici* by the development of inhibition zone and coiling of *P. indica* around the hyphae of the pathogen. Johnson *et al.* (2013) reported the antagonism of *P. indica* against *Alternaria brassicae* by antibiosis and inhibition zone development. In contradictory to the present study, Nassimi and Taheri,

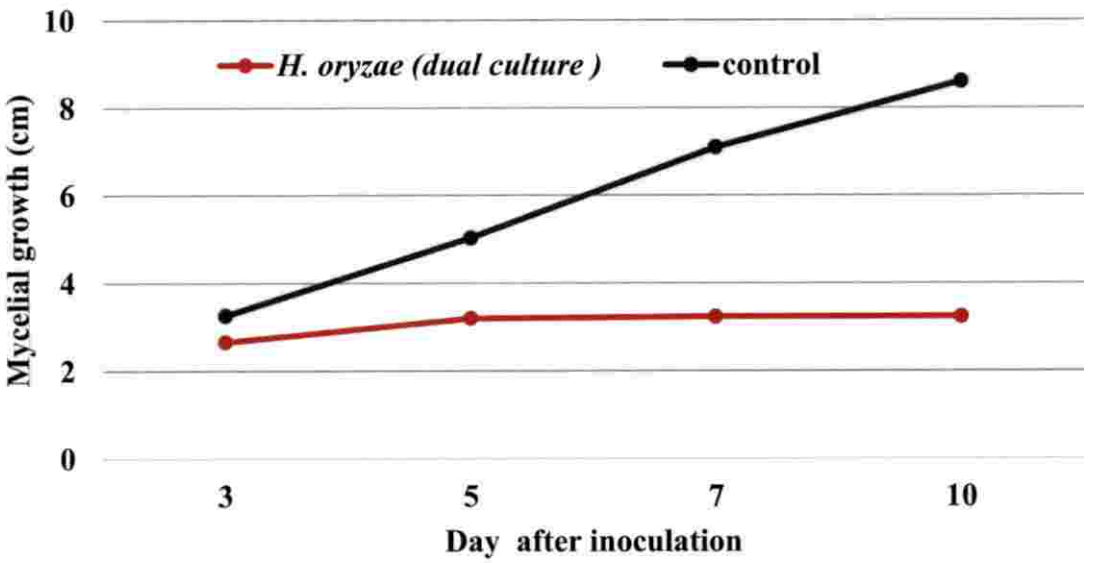

Figure 5: Mycelial growth inhibition of *H. oryzae* by *P. indica* in dual culture in PDA medium at different intervals of *H. oryzae* inoculation

(2017) noticed that *P. indica* did not exhibit direct antagonistic effect against the rice sheath blight pathogen in the dual culture.

5.4.3 *In vitro* Evaluation of *P. indica*-Primed Rice Seedlings (var. Uma) against *H. oryzae*

In vitro evaluation of *P. indica*-primed rice seedlings against *H. oryzae* revealed that *P. indica*- primed rice seedlings significantly delayed the symptom development of brown spot disease up to 24 h with a minimum lesion size of 1.43 cm on 7th DAI of *H. oryzae*. Similarly, the *P. indica*-primed rice seedlings exhibited minimum brown spot disease severity (12.4 %) in comparison with non-primed plants (100 %) on 15 DAI of *H. oryzae* (Figure 6 and 7). In addition to the disease tolerance, the beneficial fungus enhanced biomass in terms of fresh and dry weight of shoot and root in rice seedlings primed with *P. indica* and seedlings primed with *P. indica* and inoculated with *H. oryzae* compared to control and seedlings inoculated with *H. oryzae* alone. The highest shoot fresh and dry weight were recorded in plants primed with *P. indica* and inoculated with *H. oryzae* (0.32 g and 0.05 g respectively) followed by plants primed with *P. indica* alone (0.32 g and 0.04 g respectively) (Figure 8). Similarly, root fresh and dry weight were higher in plants primed with *P. indica* and inoculated with *H. oryzae* (0.13 g and 0.07 g respectively) followed by plants primed with *P. indica* alone (0.12 g and 0.06 g respectively) (Figure 9).

Evaluation of the direct effect of *P. indica* on *H. oryzae* vegetative growth and it's potential of resistance induction in rice plants were not investigated till now. Therefore, the effect of *P. indica* on rice brown spot has been demonstrated in this study for the first time by providing evidence on delayed symptom appearance and reducing the disease severity. In accordance with this finding, Knecht *et al.* (2010) reported that the *P. indica* could confer resistance to two phytopathogenic fungi namely *Rhizoctonia solani* and *Verticillium longisporum* in *Arabidopsis* seedlings. Similarly Johnson *et al.* (2013) explained the potential of *P. indica* against *Alternaria brassicae*, in pre-primed *Arabdiopsis* seedlings. *P.*

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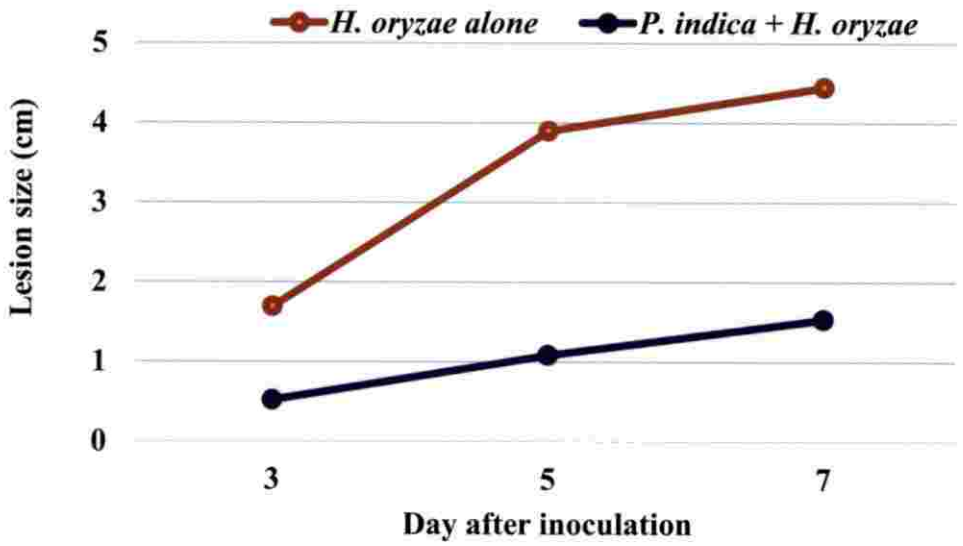

Figure 6: Lesion size of brown spot in *P. indica*-primed rice seedlings var. Uma at different intervals of *H. oryzae* inoculation

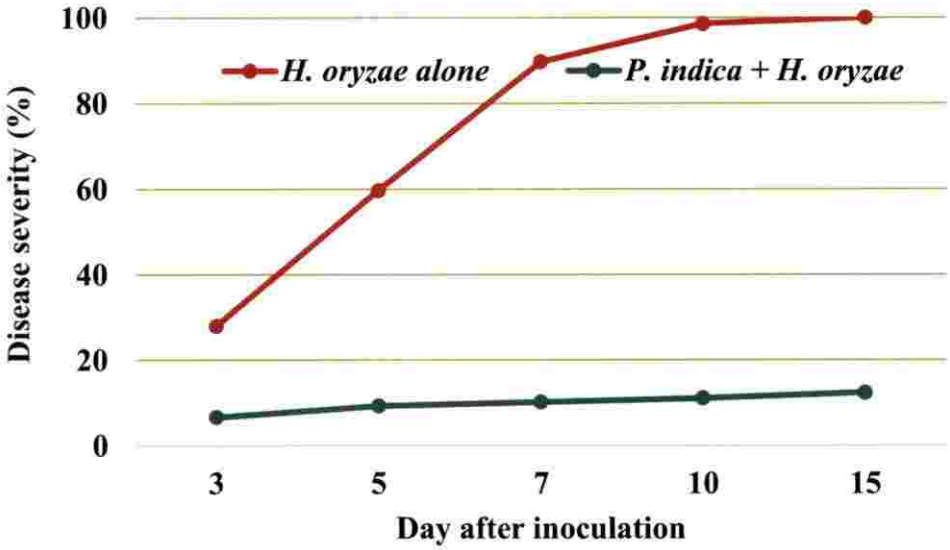

Figure 7: Severity of brown spot in *P. indica*-primed rice seedlings var. Uma at different intervals of *H. oryzae* inoculation

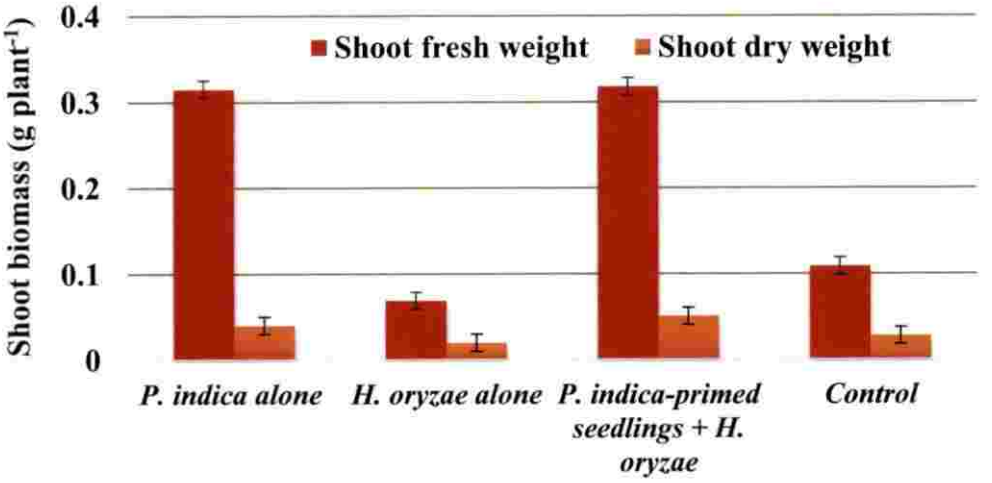

Figure 8: Shoot biomass of *P. indica*-primed rice seedlings var. Uma due to *P. indica*-priming against *H. oryzae*

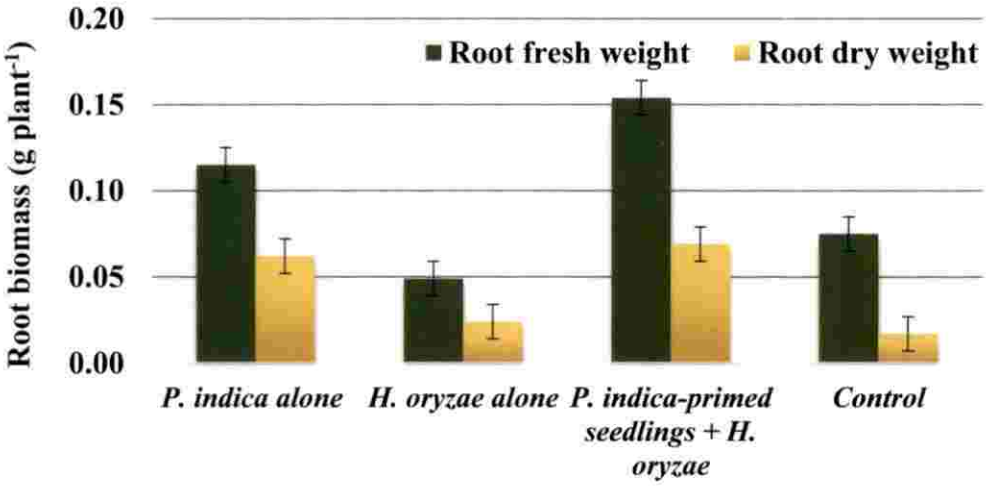

Figure 9: Root biomass of *P. indica*-primed rice seedlings var. Uma due to *P. indica*-priming against *H. oryzae*

indica colonisation resulted in remarkable disease suppression as evident in terms of reduced lesion number and size.

5.4.4 *In vivo* Evaluation of *P. indica*-Primed Rice Seedlings (var. Uma) against *H. oryzae*

In vivo evaluation of *P. indica*-primed rice plants against *H. oryzae* revealed that *P. indica*-primed rice plants significantly delayed the symptom development of brown spot disease up to 8 day with a minimum lesion size of 1.02 cm on 15th DAI of *H. oryzae* (Figure 10). Similarly the *P. indica*-primed rice plants recorded minimum brown spot disease severity (13.7 %) in comparison with non-primed plants (69.7 %) on 15 DAI of *H. oryzae* (Figure 11). These results were in accordance with the observations of Kumar *et al.* (2009) where, *Fusarium verticilloides* infection in maize roots was effectively reduced by the protective action of *P. indica.* Mousavi *et al.* (2014) noticed that rice blast disease tolerance was conferred by *P. indica*-root colonization. They further studied the mechanism of *P.indica* against *Magnaporthe oryzae* and found that beneficiary role of *P.indica* in rice against blast disease was due to boosted expression of different defence genes (PR1b, LOX, NPR1, and WRKY85). Similarly Hajipour *et al.* (2015) reported the pre-inoculation of rice plants with *P. indica* delayed crown rot (Bakanae) disease symptoms up to 14 days and the symptoms developed were mild and thus offered effective control over *Fusarium proliferatum*, the causal agent of root rot. Nassimi and Taheri (2017) also reported that the *P. indica* root colonisation of rice plants delayed the infection process of *R. solani* and decreased sheath blight severity. Decreased severity of the disease was associated with decreased levels of hydrogen peroxide (H_2O_2) and increased superoxide dismutase (SOD) activity.

Investigations on the mechanism of *P. indica*-induced resistance revealed that active nutrient uptake by *P. indica* could be involved in enhanced plant tolerance against various biotic and abiotic stress (Kumar *et al.,* 2009). Involvement of jasmonic acid signalling and role of Non-expressor of Pathogenesis-Related 1 gene (NPR1) in *P. indica*-mediated resistance was

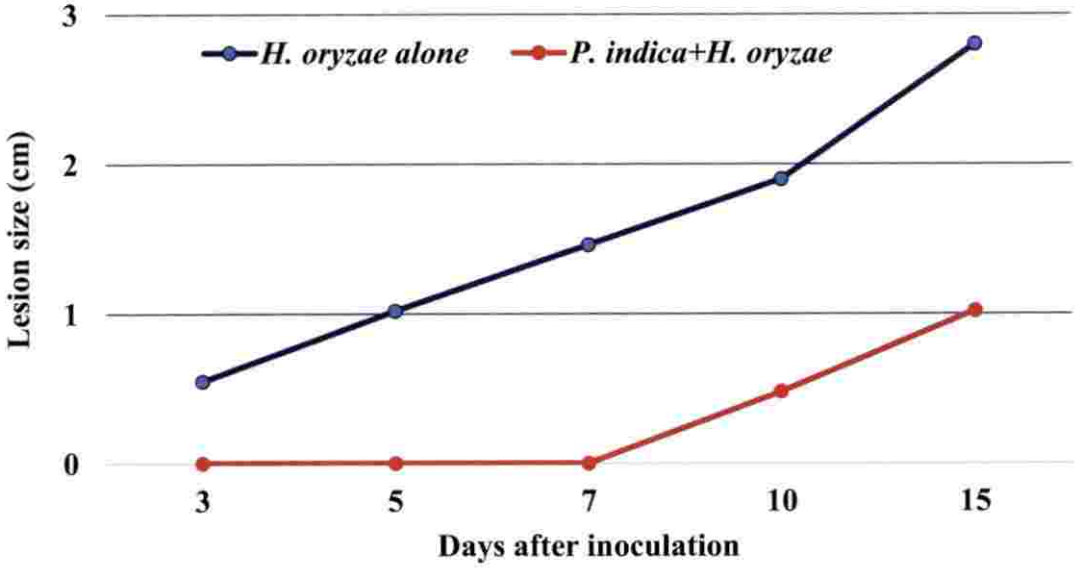

Figure 10: Lesion size of brown spot in *P. indica*-primed rice seedlings var. Uma at different intervals of *H. orvzae* inoculation

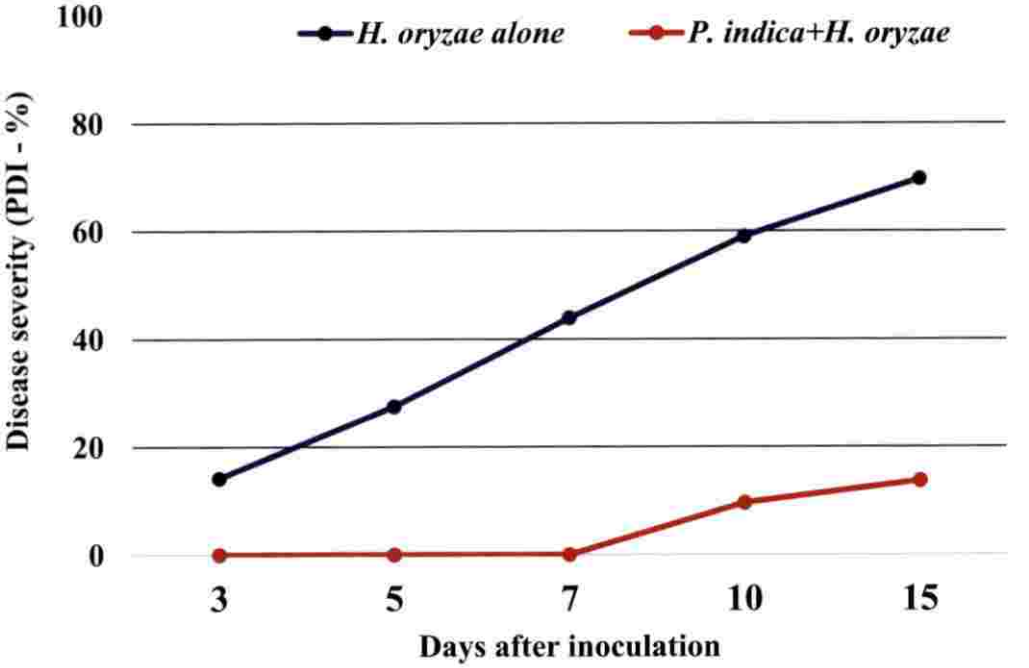

Figure 11: Disease severity of brown spot in *P. indica*-primed rice seedlings var. Uma at different intervals of *H. oryzae* inoculation

explained in Arabidopsis powdery mildew caused by *Golovinomyces orontii* by Stein *et al.* (2008).

Enhanced biomass in terms of fresh and dry weight of shoot and root was observed in rice plants primed with *P. indica* and plants primed with *P. indica* and inoculated with *H. oryzae* compared to control and plants inoculated with *H. oryzae* alone. The highest shoot fresh and dry weight were recorded in plants primed with *P. indica* alone (10.84 g and 4.22 g respectively) followed by plants primed with *P. indica* and inoculated with *H. oryzae* (9.10 g and 3.52 g respectively). The lowest shoot fresh and dry weight were recorded in plants inoculated with *H. oryzae* alone (4.54 g and 1.60 g respectively) followed by control plants (5.36 g and 1.36 g respectively). Similarly, root fresh and dry weight was higher in plants primed with *P. indica* and inoculated with *H. oryzae* (6.58 g and 2.82 g respectively) followed by plants primed with *P. indica* alone (6.20 g and 2.04 g respectively). The plants inoculated with *H. oryzae* alone recorded the lowest root fresh and dry weight (2.64 g and 1.18 g respectively) followed by control plants (2.80 g and 1.48 g respectively) (Figure 12 and 13). Similar observations were recorded by Nassimi and Taheri (2017) who reported improvement in growth parameters in terms of fresh and dry weight of shoot and root in *P. indica*-colonised plants. Enhanced biomass in the plants inoculated with *P. indica* and also in the plants inoculated with both *P. indica* and *R. solani* compared to the controls and plants only inoculated with *R. solani* was reported. These results are in support with the findings of the present study.

5.5.1. *In vitro* Evaluation of New Generation Fungicides against *H. oryzae* by Poisoned Food Technique

A study was conducted to find out the effect of new generation fungicides in controlling the mycelial growth of *H. oryzae* under *in vitro* conditions. Six fungicides belonging to triazoles and strobilurins were evaluated at four concentrations (10, 50, 100 and 250 ppm). Among the tested fungicides maximum percentage growth inhibition at lower concentration (10 ppm) was exhibited by azoxystrobin + tebuconazole (66.60) followed by pyraclostrobin + tebuconazole

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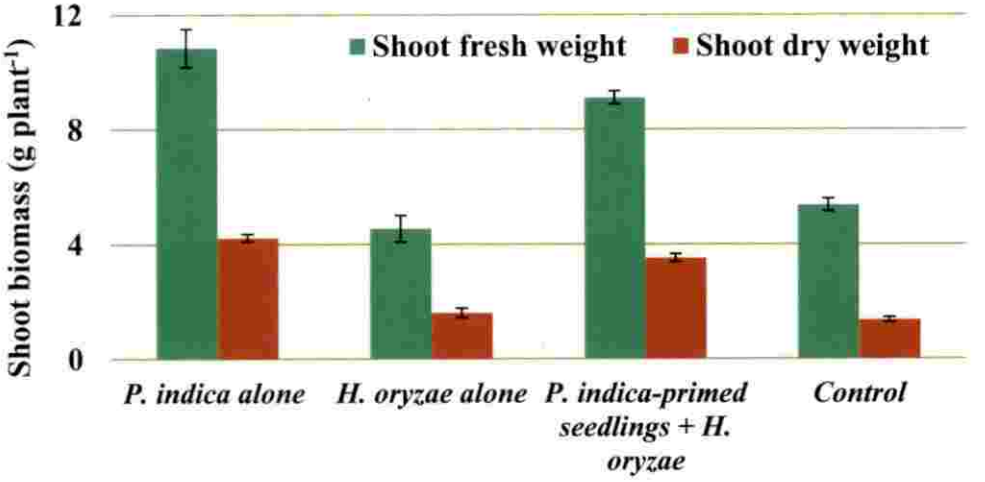

Figure 12: Shoot biomass of *P. indica*-primed rice seedlings var. Uma due to *P. indica*-priming against *H. oryzae*

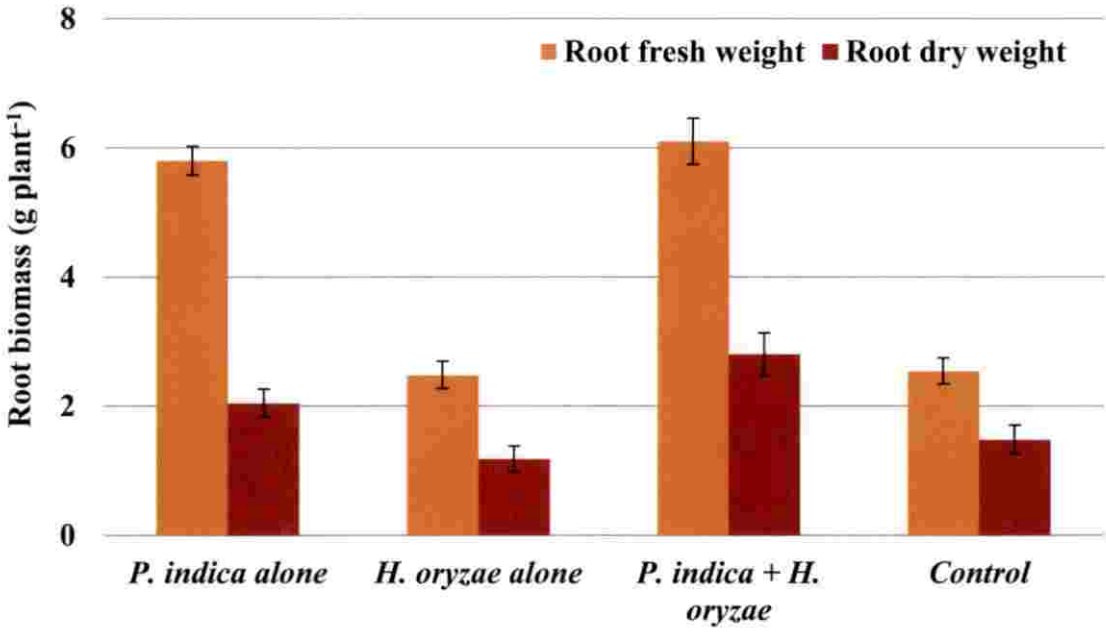

Figure 13: Root biomass of *P. indica*-primed rice seedlings var. Uma due to *P. indica*-priming against *H. oryzae*

(61.8). However on higher concentration (50 ppm) the highest growth inhibition was recorded with pyraclostrobin + tebuconazole (87.3) followed by azoxystrobin + tebuconazole (73.30). On 100 and 250 ppm concentration maximum percentage growth inhibition was recorded with pyraclostrobin + tebuconazole followed by azoxystrobin + tebuconazole (82.56 and 87.70 respectively) (Figure 18 and Figure 19).

At 250 ppm concentration, maximum inhibition to mycelial growth of *H. oryzae* was recorded in tebuconazole (Figure 14). Among the strobilurin fungicides the highest inhibition was noticed in pyraclostrobin at 250 ppm (Figure 17). Both the tested combination fungicides significantly inhibited mycelial growth of *H. oryzae* at 250 ppm. However, the complete inhibition was recorded in the combination fungicide, pyraclostrobin + tebuconazole (Figure 18). The results of the present study indicated that the best azole fungicide was tebuconazole and the best strobilurin was pyraclostrobin. Ahmed *et al.* (2002) pointed out the effect of propiconazole (Tilt 250 EC) at 500 ppm against *H. oryzae* and recorded the maximum mycelial inhibition of 95.58 per cent. Likewise, Sunder *et al.* (2005) reported the inhibitory effect of hexaconazole (0.11 ppm a.i.) and propiconazole (0.42 ppm a.i.) to the mycelial growth of *H. oryzae*. Hunjan *et al.* (2011) also revealed that trifloxystrobin + tebuconazole (0.04 %) was the best followed by tebuconazole (0.1 %) and propiconazole (0.1 %) in inhibiting the mycelial growth of the fungus. These results are in agreement with the findings of the present study where the combination fungicides pyraclostrobin + tebuconazole (100 %) followed by azoxystrobin + tebuconazole (87.7%) recoded the maximum mycelial inhibition at 250 ppm. Similarly, Mahapatra and Hemayathullah (2014) recorded the highest percent inhibition of *H. oryzae* mycelial growth (87.49 %) by propiconazole (0.1 %) and the lowest (34.72 %) by azoxystrobin (0.1 %). Similar results were reported by Kamei and Simon, (2018) where a maximum inhibition (73.04 %) of radial growth (2.73 cm) of mycelium over control was shown by propiconazole.

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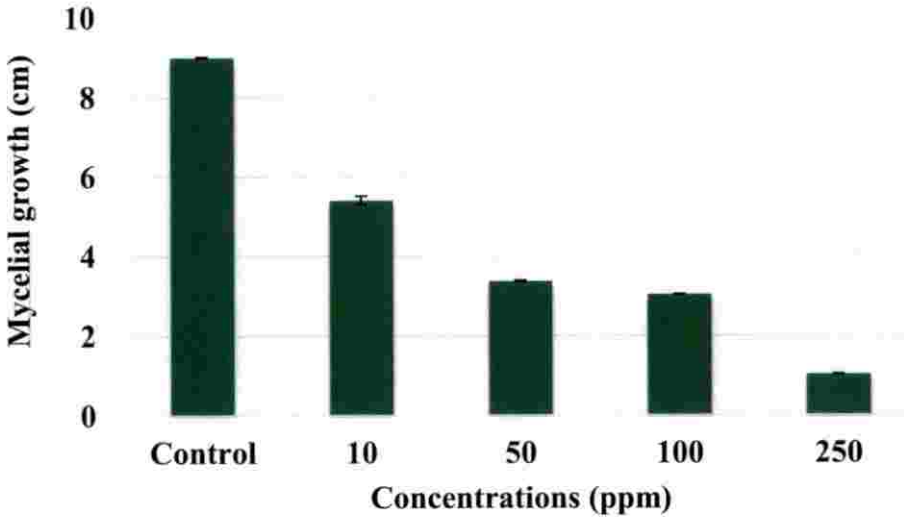

Figure 14: Effect of different concentrations of tebuconazole (ppm) on mycelial growth of *H. oryzae*

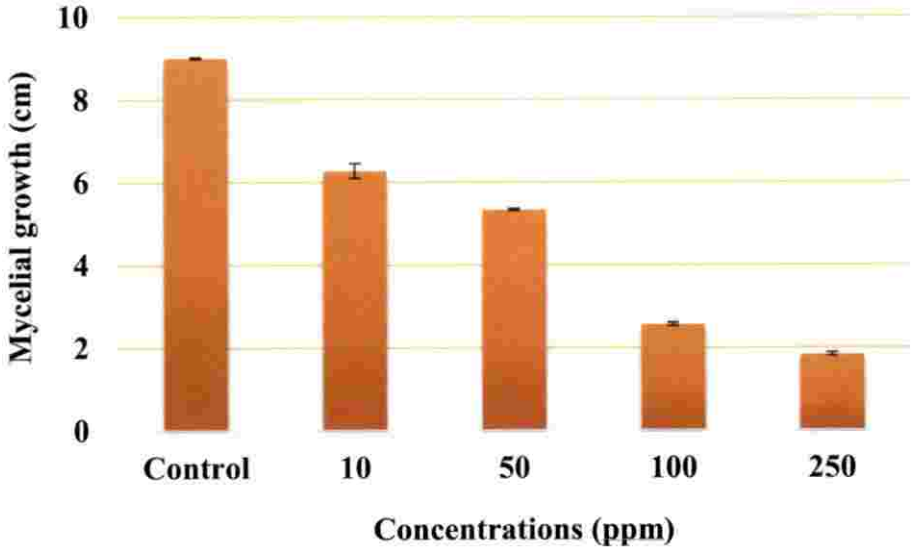

Figure 15: Effect of different concentrations of difenoconazole (ppm) on mycelial growth of *H. oryzae*

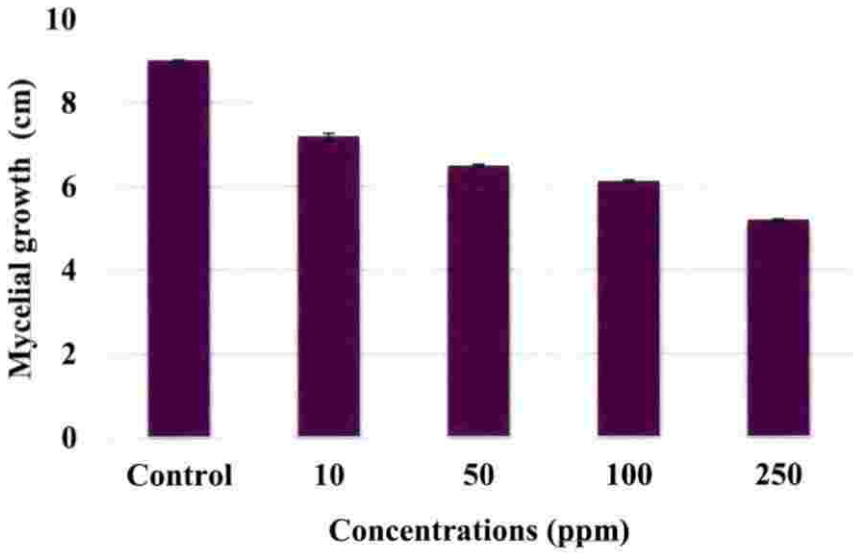

Figure 16: Effect of different concentrations of azoxystrobin (ppm) on mycelial growth of *H. oryzae*

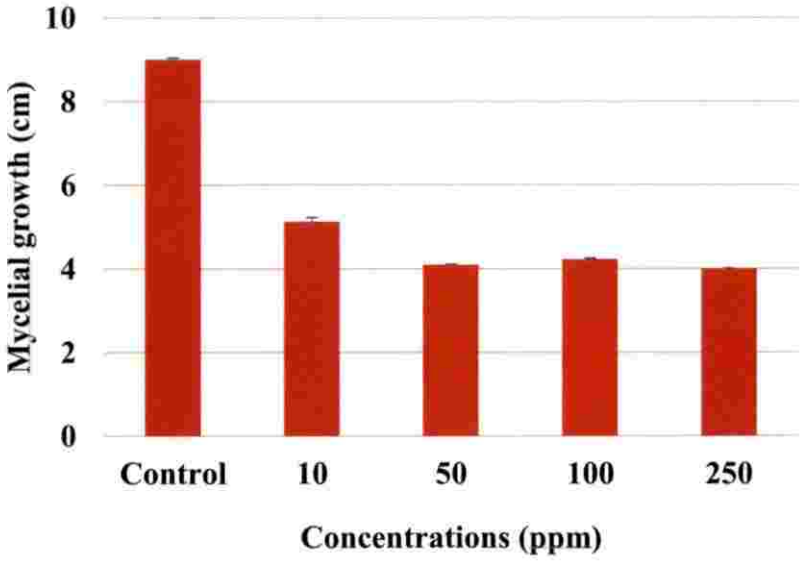

Figure 17: Effect of different concentrations of pyraclostrobin (ppm) on mycelial growth of *H. oryzae*

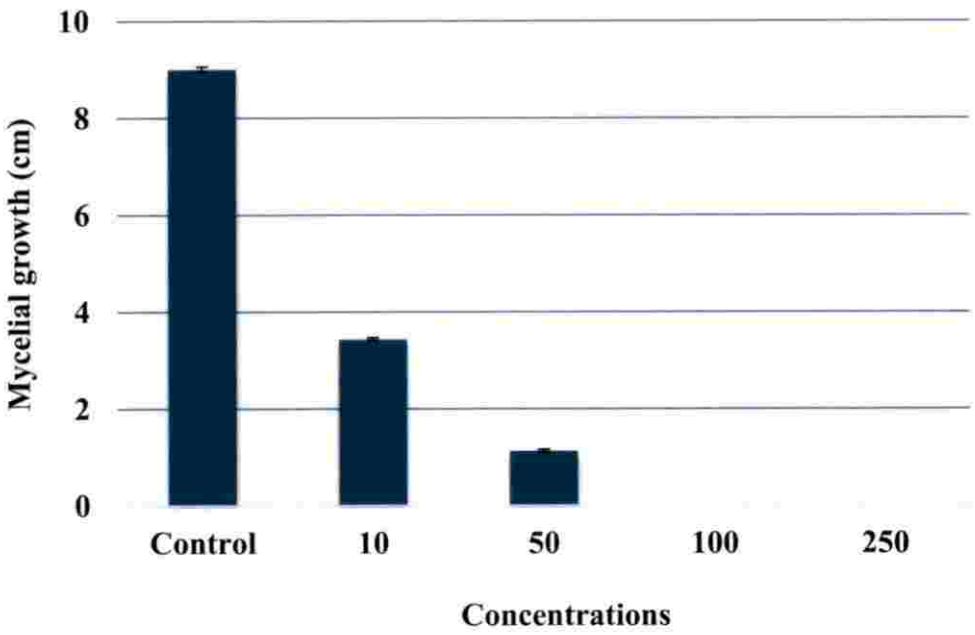

Figure 18: Effect of different concentrations of pyraclostrobin + tebuconazole (ppm) on mycelial growth of *H. oryzae*

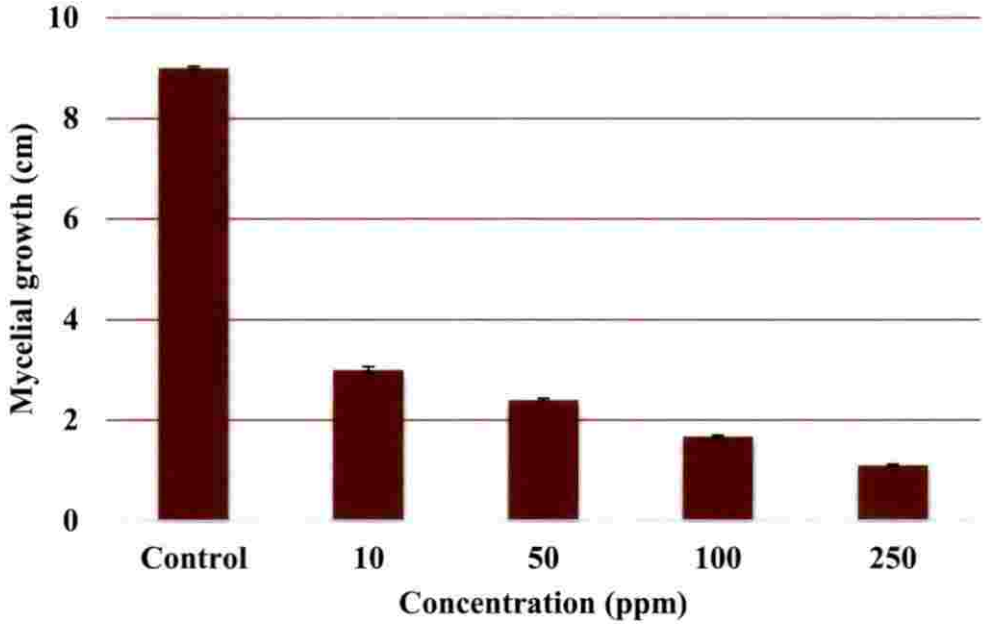

Figure 19: Effect of different concentrations of azoxystrobin + tebuconazole (ppm) on mycelial growth of *H. oryzae*

In the present study, triazoles (tebuconazole 25.9 % EC, difenoconazole 25 % EC) inhibited mycelial growth (39.97 % and 30.33 % respectively) of *H. oryzae* even at lowest concentration (10 ppm). Strobilurins also (azoxystrobin and pyraclostrobin) had a comparable effect (20.7 % and 42.93 %) over triazoles at lower concentrations. But combination fungicides azoxystrobin + tebuconazole (66.60 %) and pyraclostrobin + tebuconazole (61.80 %) had profound effect over triazoles and strobilurins (Figure 20).

5.5.2. *In vivo* Evaluation of New Generation Fungicides

A pot culture study was conducted with variety 'Uma' to evaluate the efficacy of new generation fungicides. Based on results of *in vitro* evaluation of fungicides by poisoned food technique, the treatments on recommended and higher doses were selected for *in vivo* study. Lesion size, per cent disease index and disease suppression of brown spot was calculated.

Lesion size of brown spot of rice was recorded for each treatment at different days (3, 5, 10 and 15) after spraying (DAS). Among the treatments, the lowest lesion size was recorded with trifloxystrobin + tebuconazole (0.65 cm) followed by azoxystrobin + tebuconazole (0.84 cm) and pyraclostrobin + tebuconazole (1.17 cm) on 15 DAS (Figure 21). Similar results of reduced leaf spot and stalk rot symptoms were reported by Sunder *et al.* (2010).

When the PDI values were compared at 15 day after treatments, trifloxystrobin + tebuconazole (0.05 %) recorded minimum PDI of 10.81 per cent for brown spot of rice followed by azoxystrobin + tebuconazole (17.46 %) and pyraclostrobin + tebuconazole (21.01 %) (Figure 22). Qudsia *et al.* (2017) also reported the effectiveness of tebuconazole + trifloxystrobin with a disease severity of 15.66 per cent followed by difenoconazole (17.66 %). In the present study, other treatments also showed significant disease control *viz.*, tebuconazole 0.1 per cent (31.77 % PDI), tebuconazole 0.05 per cent (37.97 %) and pyraclostrobin 0.1 per cent (41.50 %). Tebuconazole treatment recorded significant control of the disease compared to pyraclostrobin. Pyraclostrobin alone was not much effective

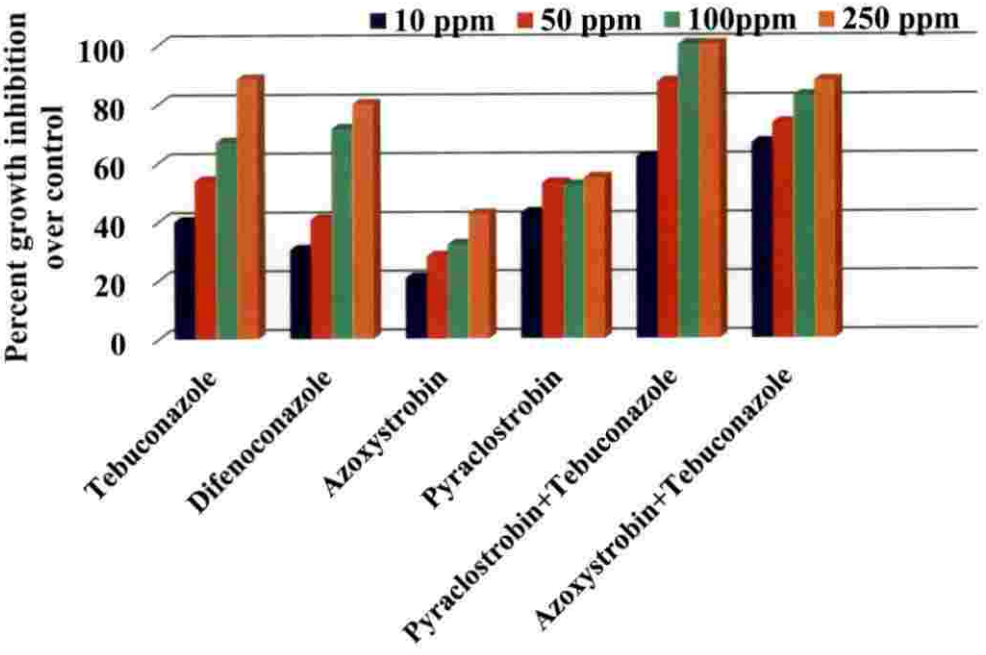

Figure 20: Per cent growth inhibition of *H. oryzae* at different concentrations of fungicides on PDA medium at 7th day after inoculation

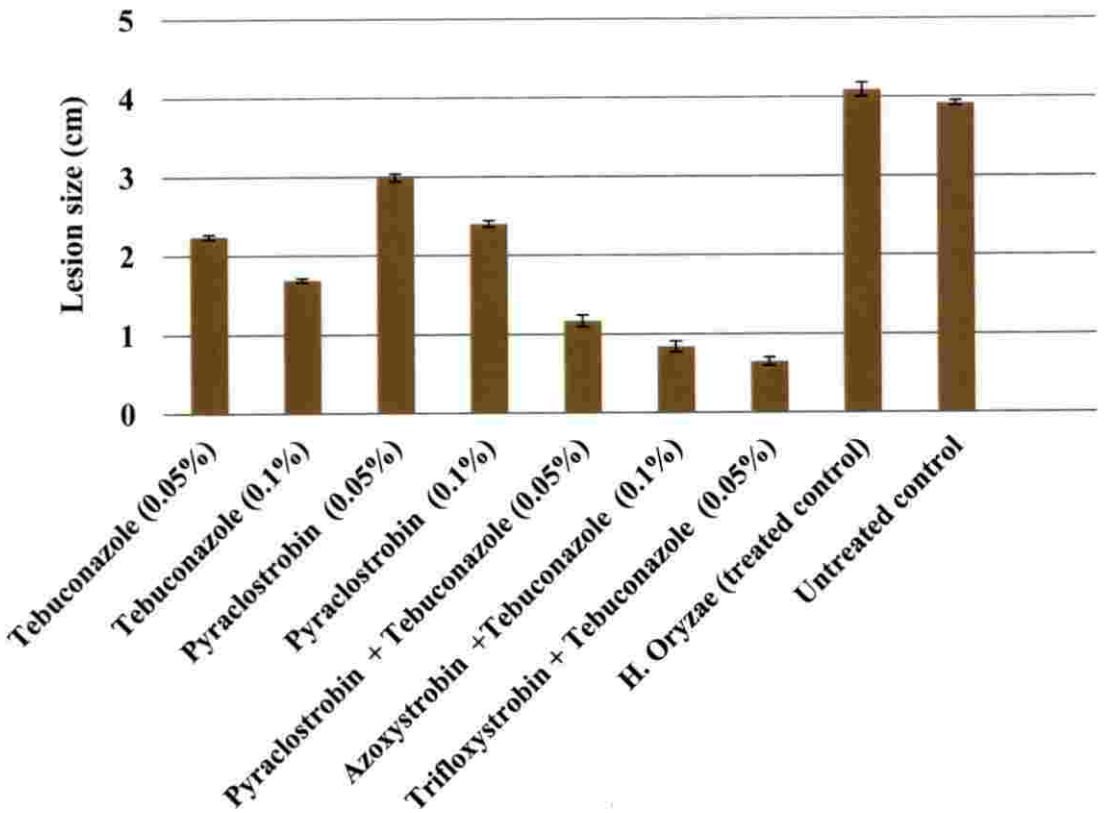

Figure 21: Lesion size of brown spot disease of rice at 15 DAS with different fungicides at recommended and double the recommended doses

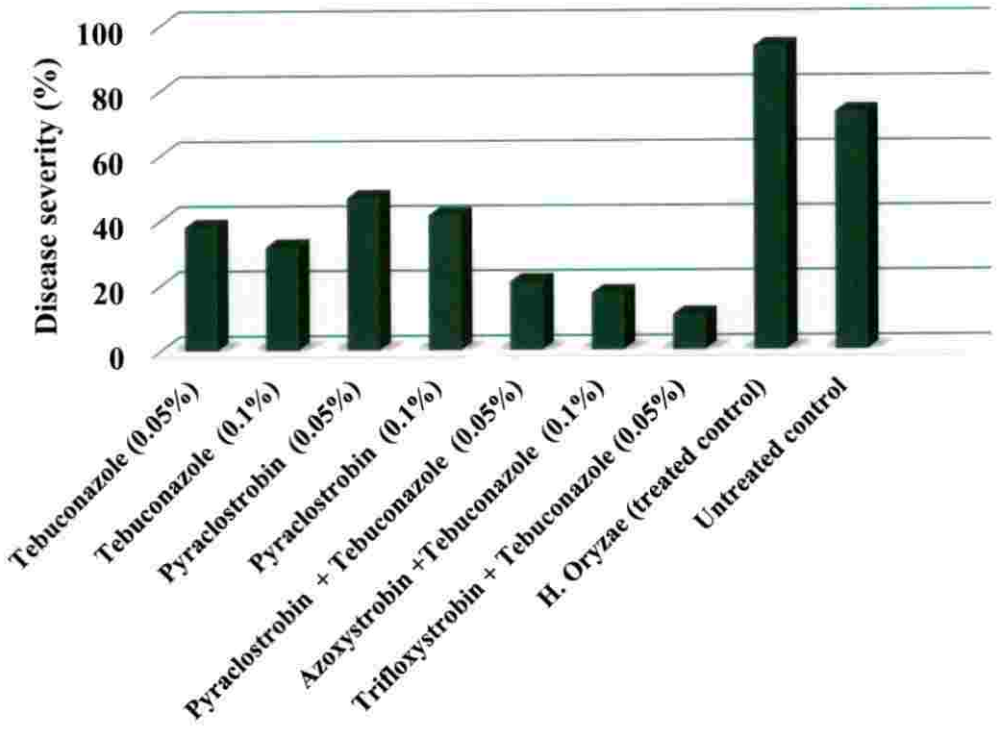

Figure 22: Disease severity of brown spot disease of rice at 15 DAS with different fungicides at recommended and double the recommended doses

under *in vivo* conditions but its combination with tebuconazole resulted higher disease reduction at recommended dosage which might be due to greater efficiency of tebuconazole to control *H. oryzae*. So the combination of pyraclostrbin + tebuconazole could be effectively used for controlling brown spot of rice, which is not commercially available at present, so need to be physically mixed.

Disease suppression was calculated based on PDI at 15th day and at recommended dose, the maximum suppression was recorded with 0.05 per cent of trifloxystrobin + tebuconazole (88.42 %), followed by azoxystrobin + tebuconazole (81.30 %) and pyraclostrobin + tebuconazole (77.52 %). At higher (double the recommended) doses of respective fungicides, disease suppression was more compared to recommended dose (Figure 23). In tandem with the outcome of the present research, Kamei and Simon (2018) found that the highest per cent reduction of brown spot disease over control was recorded in propiconazole (72.39 and 72.12) followed by propineb (69.40 and 70.83) in the recommended and double the recommended doses. Another observation obtained from the study was all the pyraclostrobin based treatments resulted in early flowering and panicle formation in rice plants.

The present study clearly indicated that higher disease incidence and severity of brown spot of rice during rainy season and higher humidity conditions. Different type of symptoms starting from seedling blight to severe grain discoloration could be observed at different survey locations. Five isolates of *H. oryzae* obtained from different locations were morphologically and pathogenically identified and found to be variable from one another. The most virulent isolate (isolate 5 from Karamana) was used during the course of the study. Evaluation of effect of fungal root endophyte *P. indica* against *H. oryzae* revealed that the endophyte colonizes the roots of rice variety on 5th day after co-cultivation. A colonization per cent of 100 was recorded on 10 DAC. Dual culture assay of *P. indica* with *H. oryzae* on PDA media confirmed that *P. indica* exhibit multiple antagonistic action against the brown spot pathogen *viz.*, lysis / inhibition zone

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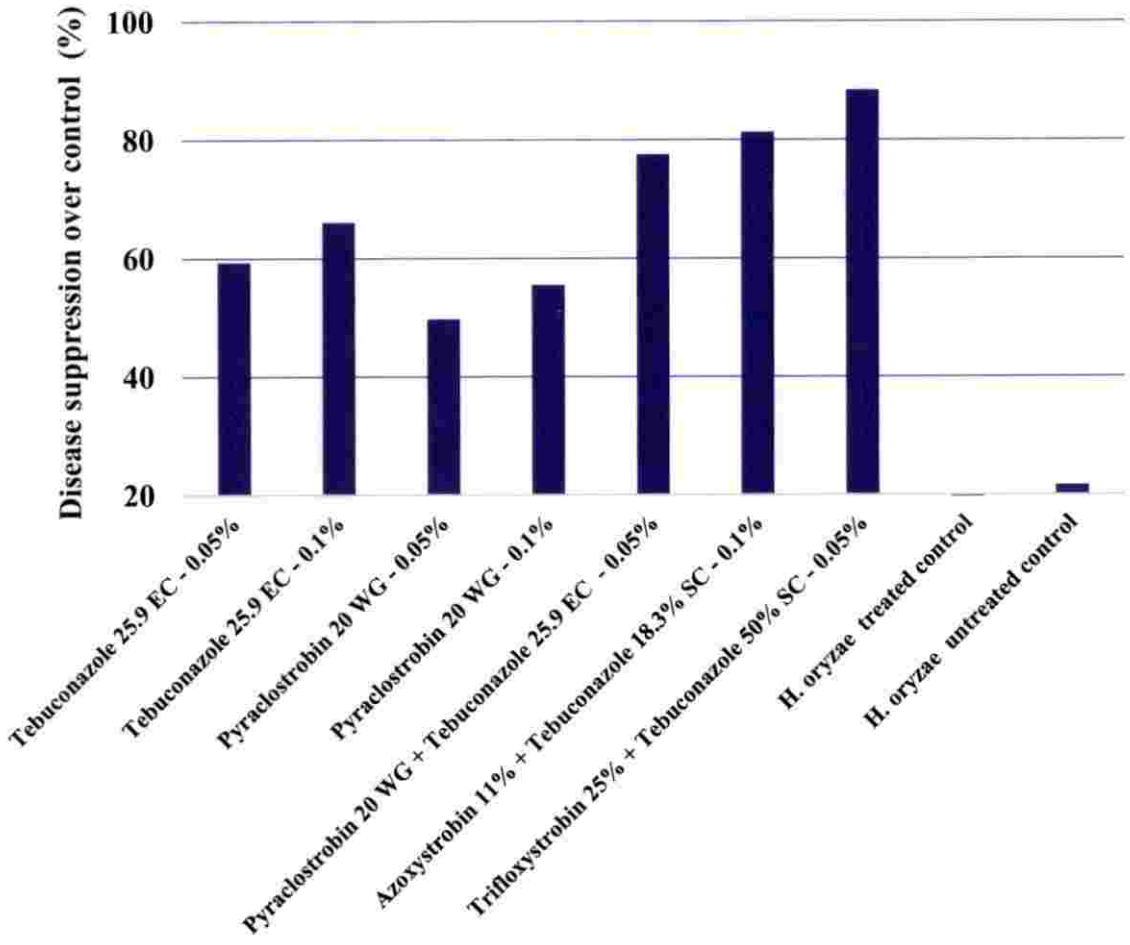

Figure 23: Brown spot disease suppression over control by different fungicides at recommended and double the recommended doses

and antibiosis. Mycelial inhibition of *H. oryzae* by *P.indica* in dual culture plate was also recorded and concluded that *P. indica* could directly inhibit the brown spot pathogen. Further the *P. indica*-primed rice seedlings and plants (var. Uma) could significantly delay the symptoms of *H. oryzae* up to eight days after inoculation of the pathogen. The primed seedlings and plants recorded the lowest lesion size and the highest disease suppression. *P. indica*-root colonization in rice seedlings resulted in enhanced growth with increased root and shoot biomass. *P. indica*-colonized roots had more number of secondary and tertiary roots with profuse root hairs. There was two-fold increase in shoot and root fresh weight and three-fold increase in shoot dry weight and 2.3 fold increase in root dry weight. Hence, it is confirmed the synergism of biotic stress tolerance and growth promotion in rice due to *P. indica*-root colonization.

Due to the presence of diverse species and races of the fungus, a completely resistant genotype of rice was not yet developed, thus the disease become a recurrent problem to rice farmers all over the globe. Spraying of conventional fungicides for management of the disease resulted problems of resistance development in the pathogen, and the complete control was thus still indomitable. The pathogen started developing resistance against many of the conventionally used fungicides recommended. So introducing new molecules like triazoles, strobilurins and their combinations will be good choice to control the disease. These are broad spectrum fungicides with high efficacy at lower doses against different diseases on diverse crops and they are eco-friendly too. Even though many fungicides of these groups are included in CIBRC recommendations, many of them are still not recommended for the management of diseases in Kerala. So by considering the results of the present experiment, multi-locational and multi-seasonal trials could be undertaken before finalizing the recommendations for management of brown spot disease in Kerala. Compatibility studies of the fungal root endophyte *P. indica* with the best pesticides used in rice cultivation have to be initiated.

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Summary

6. SUMMARY

Brown spot of rice incited by *H. oryzae* is a major rice disease prevalent under humid climate. *Piriformospora indica* is an axinically culturable beneficial root endophyte belongs to Sebacinales of Basidiomycota. Endophytic colonization of *P. indica* in roots of many plant species resulted in enhanced growth and development; and tolerance to abiotic and biotic stress. Management of the disease using conventional fungicides and resistant varieties found to be less effective due to faster resistance development of pathogen against the conventional fungicides and the rapid evolution of new pathogenic races. New generation fungicides are the synthetic compounds with highly specific mode of action and effective even at lower dozes. Since those fungicides are ecofriendly it was found as an emerging approach of disease management for many economically important plant diseases. Triazoles and strobilurins are groups of new generation fungicides which were proved to be effective against brown spot diseases of rice. Hence, the study entitled 'Management of brown spot disease of rice using fungal root endophyte *Piriformospora indica* and new generation fungicides' was conducted during 2017 - 19 at Department of Plant Pathology, College of Agriculture, Vellayani with the objectives to evaluate the effect of root endophyte *P. indica* against *H. oryzae* and the evaluation of new generation fungicides for the management brown spot of rice.

A survey was conducted in the rice growing tracts of six KAU research stations *viz*. College of Agriculture, Vellayani; IFSRS Karamana; RRS Moncombu; RARS, Kumarakom; RRS Vyttila; and RARS, Pattambi during 2017 June – 2018 January with the aim to assess the disease incidence and severity of brown spot of rice in Kerala. *H. oryzae* was found to incite the disease in five survey locations with a maximum prevalence (76.0 %) and severity (64.6 %) recorded at IFSRS Karamana. There was a complete crop failure at RARS Kumarakom due to Great Flood of Kerala 2017. Brown spot disease of rice was found to be more prevalent under humid climatic condition and rainy season with prominent symptoms of the symptoms *viz*., leaf spots, leaf blight, and panicle

blight and grain discoloration. The leaf spots appeared as round to oval brown spots on leaf lamina surrounded by yellow halo with white to grey centre. Brown necrotic lesions appeared on the panicle. Grain discoloration is the most economic loss causing symptom of the disease in which dark brown to black spots appeared on the grains that later turned into dirty lesions resulting the loss of grain quality. Pure cultures of *H. oryzae* isolates (Isolate 1 to Isolate 5) were obtained and Koch's postulates for all isolates were proved by artificial inoculation on rice variety Uma.

Regarding the morphological characters the fungus, *H. oryzae* produced white, grey, grey to black and black colour mycelia with fluffy, smooth or sparse nature of growth on PDA medium. Reverse side of culture plates exhibited greyish black, brownish black or back colour. Different isolates were recorded with either regular or irregular margin. Days taken for the coverage of petri plate varied from 5.6 to 8.93 days. The fungal mycelia was found to be hyaline and septate with a width varied from 1.49 - 2.8 µm. Different isolates of *H. oryzae* exhibited poor sporulation under laboratory conditions. Sporulation was observed in isolate 1 and isolate 5. The conidia were slightly curved with a wide center and measured 46.5 – 50 µm x 5.5 – 6.6 µm.

Among the five isolates of *H. oryzae* obtained from the survey, isolate 5 was identified as the most virulent one. The disease symptoms observed within 24 hour of inoculation of isolate 5 on detached leaf assay. It produced a mean lesion size of 2.94 cm on 5th day of leaf inoculation. On seventh day of inoculation complete leaf rotting was recorded with isolate 5 on rice variety Uma. So the isolate 5 was confirmed as the most virulent one and used for the further studies.

Evaluation of beneficial root endophyte *P. indica* against *H. oryzae* revealed that the endophyte colonizes the roots of rice variety on 5th day after co-cultivation. A colonization per cent of 100 was recorded on 10 DAC. Dual culture assay of *P. indica* with *H. oryzae* on PDA medium confirmed that *P. indica* exhibited multiple antagonistic action against the brown spot pathogen *viz.*, lysis / inhibition zone (0.65 cm) and antibiosis (0.34 cm). Regarding mycelial

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inhibition of *H. oryzae* by *P.indica* in dual culture plate there was an inhibition of 54.4 percent on 7th day of incubation of dual culture plate. On 10th day of incubation, the percent mycelial inhibition was 62.3.

In vitro evaluation of *P. indica*-primed rice seedlings against *H. oryzae* disclosed that the root priming of rice seedlings with *P. indica* could delay the brown spot symptoms by a day with a minimum lesion size of 2.6 cm on 15th DAI. Disease severity of brown spot was found to be less in *P. indica*-primed rice seedlings. A minimum disease severity of 12.4 per cent was recorded on 15 DAI. Apart from the brown spot tolerance, the primed-seedlings exhibited growth promotion. The primed-plants recorded higher shoot fresh and dry weight (0.32 g plant^{-1} and 0.05 g plant^{-1} respectively). Regarding the root growth, primed-rice seedlings had more number of secondary and tertiary roots with profuse root hairs. Root fresh and dry weight found to be higher in *P. indica*-primed rice seedlings inoculated with *H. oryzae* (0.13 g plant^{-1} and 0.07 g plant^{-1} respectively).

In vivo evaluation of *P. indica*-primed rice plants against *H. oryzae* revealed that the root priming of rice plants with *P. indica* could delay the brown spot symptoms up to eight days with a minimum lesion size of 1.02 cm on 15th DAI of *H. oryzae*. Disease severity of brown spot was found to be less in *P. indica*-primed rice plants. A minimum disease severity of 13.7 per cent was recorded at 15 DAI. Apart from the brown spot tolerance, the primed-rice plants exhibited growth promotion. There was more number of tillers and leaves in the primed-plants with increased leaf size. *P. indica* alone treated rice plants recorded higher shoot fresh and dry weight (10.84 g plant^{-1} and 4.22 g plant^{-1} respectively). Regarding the root growth, the primed-rice plants had more number of secondary and tertiary roots with profuse root hairs. Root fresh and dry weight found to be higher in *P. indica* primed rice seedlings inoculated with *H. oryzae* (6.58 g plant^{-1} and 2.82 g plant^{-1} respectively).

Thus the study concluded that *P. indica*-root priming could effectively delay the brown spot symptoms with reduced disease severity. In addition to

brown spot tolerance, *P. indica* could enhance growth promotion in the primed rice seedlings/plants.

New generation fungicides were evaluated against *H. oryzae* both *in vitro* and *in vivo* conditions. Triazole fungicides like tebuconazole, difenoconazole; strobilurins like azoxystrobin, pyraclostrobin and their combination fungicides were tested *in vitro*. The six fungicides screened against *H. oryzae* by poisoned food technique revealed that pyraclostrobin + tebuconazole at 100 ppm and 250 ppm completely inhibited the mycelial growth of *H. oryzae*. Next to these, azoxystrobin + tebuconazole and tebuconazole at 250 pm were effective and inhibited the mycelial growth of *H. oryzae* by 87.70 and 88.43 per cent respectively. Even at very low concentration (10 ppm), the new generation fungicides suppressed the growth of the pathogen.

Based on the results of *in vitro* evaluation, the best new generation fungicides were selected and tested *in vivo* against *H. oryzae*, for which a pot culture study was conducted with rice variety 'Uma'. On fifteen days after spraying, minimum lesion size of brown spot disease was recorded with the trifloxystrobin + tebuconazole – positive control (0.65 cm) followed by azoxystrobin + tebuconazole (0.84 cm) and pyraclostrobin + tebuconazole (1.17 cm). Brown spot disease severity (PDI) was recorded minimum with trifloxystrobin + tebuconazole at 0.05 per cent (10.81 %) followed by azoxystrobin + tebuconazole at 0.1 per cent (17.46 %) and pyraclostrobin + tebuconazole at 0.05 per cent (21.01%). Tebuconazole 0.1 per cent and pyraclostrobin 0.1 per cent also recorded lower PDI of 31.77 and 41.50 per cent respectively. Percentage of disease suppression over control was calculated for each treatment on the basis of PDI on 15th day. The result revealed that at recommended dosage, maximum disease suppression of 88.42 per cent was recorded by trifloxystrobin + tebuconazole (0.05 %) followed by by azoxystrobin + tebuconazole at 0.1 per cent (81.30) and pyraclostrobin + tebuconazole at 0.05 per cent (77.52).

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Thus the study concluded that the triazole and strobilurin alone and in combination were highly effective against brown spot of rice. Since these fungicides have site specific action and efficacy at lower doses, it could be used as a better alternative to the conventionally using fungicides.

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Appendices

APPENDIX - I

COMPOSITION OF STAIN USED

Lactophenol cotton blue

Anhydrous lactophenol- 67.0 ml

Distilled water – 20.0 ml

Cotton blue – 0.1 g

Anhydrous lactophenol prepared by dissolving 20 g phenol in 16 ml lactic acid in 3 ml glycerol.

Appendix – II

Potato Dextrose Agar (PDA) medium

Potato: 200g

Dextrose: 20g

Agar: 20g

Distilled water: 1 litre

Appendix - III

Murashige and Skoog medium

SI. No.	Chemicals	Stock solutions			For 1 litre
		1 litre	500 ml	250 ml	
Stock 1	NH_4NO_3	82.5 g	41.25 g	20.625 g	20 ml
	KNO_3	95 g	47.5 g	23.75 g	
	KH_2PO_4	8.5 g	4.25 g	2.125 g	
	MgSO4.7H2O	18.5 g	9.25 g	4.625 g	
Stock 2	CaCl. $2H_2O$	22.0 g	11.0 g	5.5 g	20 ml
Stock 3	Na_2.EDTA	3.7 g	1.85 g	0.925 g	10 ml
	$FeSO_4$. $7H_2O$	2.8 g	1.4 g	0.7 g	
Stock 4	$MnSO_4$. $4H_2O$	2.23 g	1.15 g	0.55 g	10 ml
	$ZnSO_4.7H_2O$	0.86 g	0.43 g	0.21 g	
	H_2BO_3	0.62 g	0.31 g	0.15 g	
	KI	0.083 g	0.04 g	0.02 g	
	Na_2MoO_4. $2H_2O$	0.02 g	0.01 g	0.006g	
	$CuSO_4$. $5H_2O$	0.002 g	0.001 g	0.0006 g	
	$CoCl_2$. $6H_2O$	0.0025 g	0.0012 g	0.0006 g	

Stock 5	Glycine	0.2 g	0.1 g	0.05 g	10 ml
	Nicotinic acid	0.5 g	0.025 g	0.012 g	
	Pyridoxim acid. HCl	0.5 g	0.025 g	0.0125 g	
	Thiamine. HCl	0.01 g	0.005 g	0.0025 g	
Inocitol					0.1 g
Sucrose					30 g
Agar					8 g

Above stock solution was pipetted out and 900 ml of distilled water, inocitiol and sucrose were added. pH was adjusted to 5.8 and make upto 1litre.

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Appendix - IV

Plant Nutrient Medium (PNM)

5 mM KNO3	- 0.5 g
2mM MgSO4. 7H2)	- 0.48 g
2mM Ca(NO3)2	- 0.472 g
Fe. EDTA	- 2.5 ml
Micronutrient mix	- 1 ml
Agar	- 10 g
Distilled water	- 1 litre

After sterilization, pH of media was adjusted by adding 2.5 ml filter sterilized 1M KH_2PO_4.

Micronutrient mix composition

70mM $H3BO_3$

14mM $MnCl_2$. $4H_2O$

0.5mM $CuSO_4$. $5H_2O$

1mM $ZnSO_4$. $7H_2O$

0.2mM Na_2MoO_4. $2H_2O$

10mM NaCl

0.01mM $CoCl_2$. $6H_2O$

Fe.EDTA

2.5 g $FeSO_4$. $7H_2O$ in 400 ml distilled water, add 3.36 g Na_2EDTA, boil the solution for 30 minutes and make up fnal volume to 450 ml.

Abstract

Management of brown spot of rice using fungal root endophyte *Piriformospora indica* and new generation fungicides

by

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ABSTRACT OF THE THESIS

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ABSTRACT

A study entitled 'Management of brown spot disease of rice using fungal root endophyte *Piriformospora indica* and new generation fungicides' was conducted during 2017-19 at Department of Plant Pathology, College of Agriculture, Vellayani with the objectives to evaluate the effect of root endophyte *Piriformospora indica* against brown spot disease of rice caused by *Helminthosporium oryzae* and evaluation of new generation fungicides *viz.*, strobilurins, azoles and their combinationsfor the management of brown spot disease of rice.

A survey was conducted in rice fields of six KAU stations during 2018-2019 to collect the infected leaf samples and to assess the disease incidence (DI) and severity (Percent Disease Intensity (PDI)). Among the surveyed locations, maximum DI (76%) and PDI (64.6%) were recorded from IFSRS Karamana whereas the disease was absent in RARS Kumarakom due to the complete crop failure. *H. oryzae* was isolated from the collected specimens; a total of five pure cultures of *H. oryzae* (Isolate 1 to Isolate 5) were obtained and Koch's postulates were proved for all the isolates in rice var. Uma.

All the *H. oryzae* isolates were screened for its virulence and pathogenicity in rice var. Uma. The isolate 5, from IFSRS Karamana, produced the symptom within 24 h of inoculation. On 5th day of leaf inoculation, isolate 5 recorded maximum PDI of 62.22 per cent with a maximum lesion size of 2.94 cm; and thus concluded as the most virulent isolate.

Dual culture assay of *P. indica* and *H. oryzae* in potato dextrose agar (PDA) medium indicated that the beneficial root endophytic fungus significantly inhibited the growth of the pathogen through multiple antagonistic properties *viz.*, lysis / inhibition zone (0.65 cm) and antibiosis (0.37 cm). Maximum growth inhibition of *H. oryzae* (54.44 %) by *P. indica* was observed on 7th day of dual culturing.

P. indica-primed rice seedlings and plants (var. Uma) could significantly delay the symptoms of *H. oryzae* up to eight days after inoculation (DAI) of the pathogen. The primed seedlings and plants recorded the lowest lesion size (67.87 and 63.6% over control respectively) at seven DAI and the highest disease suppression (87.6 and 80.3 % over control respectively) at 15 DAI. *P. indica*-root colonization in rice seedlings resulted in enhanced growth with increased root and shoot biomass. *P. indica*-colonized roots had more number of secondary and tertiary roots with profuse root hairs. There was two-fold increase in shoot and root fresh weight and three-fold increase in shoot dry weight and 2.3 fold increase in root dry weight. The above experiments were done in CRD with five replications.

In vitro evaluation of selected new generation fungicides *viz.*, tebuconazole 25.9 EC, difenoconazole 25 EC, azoxystrobin 23 EC, pyraclostrobin 20 WG, pyraclostrobin 20 WG + tebuconazole 18.3 SC, azoxystrobin (11%) + tebuconazole (18.3%) SC at 10, 50,100 and 250 ppm against *H. oryzae* in PDA by poisoned food technique revealed that all the selected fungicides significantly reduced the growth of *H. oryzae*. Combination fungicides were more effective in inhibiting the growth of *H. oryzae*. The highest inhibition was observed in pyraclostrobin 20 WG + tebuconazole 18.3 SC at different concentrations tested; and at 100 and 250 ppm, there was complete inhibition of mycelial growth of the pathogen followed by azoxystrobin (11%) + tebuconazole (18.3%) SC.

Based on the results of *in vitro* evaluation, a pot culture experiment was conducted with rice var. Uma to evaluate the efficacy of the best azole fungicide (tebuconazole 25.9 EC), best strobilurin fungicide (pyraclostrobin 20 WG) and their combinations (pyraclostrobin 20 WG + tebuconazole 18.3 SC and azoxystrobin (11%) + tebuconazole (18.3%) SC at 0.05 and 0.1 per cent against brown spot disease of rice in CRD with three replications. All the tested fungicides significantly reduced the lesion formation and development in leaves. Minimum lesion size of 0.84 cm and 1.17 cm was recorded in azoxystrobin (11%) + tebuconazole (18.3%) SC and pyraclostrobin 20 WG + tebuconazole 18.3 SC respectively. Similarly, the maximum disease suppression of 81.30 per cent and

77.50 per cent over control was recorded with azoxystrobin 11 + tebuconazole 18.3 SC at 0.1% and pyraclostrobin 20 WG + tebuconazole 18.3 SC at 0.05% respectively. The results clearly indicated the curative action of azole, strobilurin and its combination fungicides.

Thus, the present study revealed that brown spot of rice could be effectively managed by root colonization of rice seedlings with the beneficial root endophytic fungus, *P. indica* or foliar spraying of azoxystrobin 11% + tebuconazole 18.3% at 0.1% or pyraclostrobin 20 WG + tebuconazole 18.3 SC at 0.05%. The results may be subjected to multi-location and multi-seasonal field trials before recommendation. The compatibility of *P. indica* with new generation fungicides and the residue of fungicides in grains may further be studied.