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Isolation, Characterization and effect of Micro - Macronutrients on the growth of *Helminthosporium oryzae*

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ABSTRACT: Brown spot disease caused by *Helminthosporium oryzae* is considered to be a serious threat in rice cultivation in India. This disease in rice is causing a considerable yield loss in different rice growing regions of the world. In present study, *Helminthosporium oryzae* isolates were collected from five different rice growing regions of Tamil Nadu. Further, morphological characterization was studied on different media and molecular characterization of all the five isolates was done using ITS-1 and ITS-4 primers. Four macro nutrients *viz.*, Ammonium sulphate, potassium sulphate, calcium sulphate and magnesium sulphate and four micro nutrients *viz.*, ferrous sulphate, manganese sulphate, copper sulphate and zinc sulphate were tested against the growth of pathogen by incorporating these nutrients separately in Czapek's dox medium at 0.1%, 0.2% and 0.3% conc. individually. Among four micronutrients tested, $ZnSO_4$ @ 0.3% show maximum mycelial growth inhibition. Among four macronutrients tested, K_2SO_4 @ 0.3% recorded maximum inhibition of the growth of mycelium.

Keywords: Brown spot, macronutrients, micronutrients, mycelial growth, rice crop.

INTRODUCTION

Rice, second only to wheat in terms of area and production, is the primary source of food for nearly 90 percent of the world's human population, particularly in Asia. In terms of area and production, India is the world's second largest producer of rice after China. Rice is an important grain that contains a high amount of carbohydrate, protein, and fat. It accounts for more than one-fifth of all calories consumed by humans worldwide (Jatoi *et al*., 2018). The global demand for rice grain is increasing due to the continuous growth of the world population. There is a need to improve production technology in order to meet the demand. However, with the introduction of improved technologies and high yielding varieties, the crop has became susceptible to a wide range of biotic and abiotic stresses, particularly biotic stresses such as diseases (Sunder *et al*., 2014).

Brown spot of rice caused by *Helminthosporium oryzae* (Breda de Haan) Shoemaker (Teleomorph – *Cochliobolus miyabeanus*) was a major problem that eventually caused sustainable losses in both quality and quantity (Hossain *et al*., 2011). Furthermore, the disease triggered the tragic incidence of Bengal famine in 1943, which claimed the lives of 2 million people in the Bengal region prior to partition. The disease has the potential to increase crop severity by up to 90%, reducing crop growth, grain discoloration, and market quality of rice grain (Valarmathi and Ladhalakshmi 2018).

H. oryzae causes characteristic leaf spot on all the susceptible varieties of rice and the pathogen infects the rice plant at all the stages of its growth. Spots are formed on the blade and the leaf sheath. The spots vary in the shape and size. Pathogen attacks crop from seedling to milky stage. The symptoms appear as minute spots on the coleoptile, leaf blade, leaf sheath and glume, the spots are most prominent on leaf blades and glumes. On leaves, typical spots are brown in colour with grey or whitish centre resembling sesame seed with typical yellow halo over the spot (Sunder *et al.,* 2005).

There are two types of primary resistance mechanisms by which mineral nutrition can affect i.e., either by forming the mechanical barriers, primarily by the development of thicker cell walls, or by the synthesis of natural defense compounds, such as phytoalexins, antioxidants, and flavanoids, that would provide protection against pathogens (Bhaduri *et al.,* 2014; Prakash and Verma 2016; Meena *et al*., 2015a, 2016;

Priyadharsini and Muthukumar 2016; Kumar *et al.* 2017).

Soil and Foliar application of sulphur-based nutrients has proven effective to boost resistance to a variety of fungi pathogens on various crops (Wang *et al.,* 2003; Klikocka *et al.,* 2005). The use of fertiliser in Indian soil conditions increased the disease resistance, improved seed viability, and seedling viability (Cakmak *et al*., 2009). Zinc has ben mentioned by many authors in sulphate form for the treatment of rice diseases (Ramabadran and Velazhagan 1988; Reddy *et al.,* 1989; Singh *et al.,* 2009). As a result, this present research has been conducted to develop novel, more effective, and long-term disease management programs. Hence, the present study is mainly focused on the collection, morphological and molecular characterization of brown spot pathogen in rice, and its effective management by using macronutrients and micronutrients.

MATERIALS AND METHODS

Collection of samples. The infected rice leaf samples were collection from different rice growing regions of Tamil Nadu *viz*., Paddy breeding station (Coimbatore), Tamil Nadu Rice Research Institute (Aduthurai), Hybrid Rice Evaluation Centre (Gudalur), Farmer's field (Ariyalur), Farmer's field (Tiruchirappalli) from the varieties CO 52, BPT 5204, VGDM-9, CR 1009 and CO-43 respectively during the season of Rabi-2021.

Isolation of the pathogen. The fungus was isolated from a leaf sample infected with brown spot. The infected leaf samples were washed with sterile water and the infected portion of the leaf along with the green portion were cut into small pieces. These were dipped in 1 per cent sodium hypochlorite for 1 minute and then washed three times with sterile distilled water to remove the excess sodium hypochlorite. To remove excess water from the leaf surface, the leaf bits were air dried on blotter paper. These surface sterilized infected leaf pieces were placed on sterile Petriplates containing solidified PDA media which is amended with streptomycin sulphate to avoid bacterial contamination. The petri-plates were incubated for three days at 28±2°C, and the actively growing mycelium was sub-cultured. The isolated fungi were purified using the single spore method, and the purified cultures were stored at 4°C in PDA slants. The isolates were named as HO-1 to Coimbatore, HO-2 to Aduthurai, HO-3 to Gudalur, HO-4 to Ariyalur, and HO-5 toTiruchirappalli isolates.

Cultural variability. The colony morphological growth of all the five isolates are carried out on different media like potato dextrose agar (PDA) media, host extract agar (HEA) media, Czapek's dox agar (CZA) media and malt extract agar (MEA) media to know the media that is more suitable for the growth of the pathogen.

Morphological characterization. Five isolates were morphologically characterized in PDA and incubated at 28±2°C for 7 days. Colony growth, mycelial colour, sporulation, conidium size, shape, colour, and conidiophore characteristics were all observed. For 5–7 days, all five isolates were grown in PDA medium. A 9.0 mm mycelial disc from a 7-day-old culture was placed in the centre of a sterilized glass slide under aseptic conditions on a moist sterile Petri-plate and incubated at 25 ± 2°C for 3 days with 12 hours of light and 12 hours of darkness. After 3 days of incubation, the spore suspension was collected and examined under a compound microscope using sterile distilled water at 40X magnification (Kumari *et al*., 2015).

Pathogenicity test. For pathogenicity test, three different rice varieties *viz.,* CO 39, TN 1, BPT 5204 are used. Each variety is maintained at three replications. Based on cultural variability, HO-1 isolate is found to be more effective in growth, so HO-1 isolate is mass multiplied in sterilized paddy chaffy grains in 250 ml conical flask for 15 days. The spore suspension is collected by adding sterile water in the conical flask and shake vigorously and the suspension is filtered through muslin cloth. Then the spore concentration is adjusted to 5×10^5 using haemocytometer. 2-3 drops of Tween-20 is added to the spore suspension and it is sprayed on 30 days old plants. The control plants are sprayed with sterile distilled water alone. Inoculated plants are covered with transparent bag for 24 hrs (Nazari *et al*., 2015).

PDI is calculated based on the formula given below , by adopting 0-9 scale

$$\text{Per cent disease index (PDI)} = \frac{\text{Sum of all ratings}}{\text{Maximum disease grade} \times \text{Total no. of plants rated}} \times 100$$

Molecular Characterization:

DNA extraction. The brown spot pathogen isolates were grown in Potato Dextrose Broth for 15 days before harvesting the mycelial mats through filter paper. The mycelial mats were dried for 24 hours at room temperature. The DNA was extracted using the CTAB method, as described by (Saghai-Maroof *et al*., 1984). The extracted genomic DNA was electrophoresed on 0.8 % agarose gel for 30 minutes with loading dye, and the presence of genomic DNA was documented using an image analyzer.

PCR analysis. The universal primers ITS-1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') were used to screen all five *H. oryzae* isolates. The PCR amplification reaction was performed in a final volume of 30µl, which included 15 µl Master mix, 3 µl forward primer ITS-1, 3 µl reverse primer ITS-4, and 6µl sterile distilled water. Amplified by universal primers ITS-1 and ITS-4 (White *et al*., 1990) with PCR conditions which are described by Berbee *et al*., 1999. The PCR amplification was performed in a thermo cycler with the following conditions: initial denaturation at 95°C for 2 minutes, followed by 40 cycles of denaturation (95°C for 30 seconds), annealing (55°C for 30 seconds), and extension (72°C for 1 minute), and final extension at 72°C for 10 minutes, followed by 4 minutes of hold at 4°C. The PCR products were then electrophoresed for 1 hour in 1X TAE buffer in 1.2 per cent agarose gel stained with Ethidium bromide. The

gel was visualized in the gel documenting unit using a UV transilluminator.

In vitro* evaluation of effect of micronutrients and macro nutrients on the growth of *H. oryzae. Effect of certain micro nutrients *viz*., copper sulphate, ferrous sulphate, manganese sulphate, and zinc sulphate and macro nutrients *viz*., calcium sulphate, magnesium sulphate, potassium sulphate and ammonium sulphate were tested against the growth of *H. oryzae* was studied by incorporating the nutrients separately in Czapek's Dox medium. Both the micro and macro nutrients were tested at 0.1%,0.2% and 0.3% conc. (Ragavan, 2003). 15 ml of Czapek's agar medium with respective micro nutrients was poured into sterile Petri plates. The medium without any amendment served as control. Each plate was inoculated aseptically with nine mm fungal disc obtained from the actively growing region of one week old culture of *H. oryzae*. The plates were incubated at room temperature (28±3°) and the diameter of the mycelial growth was recorded when the growth in the control plates touched the periphery and was expressed in mm.

Using the formula, the efficacy was expressed as a percentage inhibition over the control.

Per cent Inhibition = C – T/ C ×100

C – Mycelial growth in control, T – Mycelial growth in treatment

RESULTS AND DISCUSSION

Cultural variability. Among the different media's tested, potato dextrose agar showed maximum radial growth (88.90 mm) in all the five isolates then followed by host extract dextrose agar (87.50 mm). In PDA and MEA media, isolate HO-4 has shown slow growth. In CZA and HEA media, isolate HO-2 has shown slow growth. The results are in accordance with Arshad *et al.* (2013), wherein they recorded the maximum growth of pathogen on Potato dextrose agar with 57.80 mm and Kumari *et al.* (2015), found the maximum growth (90 mm) of different isolates on Potato dextrose agar media (Fig. 1& 2, Table 1 & 2).

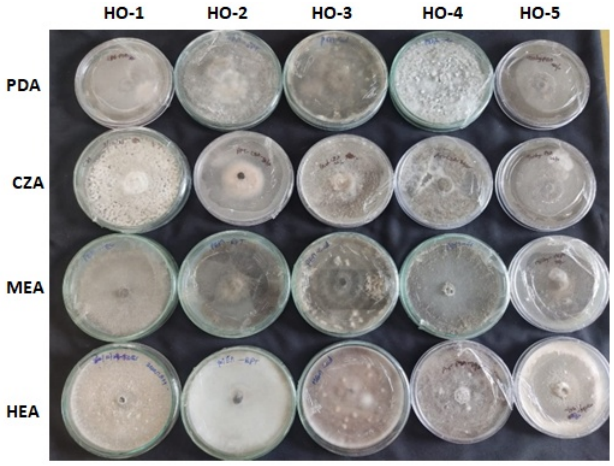


Fig. 1. Cultural variability of *H. oryzae* isolates in different media.

Fig. 2. Conidial characters of different isolates of *H. oryzae.*

Table 1: Cultural variability and mycelial growth of *H. oryzae.*

Sr. No.	Isolates	Radial growth of mycelium in different media (in mm)*				Colony character	Growth habitat	Sporulation
		PDA	CZA	HEA	MEA			
1.	HO1	89^{a}	87^{a}	88^{a}	85^{a}	Greyish black colour colony with cottony mycelial growth	Fast	+
2.	HO2	86^{b}	82^{e}	80^{e}	84^{b}	Light greyish colour with cottony growth	Fast	+
3.	HO3	85^{c}	86^{b}	87^{b}	83^{c}	Greyish black colour mycelial growth	Fast	+
4.	HO4	77^{e}	85^{c}	82^{d}	74^{e}	Greyish white colony growth	Moderate	+
5.	HO5	83^{d}	84^{d}	85^{c}	81^{d}	Greyish black colour colony growth	Fast	+
SE(d)		1.51	1.03	2.02	1.18			
CD		3.40	2.33	4.55	2.67			

* Mean of the three replication

Means in the column followed by same superscript letters are not significantly different according to DMRT

Table 2: Conidial characters of different isolates of *H. oryzae.*

Sr. No.	Isolates	Conidial characters		
		Length(µm)	Breadth(µm)	No. of septa
1.	HO1	28.02	4.86	9
2.	HO2	23.75	4.45	8
3.	HO3	25.26	4.85	7
4.	HO4	16.45	3.81	6
5.	HO5	25.58	4.73	9

Morphological characterization. All the isolates studied showed significant differences in growth and colony characters. The variation in morphological characters of different isolates indicated that isolate HO-1 showed greyish black colour colony with mycelial cottony growth, HO-2 showed Light greyish colour with cottony growth, HO-3 showed Greyish black colour mycelial growth, HO-4 showed greyish white colony growth and HO-5 showed Greyish black colour colony growth. Margin of the isolates viz., HO-1, HO-3, HO-5 showed regular pattern and HO-2, HO-4 showed irregular pattern. HO-3, HO-5 has shown flat mycelia growth, where as HO-1, HO-2, HO-4 has shown cottony mycelial growth. Sporulation is observed in all the five isolates using light microscope (40X) magnification (Fig. 3).

Molecular characterization. The full length ITS-1 rDNA region was amplified with ITS-1 (5'-TCCGTAGGTGAACCTGCG-3') and ITS-4 (5'-TCCTCCGCTTATTGATATGC-3') primers for the five isolates of *H. oryzae*. DNA amplicon was observed at the region 570 bp by checking the amplified products on 1.2% agarose gel electrophoresis and representative samples were sequenced (Fig. 4).

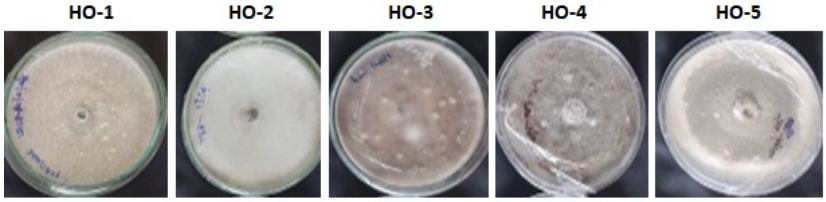


Fig. 3. Morphological variability of *H. oryzae* isolates.

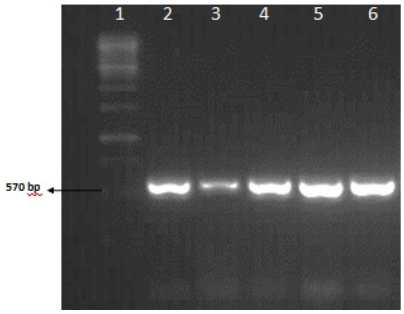


Fig. 4. PCR amplification of ITS region using Universal primers (ITS-1 and ITS-4), Lane 1- 250-10,000 bp ladder, Lane 2- HO-1,Lane-3, HO-2, Lane 4- HO-3, Lane 5- HO-4, Lane 6 –HO-5.

Pathogenicity test. Rice variety CO-39 is more susceptible to brown spot pathogen with a PDI of 32.16% at 14th day after inoculation followed by BPT-5204 with PDI of 25.78 and TN-1 with less PDI of 14.07 at 14th day after inoculation. Initially, small brown spots were appeared, later these spots enlarge and form oval shaped spots. These spots later coalesce and give dried appearance of leaves (Fig. 5, Table 3).

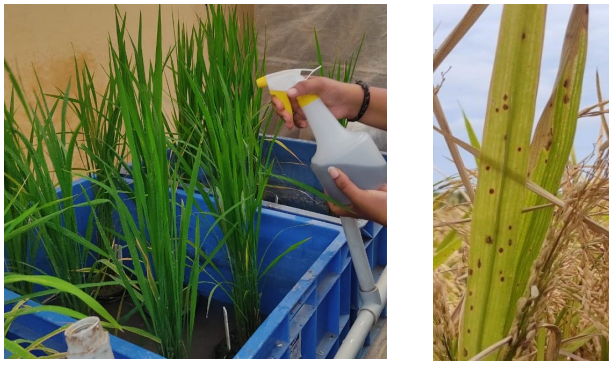


Fig. 5. Pathogenicity test: Appearance of brown spot symptoms on artificially inoculated plants.

Table 3: Pathogenicity test of different varieties of rice with PDI.

Sr. No.	Days after inoculation	Name of the variety with PDI*		
		CO 39	BPT 5204	TN 1
1.	2	0.49^{g}	0.41^{g}	0.19^{g}
2.	4	1.81^{f}	1.45^{f}	0.51^{f}
3.	6	8.03^{e}	4.55^{e}	2.45^{e}
4.	8	13.92^{d}	9.58^{d}	5.56^{d}
5.	10	21.69^{c}	15.78^{c}	9.30^{c}
6.	12	31.03^{b}	22.61^{b}	13.30^{b}
7.	14	32.16^{a}	25.78^{a}	14.07^{a}
SE(d)		0.518	0.495	0.193
C.D.(0.05)		1.122	1.072	0.417

* Mean of the three replication
Means in the column followed by same superscript letters are not significantly different according to DMRT

***In vitro* evaluation of effect of micronutrients on the growth of *H. oryzae*.** The study revealed that, the micronutrients that are tested significantly inhibited the growth of the fungus in all the concentrations. Here, there is an increased inhibition of the growth was recorded with the increase in nutrient concentration. Among these nutrients, Zinc sulphate @ 0.3% conc. has recorded the maximum inhibition of 58.89 % over control, followed by copper sulphate, ferrous sulphate, and least inhibition is recorded by manganese sulphate. Among four micronutrients evaluated, highest per cent mean mycelial inhibition was recorded in Zinc sulphate (46.7%) and the least per cent mean mycelial inhibition was recorded in manganese sulphate (17.4%) (Fig. 6, Table 4 & Graph 1).

***In vitro* evaluation of effect of macronutrients on the growth of *H. oryzae*.** The study revealed that, the macronutrients that are tested significantly inhibited the growth of the fungus in all the concentrations. Here, there was an increased inhibition of the growth recorded with the increase in nutrient concentration. Among these nutrients, potassium sulphate @ 0.3% conc. has been recorded to show maximum inhibition of 56.67 per cent over control, then followed by calcium sulphate, magnesium sulphate, and least inhibition is recorded by Ammonium sulphate. Among four macronutrients evaluated, highest per cent mean mycelial inhibition was recorded in potassium sulphate (47.4 %) and the least per cent mean mycelial inhibition was recorded in Ammonium sulphate (15.56 %) (Fig. 7, Table 5 & Graph 2).

Table 4: *In vitro* evaluation of micro nutrients against mycelial growth of brown spot pathogen.

Sr. No.	Treatment	Mycelial growth 7 days after incubation (mm)*			Percent mycelial inhibition*			Mean mycelial inhibition
		0.1%	0.2%	0.3%	0.1%	0.2%	0.3%	
1.	Ferrous sulphate	75.00^{c}	67.00^{d}	53.00^{c}	16.67^{c}	25.56^{c}	41.11^{c}	27.8^{c}
2.	Manganese sulphate	81.00^{d}	74.00^{e}	68.00^{d}	10.00^{d}	17.78^{d}	24.44^{d}	17.4^{d}
3.	Copper sulphate	62.00^{b}	54.33^{b}	46.00^{b}	31.1^{b}	40.00^{b}	48.89^{b}	40.0^{b}
4.	Zinc sulphate	57.66^{a}	49.00^{a}	36.66^{a}	35.56^{a}	45.56^{a}	58.89^{a}	46.7^{a}
5.	Control	90.00^{e}	90.00^{e}	90.00^{e}	0^{e}	0^{e}	0^{e}	0^{e}
SE(d)		1.23	0.56	1.38	0.39	0.64	0.69	0.68
C.D.(0.05)		2.77	1.26	3.12	0.87	1.45	1.57	1.53

* Mean of the three replication
Means in the column followed by same superscript letters are not significantly different according to DMRT

Table 5: *In vitro* evaluation of macronutrients against mycelial growth of brown spot pathogen.

Sr. No.	Treatment	Mycelial growth 7 days after incubation (mm)*			Percent mycelial inhibition*			Mean mycelial inhibition
		0.1%	0.2%	0.3%	0.1%	0.2%	0.3%	
1.	Ammonium sulphate	80.00^{d}	77.00^{d}	71.33^{d}	11.11^{d}	14.45^{d}	21.11^{d}	15.56^{d}
2.	Pottasium sulphate	58.00^{a}	46.00^{a}	39.00^{a}	35.56^{a}	48.89^{a}	56.67^{a}	47.04^{a}
3.	Calcium sulphate	69.00^{b}	59.66^{b}	54.00^{b}	23.30^{b}	33.3^{b}	40.00^{b}	32.20^{b}
4.	Magnesium sulphate	73.66^{c}	66.00^{c}	61.00^{c}	17.78^{c}	26.67^{c}	32.22^{c}	25.56^{c}
5.	Control	90.00^{e}	90.00^{e}	90.00^{e}	0^{e}	0^{e}	0^{e}	0^{e}
SE(d)		1.76	1.33	0.84	0.28	0.75	0.77	0.38
C.D.(0.05)		3.98	3.01	1.90	0.64	1.69	1.74	0.86

* Mean of the three replication
Means in the column followed by same superscript letters are not significantly different according to DMRT

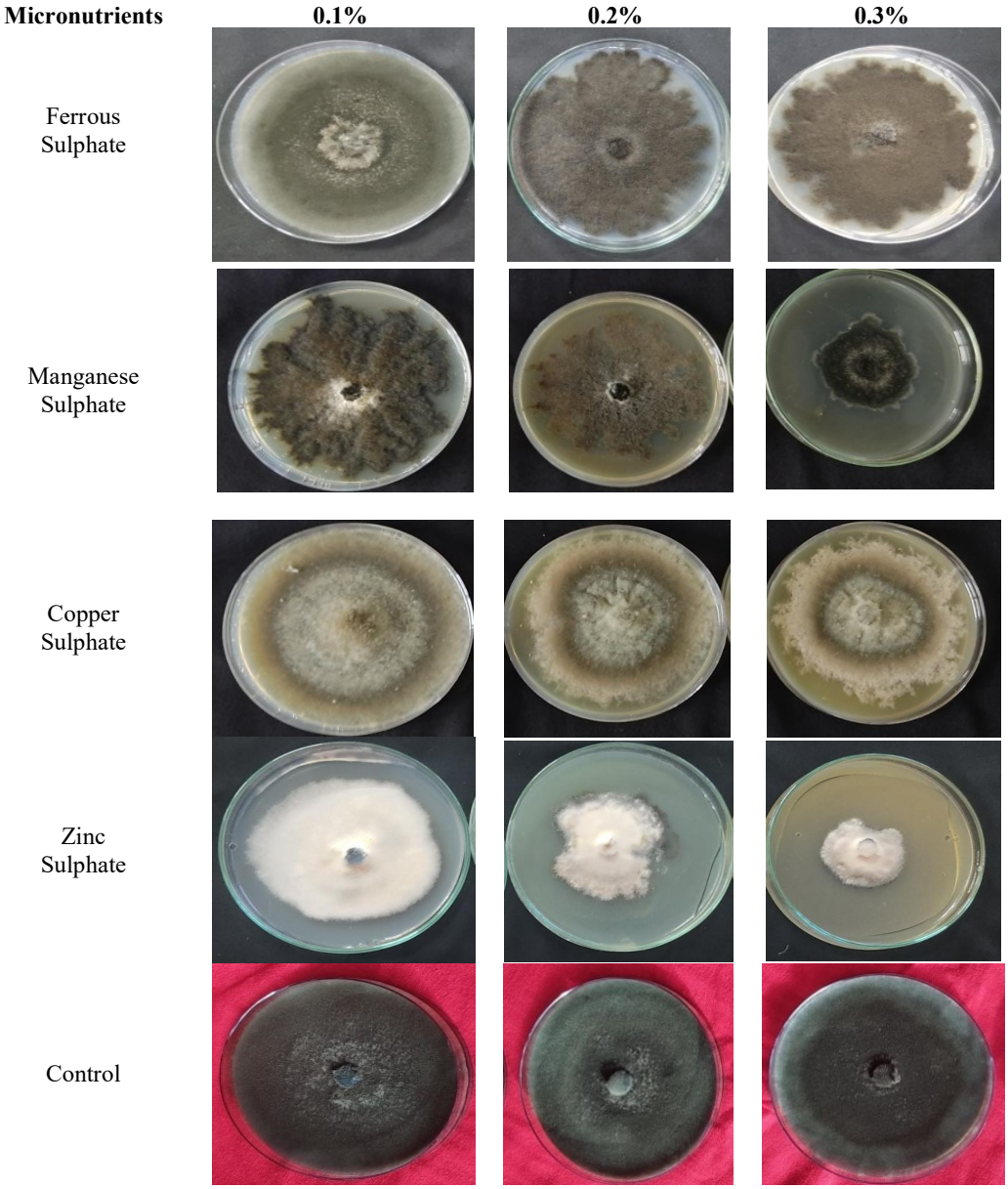
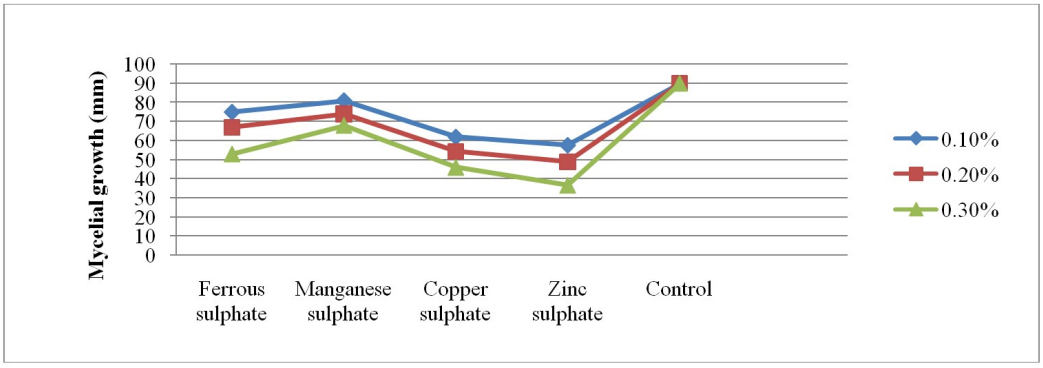
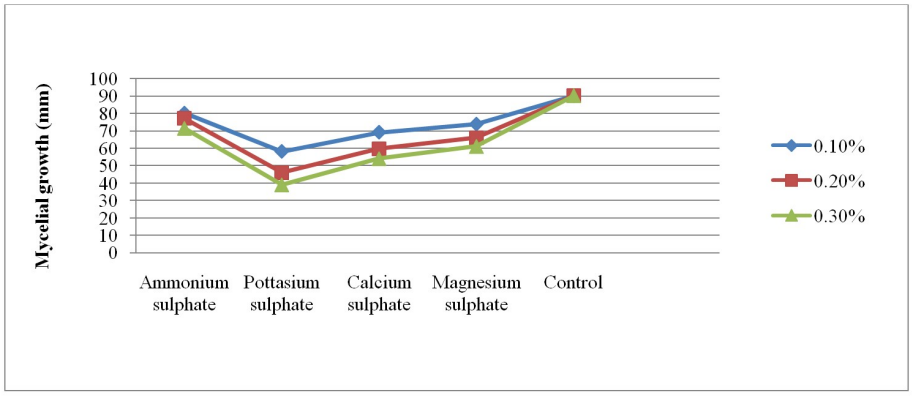


Fig. 6. *In vitro* evaluation of effect of micronutrients on the linear growth of *H. oryzae.*



Graph 1. Effect of micronutrients on linear growth of *Helminthosporium oryzae.*



Graph 2. Effect of macronutrients on linear growth of *Helminthosporium oryzae.*

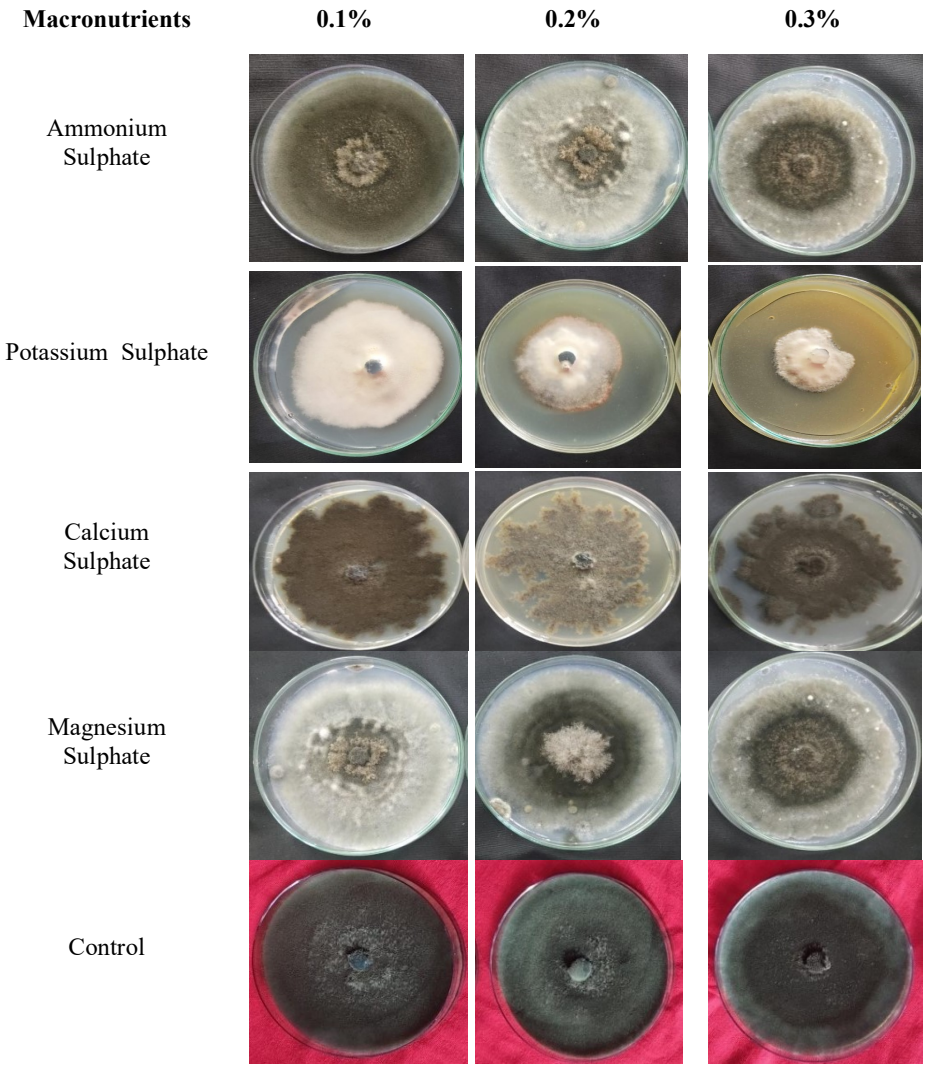


Fig. 7. *In vitro* evaluation of effect of macronutrients on linear growth of *Helminthosporium oryzae.*

DISCUSSION

In this study, brown spot infected leaves were collected from five different locations of Tamil Nadu. The brown spot pathogen was isolated and characterized based on the cultural and morphological characters. The five isolates were categorized into 2 groups: grayish black with cottony growth and grayish black colony with white spots. Mycelium is brown in colour, branched and septate and it gives rise to conidiophores from which conidia arises. Monisha *et al.* (2019) have grouped *Bipolaris oryzae* into three categories: Isolate BO-1 was having greyish with cottony mycelium, where as isolates BO-2, 4, 5 were having a mixture of grey and white mycelium with fluffy growth and Isolate

BO-3 have greyish cottony mycelium with white spots. Conidia are brown in colour, slightly curved and multi septate. Kumar *et al.,* (2011) have grouped *H. oryzae* into four different categories based on cultural characters *viz.,* black colour colony with suppressed growth, black with cottony growth of colony, black with fluffy colony growth and White with cottony growth. This was the first report by Kumar *et al.,* (2011) on the black colony character of *H. oryzae*. Kumari *et al.* (2015) categorized 52 isolates into 5 groups: Black colony with fluffy growth, Black colony with suppressed growth, Grey and cottony growth, Grey and white cottony growth and white with cottony growth. Among the different media's tested, potato dextrose agar showed maximum radial growth (88.90 mm) in all the five isolates. In pathogenicity test, among three different rice varieties, CO-39 is recorded to have more PDI which indicates more susceptible to brown spot when compared to BPT-5204 and TN-1. Next to CO-39, BPT-5204 is susceptible to brown spot pathogen and TN-1 is less susceptible than other two varieties. For the pathogenicity test, symptom development of adapted and non-adapted *Bipolaris* spp. was assessed on the resistant and susceptible cultivars of rice and corn at 3, 5 and 7 days after inoculation. Lesion size, lesion density and percentage disease severity were also taken for the test (Amorio and Cumagun 2017). This study revealed that all the nutrients which are tested have shown significant inhibition in the growth of the fungus at all concentrations when they are compared to control. Also, there is an enhanced inhibition recorded with the increase in the concentrations of the nutrients tested. Among the tested micro nutrients, zinc sulphate recorded maximum mycelial inhibition at 0.3% conc. with 58.89 per cent inhibition over control. It was followed by copper sulphate, ferrous sulphate, and manganese sulphate in the decreasing order of inhibition. With regard to the macronutrients, Potassium sulphate recorded maximum mycelial inhibition at 0.3% conc. with 56.67 per cent inhibition over control. It was followed by calcium sulphate, magnesium sulphate, and Ammonium sulphate in the decreasing order of inhibition. Addition of Ca, Mn, Cu, Zn, Fe, B and Mo at different concentrations against the pathogens of rice has showed the reduction in fungal growth (Madhiyazhagan, 1989). The inhibitory effect of calcium sulphate which is against different rice pathogens under *in vitro* conditions was also reported (Eswaran and Narayanasamy, 2000; Ragavan, 2003). As we observed in the present study, they have also reported the increased inhibition with increase in concentration of $ZnSO_4$. From different macro-micro nutrients, $ZnSO_4$ @ 3000 ppm has been shown the maximum inhibition of growth of mycelium, mycelial dry weight and also germination of spore. Silicon based nutrients like Potassium Silicate, Calcium Silicate and Sodium Silicate, potassium silicate @ 3000 ppm has been recorded the maximum inhibition of mycelial dry weight (Jaiganesh, 2019).

CONCLUSION

Hence, these findings revealed the role of micro and macronutrients play key role in management of brown spot of rice.

FUTURE SCOPE

Disease resistance of any plant is mainly genetically controlled but has a close association with the nutritional status of the plants; and thus, management by using micro and macro nutrients has always been an important regulator for plant diseases. There is a dynamic interrelation between the nutritional status of plants with pathogen and abiotic environment, and hence, proper management by chemicals and micro nutrients in cultivated crops can effectively decline the severity of most diseases. Further, with nutrient management, the decrease in the severity of diseases is more pronounced, when the crops are undernourished. Foliar spray of nutrients or fungicide application has declined the severity of diseases. In addition, there is an utmost need for the inclusion of varieties with disease resistant or tolerance in IPM practices that can effectively combined with the specific nutrient management schedules for managing plant diseases and also preserve environmental quality.

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Conflict of Interest. None.

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