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Full Length Article

Molecular Characterization of *Magnaporthe oryzae* in Punjab, Pakistan and its *In Vitro* Suppression by Fungicides and Botanicals

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Abstract

Rice blast, caused by the fungus *Magnaporthe oryzae* (anamorph: *Pyricularia oryzae*), is a disease of major economic importance in rice-growing regions worldwide, but has received little attention in Pakistan. Surveys were conducted in five rice-cultivating districts of Punjab province, Pakistan during 2016 and 2017. Disease incidence was recorded in 25 surveyed fields. Pathogen identification was confirmed as *M. oryzae* based on the morphological characterization and pathogenicity assays. BLASTn comparison of sequences of four different gene regions (ITS, actin, beta tubulin, calmodulin) of 25 isolates resulted in ≥99% homology to type specimens of *M. oryzae*. In *in vitro* and greenhouse experiments, commercially available fungicides (difenoconazole, mancozeb, tetrachlorophthalide, tebuconazole + trifloxystrobin, carbendazim, propiconazole, thiophanate-methyl) were compared with applications of botanical extracts (black pepper, clove, aloe vera, neem, and ginger) for their ability to inhibit the growth of the pathogen. The maximum incidence of rice blast, 71.25%, was observed in Jalalpur Bhattian (Hafizabad). Morphological, pathogenicity and molecular studies confirmed the *M. oryzae* as the causal organism of the rice blast disease. Difenoconazole inhibited the *in vitro* mycelial growth of *M. oryzae* more than other compounds (83.55%), followed by black pepper (70.68%). In greenhouse trials, disease severity was lowest (28.50%) for difenoconazole, followed by black pepper (33.75%). Difenoconazole and black pepper showed the most promising fungicide and botanical for the management of rice blast in Punjab province, Pakistan.

Keywords: *Magnaporthe oryzae*; Diversity; Disease Incidence; Fungicides; Botanicals

Introduction

Rice is a vital and strategic human food crop for food security worldwide (Perez-Montano *et al.*, 2014). In Pakistan, rice ranked as the second most consumed cereal crop after wheat and the third most profitable crop after wheat and cotton (Ameen *et al.*, 2014). Diseases are among the most important restrictive factors that disrupt the rice production, causing an annual yield drop probably assessed at 5% (Song and Goodman, 2001; Wang *et al.*, 2018). In Pakistan, rice is cultivated on 2,899,000 hectares and the annual production is 7,442,000 tons (P.B.S., 2017–2018). However, rice production per hectare is lower than in many other countries, in part due to ineffective disease management (Ghazanfar *et al.*, 2009). The fungus *Magnaporthe oryzae* infects to all portions of the rice plant but the greatest loss occurred when the necks and panicles are attacked. Symptoms are more acute for neck blast, which is characterized by infection at the base of panicle and subsequent rot (Xiao *et al.*, 2017). Rice blast losses in individual fields can be severe; for example, 75%, 50% and 40% grain reduction in India, Philippines and Nigeria, respectively (Padmanabhan, 1965; Awoderu and Esuruoso, 1974; Ou, 1985). Blast causes at least US$55 million production losses annually in South and Southeast Asia (Sukanya *et al.*, 2011). In Pakistan, during the past 20 years, rice blast has occurred frequently in the Punjab province districts of Toba Tek Singh, Faisalabad, Vehari (Arshad *et al.*, 2008).

Misidentification of fungal pathogens has traditionally slowed efforts to combat major crop diseases in Pakistan, but DNA-based methods have improved the speed and accuracy of pathogen identification. For *M. oryzae*, the internal transcribed spacer (ITS), β-tubulin, calmodulin and actin gene regions have been used to reliably confirm the identify of isolates (Hirata *et al.*, 2007). Using multi-gene phylogenetic analysis, researchers have characterized diversity within collections of *M. oryzae* isolates from several major rice-producing countries (Choi *et al.*, 2013; Abed-Ashtiani *et al.*, 2016), but there are no reports that assess the diversity of this important pathogen in Pakistan.

Several synthetic chemical fungicides, including difenoconazole, mancozeb, tetrachlorophthalide, tebuconazole + trifloxystrobin, carbendazim, propiconazole,

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thiophanate-methyl are commonly sprayed to manage the blast of rice (Anwar *et al.*, 2002; Gohel *et al.*, 2008; Ghazanfar *et al.*, 2009). However, intensive programs of fungicide spraying can endanger human health, degrade environmental health, and accelerate the development of fungicide resistance, so there are persuasive reasons to seek alternative approaches to supplement or replace synthetic chemical fungicides. Botanical extracts offer a much more favorable safety profile, and several have shown promise in suppressing crop diseases (Iftikhar *et al.*, 2010; Babar *et al.*, 2011). Herbal medicinal plants such as *Aloe vera, Piper nigrum, Syzygium aromaticum, Zingiber officinale* and *Azadirachta indica* have proven antifungal properties. These extracts incorporate a wide range of enzymes which suppress fungi and can slow the development of rice blast (Hasan *et al.*, 2012; Abdel-Shafi, 2013; Uda *et al.*, 2018).

The objectives of the current study were to i) characterize the rice blast fungus from core rice producing areas of Punjab, Pakistan, and ii) compare the efficacy of several botanical extracts and synthetic chemical fungicides for suppression of rice blast.

Materials and Methods

Survey and Sample Collection

A detailed survey was conducted during 2016 and 2017 to record the rice blast disease incidence in farmer fields of Punjab province, Pakistan. Five major rice-cultivating districts were included: Nankana, Narowal, Hafizabad, Sialkot and Gujranwala were selected for these surveys. In each district, five villages were arbitrarily selected based on previous history of rice blast for disease incidence rating and sampling (Fig. 1). The stratified sampling design with five arms were used for sampling and disease incidence rating. The incidence was recorded in 1-meter radius at five different ends of the sampling arms located at 100 feet distance. The coordinates of each visited area were also recorded using the GPS device (Garmin Montana 680T GPS, U.S.A.). The data of disease incidence were recorded by using the formula;

$$\text{Disease Incidence (\%)} = \frac{\text{Number of diseased plants}}{\text{Total number of plants}} \times 100$$

As, the disease appears during all stages of the rice plant, the leaf/panicle samples of rice blast at panicle emergence stage were collected on visual symptomatic basis. The samples were brought back to the laboratory at University of Agriculture Faisalabad (U.A.F.). The samples were stored at 4°C refrigerator till further processing.

Isolation and Identification of the Pathogen

Segments (5 mm^2) of leaf tissue from the edges of lesions were excised using a sterilized scalpel followed by immersed in 1% sodium hypochlorite solution for 1 minute to remove any secondary pathogens. The samples were rinsed twice with distilled water and dried on autoclaved filter paper disc and transferred to potato dextrose agar (PDA) medium. The petri dishes were incubated at 26°C under 12-h per day illumination for 6–9 days and examined for fungal growth. So, purified cultures were examined under the microscope, and morphological characteristics (the colony color, conidia shape, conidia color and size, conidial septations, hyphal septations and color) were noted.

Pathogenicity Assay

Rice seedlings (Basmati-385) were grown in earthen pots that are filled with autoclaved soil. Immediately before inoculating, thirty days old rice plants were sprayed with sterile distilled water and covered with polyethylene bags for 24 h to provide a high-humidity microenvironment. The spore suspension (2×10^5 spores/mL) was made in sterile distilled water from 10 days old fungal cultures of *M. oryzae*. The infected leaf samples were collected from (Kot Hira Das) Nankana district, grown on PDA plates at 25°C and pathogen was identified based on their morphological keys. The spore suspension was sprayed onto the healthy plant leaves and again covered with plastic bags. The bags were removed, after 48 h. of incubation and the plants were further transferred to the greenhouse. The control plants were kept by spraying the plants with only sterile distilled water and incubating as for inoculated plants. Observations were made for symptom development after one week of interval. To complete the Koch's postulates, the pathogen was re-isolated from diseased leaves and morphological traits were compared with original cultures.

PCR Amplification and DNA Sequencing

The DNA was extracted by using the CTAB method (Moller *et al.*, 1992) and further quantified by Nanodrop 2000 spectrophotometer (Thermo Scientific, U.S.A.). Amplification of DNA product was made using the PCR green master mix (buffer, dNTPs, thermostable hot-start DNA polymerase) and forward and reverse primers. For the ITS region, the primers ITS1F and ITS4R (White *et al.*, 1990), for beta tubulin gene the primers bt-1a and bt-1b (Glass and Donaldson, 1995), for actin gene the primers ACT-512-F and ACT783-R (Carbone and Kohn, 1999) and for calmodulin gene the primers CAL-228-F and CAL-737-R (Carbone and Kohn, 1999) were used. Polymerase chain reaction (PCR) amplification was performed in a total reaction volume of 30 μL. Amplifications were carried out in a thermocycler programmed for 94ºC for 2 min. followed by 34 cycles at 94ºC for 30 s, 53ºC annealing temperature for 30s, and 72ºC for 1 min; and a final extension for 10 minutes at 72ºC (Hirata *et al.*, 2007). PCR products were examined by electrophoresis in 1% agarose gel and analyzed by staining with gel green. Further, the amplified products were viewed under ultraviolet trans-illuminator

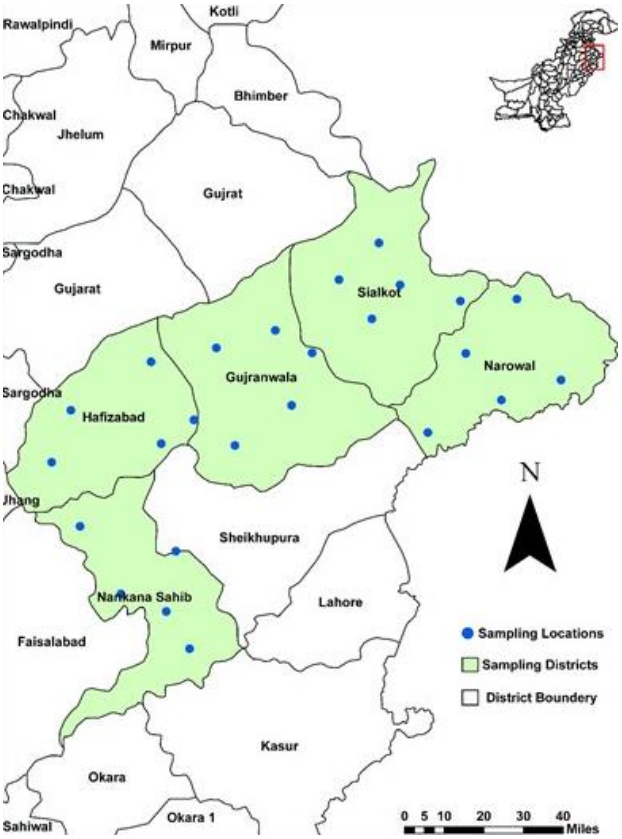

Fig. 1: The map represents the locations in Punjab, Pakistan from where the infected disease samples of rice blast were collected, and the data of disease incidence were examined

and photographed by using the Gel Doc™ EZ imager. The purified PCR products were sequenced in both the directions. Homology examinations for resultant sequences were completed by relating with those submitted in GenBank Nucleotide database, introduced by the National Center for Biotechnology Information (NCBI) using BLAST analysis (Boratyn *et al.*, 2013). All the generated sequences in this study were submitted to GenBank and assigned with accession numbers.

In vitro Assessment of Fungicides and Plant Extracts

The poisoned food technique was used to assess the efficiency of seven commercial fungicides difenoconazole (score 250 EC), mancozeb (dithane M-45), tetrachlorophthalide (rabicide 30 WP), tebuconazole + trifloxystrobin (nativo), carbendazim (bavistin), propiconazole (tilt 250 EC), thiophanate-methyl (topsin-M) and five plant extracts black pepper (*Piper nigrum*), clove (*Syzygium aromaticum*), aloe (*Aloe vera*), neem (*Azadirachta indica*) and ginger (*Zingiber officinale*) under *in vitro* conditions. The plant extracts were prepared in ethanol solvent by following the method Uda *et al.* (2018).

Three fungicide doses (100 ppm, 200 ppm and 300 ppm), and three concentrations of plant extracts (5%, 10% and 15%) were also tested to suppress the growth of *M. oryzae*. The experiment was conducted in factorial arrangements in a completely randomized design (CRD) with three replications. Concentrations of fungicides and botanicals and days of assessment of growth inhibition (%) were treated as different factors. Fungicides and plant extracts were diluted in cooling potato dextrose agar (PDA). A sterilized cork borer (8 mm) was used to excise plugs from the pure culture of actively growing *M. oryzae* colonies; the plugs were shifted to the center of each amended PDA plate. Control plates grew the transferred plugs on non-amended PDA plates. All plates were incubated at 26°C and fungal mycelial growth was recorded after 3, 5 and 7 days. The mycelial radial growth inhibition (%) was recorded by using the following formula:

$$\text{Inhibition of the mycelial radial growth (\%)} = \frac{C - T}{C} \times 100$$

Where C and T denoted the mean colony diameter of fungus (mm) in control and amended PDA plates, respectively.

Greenhouse Evaluation of Fungicides and Plant Extracts

The single most suppressive concentration of difenoconazole (300 ppm) and black pepper (15%) obtained from *in vitro* experiments were assessed in greenhouse trials. Rice plants ('Basmati-385' and 'Basmati C-622') were grown in earthen pots (22.5 cm) filled with autoclaved soil. The plants were treated with the spore and mycelial suspension of *M. oryzae* (2 x 10^5 spores/mL) that was prepared by using sterile distilled water. All the obligatory necessities such as 12 h period of light and dark and >80% relative humidity was maintained for the pathogen establishment. After the onset of rice blast foliar symptoms, the most effective concentration of fungicide and plant extract were evaluated. The experiment was designed using factorial under CRD with four replications of each treatment. The individual concentration of difenoconazole and black pepper and days of assessment of growth inhibition (%) were treated as different factors. Data for disease severity was recorded for five weeks using the rice blast evaluation scale of the International Rice Research Institute (IRRI, 1996).

Data Analysis

Data were analyzed using "Statistix 8.1" statistical software (McGraw-Hill, 2008). Spatial maps were made using ArcGIS Pro 2.2.4. First, disease incidence for each sampling location was mapped using graduated symbols, where the symbol size corresponds to the disease incidence. After that, data for all locations (Nankana, Narowal, Hafizabad, Sialkot and Gujranwala) were pooled for each district and the

calculated mean values were mapped to visualize rice blast incidence per district. Analysis of variance (ANOVA) at 5% level of significance was used to analyze the efficiency between fungicides and plant extracts. Tukey's HSD test was applied for statistical comparison among treatments.

Results

Survey

To investigate the pathogenic diversity associated with the blast of rice the five-prime rice cultivating zones (Nankana, Narowal, Hafizabad, Gujranwala and Sialkot) were surveyed for sample collection and to examine the disease incidence. The maximum mean incidence of rice blast 67.50% was observed in five visited areas of Hafizabad district (Ramke Chattha: 63.75%, Jalalpur Bhattian: 71.25%, Bugga: 60.00%, Hanjrawan Wala: 75%, Pindi Bhattian: 67.50%) followed by 60.75% in Nankana district (Morar Kalaan: 66.25%, More Khunda: 62.50%, Kot Hira Das: 58.75%, Chak 9 GB: 55.00%, Safdarabad: 61.25%). The least incidence (43.25%) of blast disease was observed in the areas of Gujranwala district (Gagewali: 46.25%, Pir Kot: 42.50%, Verpal Chatha: 38.75%, Korotana: 45.00%, Nowshera Virkan: 43.75%; Fig. 2 and 3).

Pathogen Isolation and Identification

For morphological and molecular studies, the pathogen was isolated from the leaf or panicle samples on PDA medium. The observed colony color of the fungus is greyish white having circular smooth margins with raised mycelium. Conidia were small, pyriform shaped with two septations. The average size of the conidia was ranged from 22.7x8.2 µm. Hyphae of the fungus were septate and hyaline, which conclude the pathogen as *M. oryzae* (Fig. 4).

Pathogenicity Assay

Pathogenicity assays confirmed that *M. oryzae* was the causal pathogen of blast disease in surveyed districts. Disease symptoms were noticed after 2 weeks of inoculation. The experimental symptoms were identical to those observed at the time of that samples were collected from farm fields. The fungus was successfully re-isolated from the inoculated plants, fulfilling the Koch's postulates.

Molecular Identification of the Pathogen

The ITS region, beta tubulin, actin and calmodulin genes of *M. oryzae* were successfully amplified with fragment of 510 bp, 500 bp, 280 bp and 520 bp, respectively (Fig. 5). BLASTn searches based on the four gene regions showed that the isolates used in this study were ≥99% similar to those of *M. oryzae* in GenBank (NCBI). Sequences of all the isolates were submitted to NCBI GenBank to get the accession numbers (Table 1).

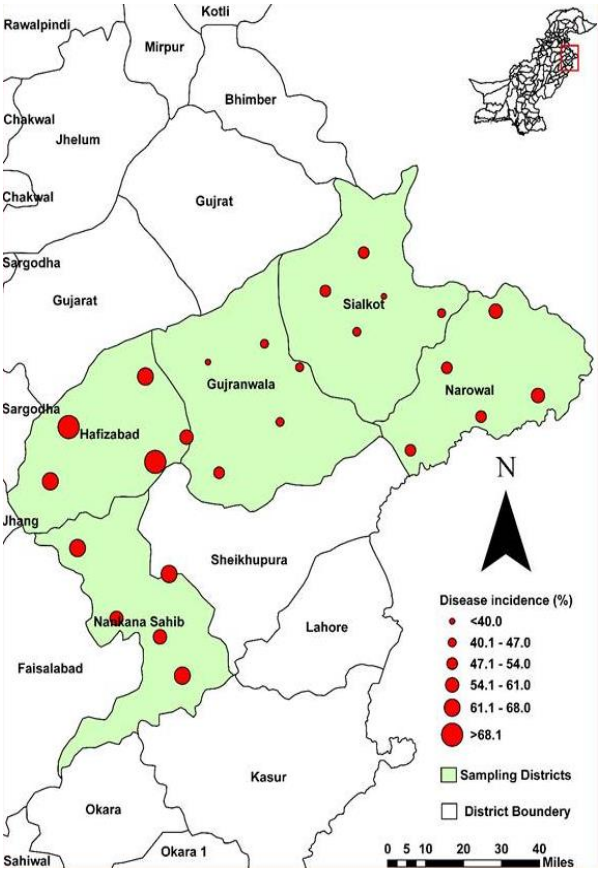

Fig. 2: Map depicting the geographical location of surveyed areas in Punjab, Pakistan to examine the incidence of rice blast. From each visiting district, further five villages were selected to collect data for disease incidence. The villages in district Hafizabad represent the higher disease incidence as compared to the other villages

In vitro Suppression of *M. oryzae* Mycelial Growth

Difenoconazole (Score 250 EC) was the most suppressive fungicide with 83.55% growth inhibition of *M. oryzae* at 300 ppm. Mancozeb (Dithane M-45) was the second most suppressive at 78.75% inhibition, followed by tetrachlorophthalide, tebuconazole + trifloxystrobin, carbendazim, propiconazole, thiophanate-methyl. Generally, with increasing concentrations of fungicides, the colony development of *M. oryzae* was gently decreased (Fig. 6).

After 7 days of incubation at 15% concentration, black pepper inhibited *M. oryzae* mycelial growth by 70.68%, followed by ginger (65.96%). Neem was the least suppressive plant extract with growth inhibition is 48.20% (Fig. 7).

Greenhouse Evaluation of Fungicide and Plant Extract

The most suppressive fungicide (difenoconazole at 300

Table 1: Details of the visited rice growing areas of Punjab, Pakistan. From each visited district of Punjab, further the five villages were selected arbitrarily, and the data of coordinates were recorded. Diseased samples were collected from leaves/panicles portions. Besides the molecular analysis the sequences of ITS, actin, beta-tubulin and calmodulin genes were submitted to NCBI for the accession numbers

Districts	Villages	Coordinates	Plant portion	ITS	Actin	Beta-tubulin	Calmodulin
Nankana	Morar Kalaan	31°42'13.9"N 73°24'13.6"E	Leaves/panicles	MH424730	MH898678	MH539652	MH932588
	More Khunda	31°19'19.1"N 73°48'11.7"E	Leaves	MH424731	MH898679	MH539653	MH932589
	Kot Hira Das	31°26'18.0"N 73°43'04.8"E	Leaves	MH424732	MH898680	MH539654	MH932590
	Chak 9 GB	31°29'36.0"N 73°33'08.6"E	Leaves/panicles	MH424733	MH921396	MH539655	MH932591
	Safdarabad	31°37'35.5"N 73°45'10.3"E	Leaves	MH424734	MH921397	MH539656	MH932592
Narowal	Jarpal	32°09'31.3"N 75°09'30.1"E	Leaves	MH424735	MH921398	MH539657	MH936345
	Darman	32°24'33.0"N 74°59'50.1"E	Leaves	MH424736	MH921399	MH539658	MH936346
	B. Chada Kalan	32°14'27.6"N 74°48'39.7"E	Leaves/panicles	MH424737	MH921400	MH539659	MH936347
	Baddomalhi	31°59'44.1"N 74°40'21.3"E	Leaves	MH424738	MH921401	MH539660	MH936348
	Jassar	32°05'44.2"N 74°56'28.3"E	Leaves	MH424739	MH921402	MH539661	MH936349
Hafizabad	Ramke Chattha	32°12'54.3"N 73°39'45.2"E	Leaves/panicles	MH424740	MH921403	MH539662	MH936350
	Jalalpur Bhattian	32°03'53.3"N 73°22'12.1"E	Leaves	MH424741	MH921404	MH539663	MH936351
	Bugga	32°02'05.4"N 73°49'09.0"E	Leaves/panicles	MH424742	MH921405	MH539664	MH936352
	Hanjrawan Wala	31°57'40.1"N 73°41'59.4"E	Leaves	MH424743	MH921406	MH539665	MH936353
	Pindi Bhattian	31°54'11.9"N 73°18'00.4"E	Leaves	MH424744	MH921407	MH539666	MH936354
Sialkot	Kotli Loharan	32°34'56.3"N 74°29'38.4"E	Leaves	MH424745	MH921408	MH539667	MH936355
	Sambrial	32°28'07.8"N 74°20'53.5"E	Leaves	MH424746	MH921409	MH539668	MH936356
	Seoki	32°20'52.1"N 74°28'05.3"E	Leaves	MH424747	MH921410	MH539669	MH936357
	Chobara	32°24'11.3"N 74°47'28.0"E	Leaves	MH424748	MH921411	MH539670	MH936358
	Dheera Sandha	32°27'10.7"N 74°34'16.2"E	Leaves	MH424749	MH921412	MH539671	MH936359
Gujranwala	Gagewali	32°14'33.9"N 74°15'00.8"E	Leaves	MH424750	MH921413	MH539672	MH936360
	Pir Kot	32°18'44.3"N 74°06'57.8"E	Leaves/panicles	MH424751	MH921414	MH539673	MH936361
	Verpal Chatha	32°15'29.0"N 73°54'03.4"E	Leaves	MH424752	MH921415	MH539674	MH936362
	Korotana	32°04'47.9"N 74°10'31.1"E	Leaves	MH424753	MH921416	MH539675	MH936363
	N. Virkan	31°57'19.7"N 73°58'07.4"E	Leaves	MH424754	MH921417	MH539676	MH936364

ppm) and plant extract (black pepper at 15% concentration) in *in vitro* trials were evaluated in the greenhouse trials. The lowest disease severity (28.50%) was observed on both rice cultivars (Basmati-385 and Basmati C-622) that had been sprayed with difenoconazole, followed by black pepper (33.75%); in contrast, positive control plants averaged 54.75% severity (Fig. 8).

Discussion

In Pakistan, the pathogen of rice blast was previously diagnosed using classical methods like pathogen morphology and based on symptoms. In the present studies, the surveys from the different geographical locations from Punjab, Pakistan was done for sample collection and to examine the incidence of this important disease. For an accurate estimation, the pathogen of this disease was diagnosed using the morphological, pathogenicity and molecular assays which is proved to be *M. oryzae*.

In the last few years, it was noted that the prevalence of rice blast like symptoms in Pakistan has been expanded to the areas (Nankana, Narowal, Hafizabad, Sialkot, and Gujranwala) where it was not appeared as tremendously. This might be because this pathogen migrates to more suitable climatic conditions (25 ± 2^{o}C temperature; >80% relative humidity) in these areas and rapidly expand. In the current studies, the maximum (mean) disease incidence of rice blast 67.50% was observed in five visited areas of Hafizabad district followed by 60.75% in Nankana district. According to Hafiz (1986), Bhatti and Soomro (1994) and Jiskani (1999) blast, bacterial blight, false smut, brown spot and stem rot are occasionally examined the most critical diseases at various rice producing areas of Punjab, Pakistan. Iram *et al.* (2003) reported that the incidence of blast disease in the areas of Gujranwala, Sialkot, Narowal, and Sheikhupura (Pakistan) was 0, 0, 1.33 and 4% which is very low as compared to the current situation. Therefore, to examine the most important and infectious diseases of rice, the identification of responsible pathogens, their control measures, and some other relevant information are crucial; therefore, the growers may shelter their crops from these plant ailments, research workers may designate their plans and extension employees may also be vigilant.

Morphological studies provide the preliminary validation of the pathogen based on the criteria of isolated fungus, colony color and morphology, conidia shape, color, size and septations, hyphal color and septations. The growth pattern of the isolates found in the current study was identical to those noticed by Vanaraj *et al.* (2013). These exploratory identifications keys were expedient for diagnosing the pathogen of rice blast. DNA barcoding has been recommended as the most appropriate method for the identification of fungal pathogens due to low accuracy and overlapping of few morphological characters. Molecular approaches such as the DNA barcoding of different genes (ITS, beta-tubulin, actin and calmodulin) is considered as

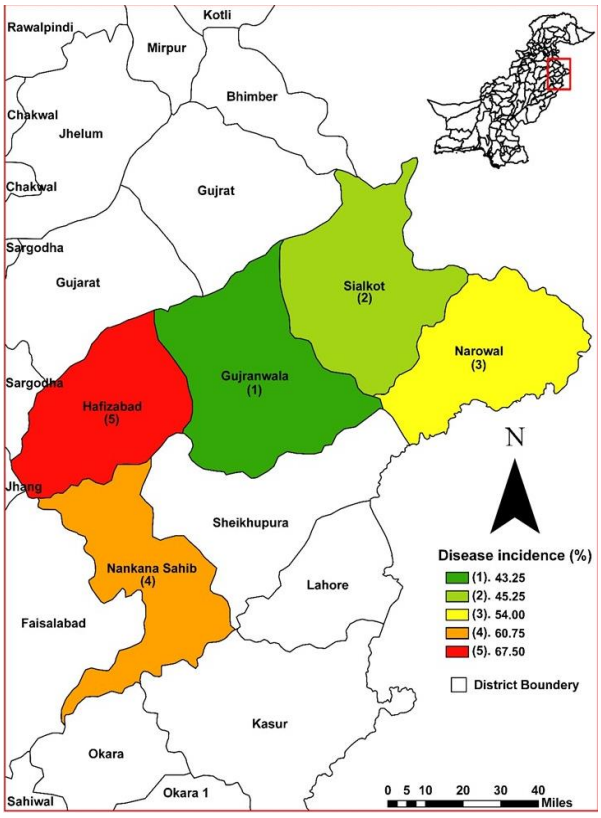

Fig. 3: Map representing the mean incidence of rice blast in surveyed districts of Punjab, Pakistan. District Hafizabad represented the higher disease incidence followed by the Nankana Sahib district

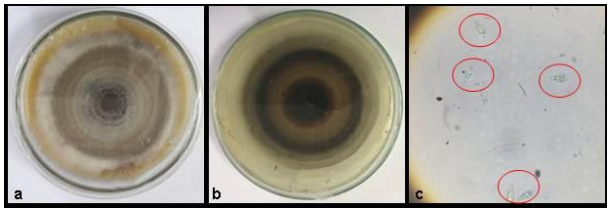

Fig. 4: (a-b). Front and back view of the fungal isolates of *Magnaporthe oryzae*. The isolates were greyish white in color having circular smooth margins with raised mycelium. **(c).** The conidia were small and pyriform shaped with two septations

the best alternative way of identification of *Magnaporthe* isolates at species level or lower than the species level (Hirata *et al.*, 2007; Nasehi *et al.*, 2014). A deep information about the pathogenic diversity is necessary to be able to understand its influence on disease epidemiology and its management (Scheuermann *et al.*, 2012). The status of rice blast has been known across the globe but the fundamental knowledge about the pathogenic diversity and the factors disturbing the genetic structure of the pathogens is still uncertain in Pakistan.

Based on the above facts, there was a dire need to identify or diagnose the molecular diversity among the

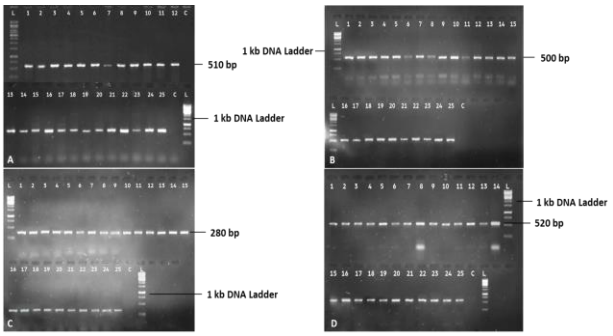

Fig. 5(A): Amplicons of *Magnaporthe oryzae* isolates with ITS1-ITS4 primers which attain the size of 510 bp. **(B).** Amplification with primers Bt1a and Bt1b, which attain the size of 500 bp. **(C).** Amplificons from primers ACT-512F and ACT-783R (280 bp). **(D).** Amplificons from primers CAL-228Fand CAL-737R (520 bp). **L**: 1 kb DNA ladder and **C**: is the negative control

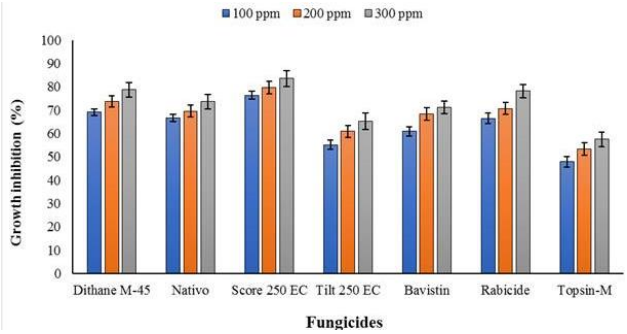

Fig. 6: Comparative effect of fungicides (Rabicide 30 WP, Score 250 EC, Topsin-M, Nativo, Dithane M-45, Tilt 250 EC and Bavistin) and concentrations on growth inhibition (%) of *Magnaporthe oryzae*. The bars above each fungicide line donated as the standard errors. HSD = 1.54; $P \leq 0.05$

population of *M. oryzae* which can be helpful in managing this crop disorder. The analysis of calmodulin, ITS, beta-tubulin, and actin gene regions concluded that the pathogen of rice blast from Punjab, Pakistan was *M. oryzae*. Our results were closely relevant to the findings of Abed-Ashtiani *et al.* (2016) with his work on the characterization of *M. oryzae* collected from rice in Malaysia. They identified the rice blast pathogen as *M. oryzae* based on the multigene phylogenetic analysis (ITS, beta-tubulin, actin, and calmodulin genes). Similarly, Lau (2013) worked on the identification and characterization of rice blast fungus (*M. oryzae*) based on the morphology and with molecular examinations. He stated that misidentification of various fungal isolates occurred due to the characterization on the basis of morphology, but ITS sequencing achieved more accurate recognition of all isolates at species level. Based on his findings, the ITS sequencing technology is a reliable and rapid molecular tool for the characterization of pathogenic fungal isolates.

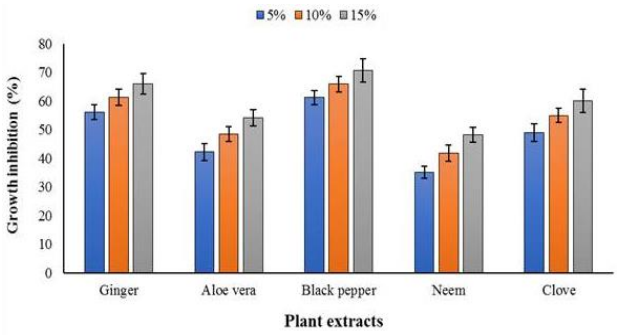

Fig. 7: Comparative effect of plant extracts **(**Black pepper; Aloe Vera; Clove; Ginger; Neem) and concentrations (5, 10 and 15 percent) on growth inhibition (%) of *Magnaporthe oryzae*. All the botanicals significantly inhibit the growth of *M. oryzae*, but black peeper was found the most effective plant extract followed by ginger. The bars above each botanical donated as the standard errors. The critical value for comparison among the treatments is 1.17 with alpha 0.05

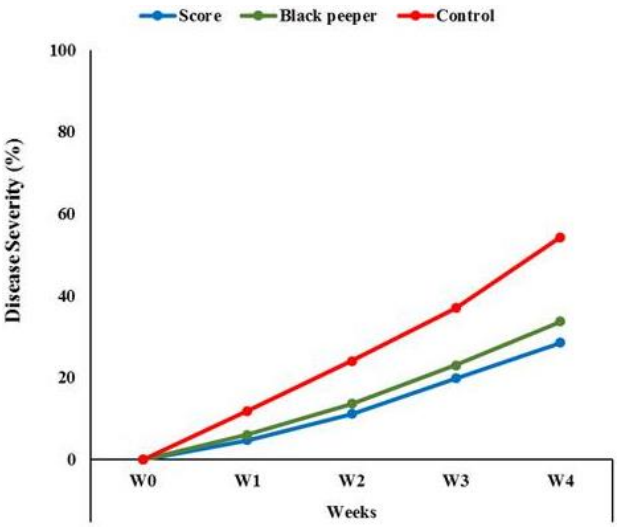

Fig. 8: Comparative effect of fungicide and plant extract on percent disease severity during different weeks of interval. $p \leq 0.05$

Score (difenoconazole) was found to be the most effective fungicide with 83.55% growth inhibition of *M. oryzae.* The active ingredient in score is difenoconazole which disrupts the biosynthesis of sterols in the cell membrane during penetration and haustoria formation. Therefore, it inhibits the development of the pathogens (Nithyameenakshi *et al.*, 2006). Our results are in line with those stated by Ghazanfar *et al.* (2009) when he evaluated the efficacy of different fungicides (score, rabicide, nativo, cuproxit, armure, tilt, filia and wsh 004) against the mycelial growth of *M. oryzae* at different concentrations. He concluded that all the evaluated fungicides demonstrated to be effective against the growth of *M. oryzae* but score, nativo and rabicide proved to be remarkable in all the three weeks in minimizing the fungal growth more in third week with 12.85%, 11.46%, and 12.15%. Among the botanicals, it was noticed that the extracts of *P. nigrum* have the antifungal and antibacterial properties which inhibit the penetration of seed-borne pathogens (Dubey *et al.*, 2000). Similarly, gingerol is the highly active element observed in ginger and showed that this extract has antimicrobial activity and medicinal properties as reported other than that as an anti-lipid, analgesic, antioxidant, anti-diabetic and anti-tumor (Uda *et al.*, 2018). Our results are in line with the Sukanya *et al.* (2011) who estimated the efficiency of oleoresin and essential oils from *curcuma domestica, piper nigrum* and *coriander sativum* for the management of *P. oryzae* in rice. Their results indicated that pepper oil gave maximum inhibition of mycelial growth as 5.9%, 5.5%, 4.4%, and 7.0% inhibition at 100, 200 and 300 ppm as compared to the control, respectively.

However, in the current study the pathogen of rice blast was investigated in detail in Punjab, Pakistan, but there is a still need to do some extra work in the future. We surveyed, only five districts, the higher incidence of rice blast implies that the disease is significantly prevalent in the rice cultivating region of Punjab, Pakistan. Future studies must be conducted to monitor and map the status of this disease in all rice-cultivating regions of the country. Obtained information will help to evaluate yield losses in rice crop due to this disease which in turn will support the scientific community to devise new means to save this valuable crop and will guide recommendations for the sustainable application of management tactics in growers' field. Besides, the analysis with molecular markers, i.e. ISSR and RAPD were further required to find out the diversity in more details. The fungicides and plant extracts should be evaluated in field trials at different geographical locations.

Conclusion

The identified pathogen of rice blast in core rice growing areas of Punjab, Pakistan is *M. oryzae* with maximum disease incidence were noticed in Hafizabad regions. The pathogen was confirmed through morphological, pathogenic and molecular assays by keeping the four different genes: ITS, actin, beta-tubulin and calmodulin with the size of 510 bp, 280 bp, 500 bp, and 520 bp respectively. To overcome the incidence of this disease, score and black pepper were found the most effective fungicide and plant extract under *in vitro* experiments with a maximum inhibition rate of 83.55% and 70.68% respectively. However, under greenhouse trails, maximum disease severity was observed in case of fungicide and plant extract was 28.50% and 33.75% after the four weeks when compared with the control plants (54.75%) which conclude the effectiveness of these chemicals for the long-term management of *M. oryzae*.

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