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Chemotherapy of rice blast (*Pyricularia oryzae*) under field conditions

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Abstract

Rice blast disease (RBD) is a potential threat in the rice belt of Punjab, Pakistan. The current research was planned on the objective to evaluate fungicides and their doses against *P. oryzae in-vitro* and *in-vivo*. Currently, management practices are inadequate to control RBD; subsequently, the blast is dominating in rice-growing areas of Pakistan. As RBD has a wide host range hence, eradication and crop rotation are of minute importance to control this disease. Henceforth, there is a persistent need to devise a substitutive approach for blast management. RBD is largely managed by three methodologies, viz., cultural practices, chemical control and by using resistant varieties. During the current study, six fungicides were evaluated in vitro against *P. oryzae* at three different doses 100ppm, 200ppm, 300ppm using the food poison technique. Amongst six fungicides, Nativo, containing trifloxystrobin 25% + tebuconazole 50%, and Azomide Super, containing difenoconazole and cyprodinil, proved to be best, at 200ppm, controlling *P. oryzae in-vitro* and *in-vivo*. *In-vivo*, Nativo and Azomide Super fungicides reduced RBD severity significantly to 87% and 83%, respectively. The current study revealed two fungicides, Nativo and Azomide, effective against *P. oryzae* at 200 ppm.

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Introduction

Rice (*Oryza sativa* L.) is one of the most significant grains in the world, and its worldwide consumption is about 50% (Luo *et al*., 1998). Except for sandy soil, rice may be planted in every type of soil. It may be grown on salt-affected soil where other crops are unable to thrive. Rice production in Pakistan increased from 7.414 million tonnes to 8.419 million tonnes in 2020 (GOP, 2020). Many varieties are cultivated in Pakistan, including Super Basmati, Basmati 2000, Basmati Pak (karnal Basmati), Basmati 370, Basmati 515, Basmati Kissan, and many others, but the yield is very low compared to other countries, and this low production is attributed to several biotic and abiotic factors. The major biotic component is a disease, which causes $5 billion in annual crop losses (Asghar *et al*., 2007). Fungi, viruses, bacteria and nematodes are responsible for more than 70% of diseases (Zhang *et al*., 2009). Globally, fungal infections are expected to limit yearly rice yield by 14% (Agrios, 2005). RBD is one of the most economically significant diseases caused by a fungal pathogen. RBD occurs in practically all rice-growing countries, resulting in annual output losses of up to 30%, which are enough to feed 60 million people (Skamnioti *et al.,* 2009). *P. oryzae* is the fungus that causes rice blast disease (Koutroubas *et al.,* 2009). This fungus can attack rice at any stage of growth, beginning at the seedling stage, and causes severe leaf damage (Wilson and Talbot, 2009). In certain rice cultivars, it can cause significant damage under optimal conditions (25–30°C and 80–95%) (Nizolli *et al.,* 2021). Owing to the attack on rice leaves and panicles, it affects both vegetative and reproductive stages (Seebolds *et al*., 2004). During the vegetative and reproductive phases, leaf blast develops elliptical lesions on the leaves (Bastiaans, 1991). Grain sterility is caused by the neck blast, which reduces grain size, yield, and quality (Khan *et al.,* 2014).

RBd can be controlled using an array of cultural, biological, and chemical methods (Kurschner *et al.,* 1992). Adjustment of sowing time, optimum supply of nitrogen fertilizers, use of resistant cultivars, application of fungicides, and maintaining a high level of moisture were all used in cultural techniques (Ribot *et al.,* 2008). Further, soil with a high level of organic matter and biological activity reduces the risk of RBD infection (Luong *et al.,* 2003). The use of fungicides remained a recommended and effective method to control RBD. Isoprothiolane applications increased both grain and straw yield of rice. Mancozeb has been proved to be effective against blast at 1000 ppm and 10,000 ppm (Jamal-u-Ddin *et al.,* 2012). The current study was planned with the objective to evaluate fungicides, both *in-vitro* and *in-vivo*, at different doses to control *P. oryzae*.

Materials and methods

Collection of diseased leaf samples

Diseased leaf samples exhibiting clear symptoms of RBD were taken from the fields of Rice Research Institute (RRI), Kala Shah Kako (KSK), Punjab, Pakistan, during 2017, and stored at 4°C in a refrigerator. The samples were then used to isolate and purify *P. oryzae* (Wei *et al.,* 2020).

Isolation, identification and preservation of P. oryzae

Potato agar dextrose medium (PDA) was used for the isolation and purification of fungus *P. oryzae*. Sliced potatoes were boiled in 1 liter of water for half an hour (30 mins.) and decanted through cheesecloth to obtain 200 mL potato dextrose. Then added agar (20 g) and glucose (20 g) to 200 mL potato dextrose and shook the material vigorously to mix the ingredients in the potato dextrose. The 200 mL potato dextrose was shifted to a 2000 mL conical flask and the remaining volume was completed by pouring distilled sterilized water to make 1000 mL PDA. The resulting medium was kept at 121 °C at 15 pascals for 15-20 minutes in an autoclave for sterilization. Sterilized PDA medium was poured into the sterile Petri-plates in a laminar flow chamber. For sterilization of glassware, a hot air oven was used at 180 °C for 2 hours.

For isolation of fungus *P. oryzae*, the tissue segment method was used. Disease-infected leaves were

chopped into pieces about 3-4 cm. For surface sterilization, these small pieces were dipped into 0.5% NaOCl solution for one minute, washed thrice with distilled water and dehydrated with a sterilized paper towel under aseptic conditions in a laminar flow chamber. Three to four samples were placed on PDA containing Petri-plates and placed in an incubator at 20 ± 2°C for a period of fifteen days. As the colonies of *P. oryzae* were developed in Petri-plates on PDA, they were isolated. After that, the single spore method was used to purify the cultures and maintained them at 4°C for future use (Agrawal *et al.,* 1989). *P. oryzae* was identified on the basis of its morphology using the manual of illustrated genera of fungi imperfecti (Barnett and Hunter, 1998).

Mass culture preparation of P. oryzae inoculum

The leaves of rice were dipped in distilled sterilized water for twelve hours under shade (Agrawal *et al.,* 1989). These soaked leaves were then shifted to conical flasks (at the rate of 250 g/1liter flask). Openings of these conical flasks were closed with cotton plugs and placed in an autoclave at the temperature of 121°C at 15 pascals for 30 minutes. The leaves were autoclaved in order to remove contaminants. Six-mm agar plugs (4 in numbers) were picked from fresh cultures of *P. oryzae* and placed on the autoclaved leaves present in 1 liter of conical flasks. To avoid contamination, 25-mg streptomycin was also spread on the autoclaved leaves in conical flasks. After that, cotton plugs were tightened and conical flasks were placed in an incubator at 20 ± 2 °C for seven days to enhance the growth and development of pycnidial cultures of *P. oryzae* (Khan *et al.,* 2001).

In-vitro evaluation of fungicides against P. oryzae

Six fungicides were evaluated against *P. oryzae* at three different concentrations (100ppm, 200ppm, 300ppm) by using the food poison technique. The concentrations were made by mixing 100, 200 and 300-mg active ingredients of fungicides in 1000ml distilled sterilized water by using Borum and Sinclair technique (1968). PDA medium was prepared and sterilized in an autoclave at 121°C at 15 pascals for 15 minutes. Petri-dishes were sterilized in the oven at 180 °C for two hours. Molten PDA was mixed with desired concentrations of fungicides and then poured in sterilized Petri-dishes in a laminar flow chamber to avoid the contamination of saprophytes. In the case of control, only molten PDA medium was poured into Petri-dishes. After solidification, a 5mm mycelial plug of *P. oryzae* was placed in the center of each Petri plate. The plates containing the inoculum were then shifted to an incubator having a temperature of 20 ± 2°C under dark conditions until the appearance of full colony growth of *P. oryzae* in the control plates. After that, colony growth diameter of *P. oryzae* was taken in treated and control plates to measure percent colony growth inhibition of *P. oryzae* with the formula given by Ghazanfar *et al.* (2009):

$$\text{Inhibition percentage} = \frac{C - T}{C} \times 100$$

Where;

C = *P. oryzae* mycelial growth in control plates

T = *P. oryzae* mycelial growth in treatment plates

A completely randomized design (CRD) was used during in-vitro bioassays of fungicides. Each treatment had three replications. To evaluate the fungicidal treatments effects, i.e., individual and interaction, under lab. conditions, the data were subjected to an analysis of variance (ANOVA) test. Duncan's Multiple Range (DMR) test at $P < 0.05$ was employed to compare the homogeneity of means.

In-vivo evaluation of fungicides against RBD

The fungicides and the concentrations found best under in-vitro experiments were also tested under field conditions. For this purpose, one highly susceptible variety (C-622) was sown in a randomized complete block design (RCBD), keeping control untreated. Each block was comprised of six rows of the susceptible cultivar (C-622), five rows were for the application of treatments, and one was kept as control. On each block, the inoculum of *P. oryzae* (spore suspension 10^6 conidia/mL) was kept on spraying until the epidemic conditions were created in each block. As epidemic conditions appeared, desired formulations of treatments were applied using knapsack sprayer. While plants that served as

control were sprayed with sterilized water. Percent disease severity index (PDI) was calculated by recording RBD severity on randomly selected 10 plants. The RBD severity data were recorded after the one-week interval and kept on recording up to three weeks after the application of treatments. Percent PDI was determined by the following formula used by Ghazanfar *et al.* (2009):

$$\text{PDI (\%)} = \frac{\text{Sum of individual rating}}{\text{No. of observations} \times \text{Maximum examined disease scale}} \times 100$$

To evaluate the interactive effects of the fungicides, data were subjected to ANOVA, and DMR test was used to note the differences between the effects of different treatments.

Results

In-vitro evaluation fungicides against P. oryzae

ANOVA for the percent inhibition of *P. oryzae* showed significant differences among the treatments, concentrations and days intervals (Table 2). Among all the fungicides, Nativo showed the highest percent inhibition of the hyphal growth of *P. oryzae* with the mean value of 66.80%, followed by Azomide Super (61.86%) as compared to other fungicides and control.

Table 1. Fungicides used against *P. oryzae*.

Sr. No	Common name	Chemical Name	Formulation	Source
1.	Amistar Top	125 g/l difenoconazole + 200 g/l Azoxystrobin	325 SC	Syngenta
2.	Filia	Propiconazole + Tricyclazole	525 SC	Syngenta
3.	Nativo	Tebuconazole 50% + trifloxystrobin 25%	75 WC	Bayer crop science
4.	Azomide Super	Difenoconazole+ Cyprodinil	400 SC	Suncrop
5.	Switch DF 80 WG	Cyprodinil&fludioxonil	80 WG	Syngenta
6.	Armure	Propiconazole + Difenoconazole	300 EC	Syngenta

Table 2. ANOVA for the effect of different fungicide concentrations on percent inhibition of *P. oryzae*.

Source	Degrees of freedom	Sum of squares	Mean squares	F-value	Prob.
Days (D)	2	2662.8	1331.4	933.88**	0.0001
Fungicides (F)	6	80831.6	13471.9	9449.68**	0.0001
Concs. (C)	2	417.8	208.9	146.53**	0.0001
D × F	12	499.0	41.6	29.17**	0.0001
D × C	4	19.2	4.8	3.36*	0.0120
F × C	12	536.0	44.7	31.33**	0.0001
D × C × F	24	82.1	3.4	2.40**	0.0009
Error	126	179.6	1.4		
Total	188	85228.0			

NS = Non-significant (P>0.05);

* = Significant (P<0.05);

** = Highly significant (P<0.01).

The lowest percent inhibition was exhibited by Switch-DF with a mean value of 46.50%. Moreover, it was also observed that Nativo showed the maximum percent inhibition at 3rd, 5th and 7th day with the mean value of 61.67, 65.19 and 73.56%, respectively (Table 3).

In-vivo evaluation of fungicides against RBD

ANOVA showed the effect of fungicides significant (P < 0.05) at 200ppm on PDI of RBD (Table 4). The PDI was recorded lowest of fungicide treated rice plants compared to control. Under field conditions, Nativo significantly (P<0.05) reduced RBD severity, PDI 12%, compared to other fungicides and control.

The PDI of Azomide Super and Switch-DF treated rice plants was not significantly different (P<0.05) at 200 ppm concentration. Amistar-Top treated rice plants showed significantly (P<0.05) high PDI 25% compared to other fungicides (Table 5).

Table 3. Effect of fungicides on percent inhibition of *P. oryzae* on different days.

Fungicides	Days			Mean
	Day 3	Day 5	Day 7	
Amistar Top	51.59±1.94f	56.09±2.09de	61.52±1.74c	56.40±1.33C
Filia	50.57±0.57fg	56.05±0.73de	62.50±0.37c	56.37±1.01C
Nativo	61.67±1.10c	65.19±0.82b	73.56±0.42a	66.80±1.08A
Azomide Super	56.89±0.50d	62.14±0.72c	66.54±0.63b	61.86±0.85B
Switch-DF	41.51±0.69j	46.58±0.79h	51.42±0.50f	46.50±0.88E
Armure	43.69±0.29i	49.21±0.45g	54.67±0.31e	49.19±0.90D
Control	0.00±0.00k	0.00±0.00k	0.00±0.00k	0.00±0.00F
Mean	43.70±2.43C	47.89±2.62B	52.89±2.89A	

Means sharing similar letters in a row or in a column are statistically not significant (P>0.05). Small letters represent comparison among interaction means and capital letters used for overall means.

Discussion

This research was a start-off to ameliorate our understanding of the efficacy of different fungicides and their doses to manage RBD effectively. Our study revealed that all fungicides we evaluated in the present study reduced colony growth/ disease severity compared to the control. We found Nativo fungicide at 200ppm most effective both in-vitro and in–vivo. This is congruent with already conducted research (Ghazanfar *et al.,* 2009; Deepan *et al.,* 2018; Singh *et al.,* 2019; Balgude *et al.,* 2019). Nativo is a systemic, wide-ranging fungicide containing tebuconazole and trifloxystrobin and being used as curative and protective fungicides. This fungicide not only controls diseases but also improves crop yield. In rice crop, it increases the quality of the yield by decreasing the incidence of dirty panicles (https://www.cropscience.bayer.in/ProductsH/Brands/Crop-Protection/Fungicide-Nativo).

Nativo is a demethylation inhibitor fungicide and its effectiveness against *P. oryzae* may be elucidated owing to its quick absorption and systemic translocation in plants, which enable its early assimilation in adequate amounts in plant tissues to encounter mycelial growth (Pathan *et al.,* 2020).

Table 4. ANOVA for the effect of fungicides on PDI of RBD**.**

Source	Degrees of freedom	Sum of squares	Mean squares	F-value	Prob.
Replication	2	0.44	0.220	0.15NS	0.8658
Fungicide	6	5369.76	894.960	593.97**	0.0001
Error	12	18.08	1.507		
Total	20	5388.28			

NS = Not-significant (P>0.05);

**= Highly significant (P<0.01).

The in-vitro effectiveness of Nativo against *P. oryzae* may also be due to its ability to inhibit spore germination (Avozani *et al.,* 2014). It has been evidenced that Nativo fungicide plays a role in inhibiting sterol biosynthesis in the membranes of fungal pathogens by interjecting the activity of C14-demethylase. Nativo fungicide is also reported to inhibit the activities of enzymes involved in mitochondrial respiration (Kongcharoen *et al.,* 2020). Nativo under filed conditions controlled RBD up to almost 87%, which is in agreement with previous research. Kongcharoen *et al.* (2020) evaluated the efficacy of fungicides containing tebuconazole and trifloxystrobin and found more than 60% control of RBD under field conditions. Similarly, Ahmad *et al.* (2020) checked the efficacy of *P. oryzae* in-vivo and found Nativo as effective. They further found that Nativo controlled 90% RBD under field conditions. Ghaznafar *et al.* (2009) found Nativo fungicide most effective against RBD under field conditions. They noted a reduction in disease severity of blast around 80%.

Table 5. Effect of different fungicides at 200 ppm concentration on PDI of RBD.

Fungicide	Mean±SE
Amistar-Top	24.55±0.60B
Filia	21.40±0.42BC
Nativo	12.91±0.24E
Azomide Super	16.93±0.97D
Switch-DF	17.99±0.20CD
Armure	20.82±0.30C
Control	63.40±1.19A

Means sharing similar letters are statistically not significant (P>0.05).

The second most effective fungicide was Azomide super which is the combination of difenoconazole and cyprodinil. This fungicide, which is systemic in nature, has been used to control different plant diseases successfully. Previous research has shown that this fungicide produces less resistance in the fungi (Yang *et al.,* 2019). Hence, its use to control RBD will be proved effective. The active ingredients difenoconazole and cyprodinil are site-specific and control fungi by attacking CYP51, altering sterol-biosynthesis pathways, increasing active efflux by using ABC transporters and bringing changes in integrity and cell composition of the cell membrane (Villani *et al.,* 2016; Kasmi *et al.,* 2018). Therefore, it can be concluded that Azomide super has controlled *P. oryzae* on similar lines. Azomide super in this study controlled RBD up to 83%. This is in line with the findings of Ahmad *et al.* (2020). They used the fungicide Recado 32.5% SC, having the same formula of Azomide super, and controlled RBD up to 82% with foliar applications. Similarly, Singh *et al.* (2019) found the effectiveness of active ingredient difenoconazole more than 80% against RBD employing foliar applications.

Conclusion

The key research revelation from this research is that systemic fungicide, containing tebuconazole and trifloxystrobin, and difenoconazole and cyprodinil, as active ingredients, are the most effective curative fungicides to control RBD. In addition, this study engendered a necessary foundation for further investigations to optimize the formulation/dose of fungicides for the effective protection of RBD.

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