IRSTI 68.37.31; 68.37.13 https://doi.org/10.26577/IJBCh2023v16i2a4

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(Received September 23 2023; received in revised form October 13 2023; accepted October 26 2023)

Synthetic fungicides for controlling brown leaf spot of rice caused by *Bipolaris oryzae*

Abstract. Rice (*Oryzae sativa* L) is suffering from several biotic and abiotic factors. Among biotic factors, brown leaf spot of rice (BLS) is potentially devastating disease of rice causing the severe yield losses up to 100%. The current study was designed to evaluate most effective synthetic fungicides management strategy towards BLS disease. For this purpose, 12 fungicides were screened out under *in vitro* conditions and four most effective fungicides were further demonstrated against the targeted pathogen with three different concentrations (100, 150 and 150 ppm) by using poisoned food technique. The results revealed that propiconazole and thiophanate methyl showed the strong inhibitory effect against *B. oryzae* followed by contaf plus and bavistin respectively. The promising fungicides (propiconazole and thiophanate methyl) under lab conditions were further evaluated *in vivo* against BLS disease by using 3 types of applications i.e. Pre-inoculation, Post-inoculation and after symptoms appearance. Findings showed that, Post-inoculation was found most effective as compared to other applications, furthermore combination of propiconazole and thiophanate methyl showed significant reduction in disease incidence percent as compared to solo applications. The reduction in disease incidence percent in all application methods suggested that these synthetic fungicides could be used against BLS of rice.
Key words: Propiconazole, Thiophanate methyl, Pre-inoculation, Contaf Plus, Bavistin.

Introduction

Rice (*Oryzae sativa* L) is a major cereal crop that is consumed as a staple diet by more than half of the world's population. Asia produces and consumes about 90% of the world's rice, which is a symbol of a culture's ability for survival [1,2]. Rice is also considered as the "Queen of cereals" [3]. Currently, rice is growing worldwide except Antarctica [4]. It is an important crop for containing a high amount of carbohydrates, protein, and fats. More than 1/5 of the global calorie intake for humans is provided by it [5]. It contains vitamins and minerals, and it is particularly rich in potassium, vitamin E and vitamin B (thiamin, niacin) [6]. Worldwide, its production leads 500 million tones over an area of 167.24 million hectares, while in Pakistan, it is grown on 3.3 million hectares with production of 8.4 million tonnes [7]. Punjab and Sindh are the major rice-growing provinces of Pakistan and these are accounted for the majority of the country's production [8]. There is a potential demand for rice production to be increased as continuously rising in world's population. Rice crop production is estimated to be increased up to 852million tons by the year 2035, as compared to its current production [9]. Therefore, it is necessary to boost production technology to meet the demand. However, with the development of more advanced techniques and high-yielding varieties, the crop is more vulnerable to biotic and abiotic factors.

Pakistani basmati rice varieties are famous throughout the world for their unique aroma but unfortunately, these varieties are tragically also susceptible to several diseases, including paddy blast, bacterial leaf blight, and brown leaf spot of rice [6]. Among a number of rice-related diseases, the brown spot of rice, which is brought on by the plant-pathogenic fungi *Bipolaris oryzae*, *Helminthosporium* *oryzae,* and *Drechslera oryzae,* is one of the most devastating disease causing both qualitatively and quantitatively losses [10]. It is named as "chronic and orphan disease" because it affects millions of hectares of rice annually [11]. The pathogen of brown leaf spot disease of rice *(Bipolaris oryzae)* was responsible for the great "Bengal Famine 1942-43" causing the starvation death of 2 million people as a result of destruction of 50-90% of rice crops [12]. The pathogen attacks both at seedling and maturity stage [10]. As the pathogen is seed-borne [13], it causes blight on seedlings and can cause 10–58% seedling mortality because it spreads through heavily infected seeds [12]. The characteristic symptoms appear as small, circular, dark brown to purple-brown spots on leaves, panicles, glumes, and grain. Fully developed lesions are circular to oval with a light brown to grey center, surrounded by a reddish-brown margin, and ultimately kill the leaf [10]. When the infection is severe, grey mycelial growth can be seen between the sheaths and stalks and the neck turns into black color [14], and when the conditions are favorable, inadequate grain filling takes place [15].

Primarily, the weeds and the contaminated soil are the major sources for the pathogens survival. The pathogen enters the host plant by direct penetration or through the stomata [16]. It requires a temperature of 20°C and >80% relative humidity for the development of infection [17]. The disease becomes more severe when there is an excessive usage of nitrogen fertilizer. Under unfavorable environmental conditions, the rate of disease incidence become very high leading to seed discoloration, poor grain quality and yield losses [18]. Several management strategies for controlling the disease are in practices such as the use of resistant varieties, synthetic fungicides, biological and cultural control practices [19]. No doubt, use of biocontrol agents are environment friendly disease management approach [20], but the use of bio-agents in foliar spraying cannot completely manage the disease. The best strategy to manage the disease is the use of resistant varieties. The use of resistant varieties is safe, efficient, and cost-effective for controlling the rice diseases but due the lack of resistant varieties farmers mostly prefers the use of synthetic chemicals as this is the most effective management strategy for preventing brown spot disease, highly recommended method and widely used throughout the world [19].

Several studies on the use of synthetic chemicals to control the brown leaf spot disease have been reported. [21] examined four different fungicides including Carbendazim 50WP, Carboxin 50 WP, Propiconazole 25 EC and Hexaconazole 25 EC @500ppm concentration. Among all, propiconazole was found to be effective fungicide that prevented 96.58% of fungus growth under lab conditions. [22], also evaluated different fungicides against the brown leaf spot of rice. All tested fungicides showed the best result against the *Bipolaris oryzae* under *invitro* conditions. But Bavistin performed excellent result at a concentration of 1500ppm as compared to other fungicides. Another experiment was performed in which [23] used six fungicides against brown leaf spot of rice. Among different fungicides, two (Propiconazole (1 ml per l) and Hexaconazole (2 ml per l) were found to be effective in reducing severity from 22.34% to 5.19 and 7.98% respectively, and significantly increased grain yield. [24] reported in their study about the antifungal response of different synthetic fungicides under *invitro* conditions. The most effective among chemicals was Propiconazole, while tebuconazole+trifloxystrobin and pyraclostrobin+ epoxyconazole also showed significant results at 0.75 ppm concentration.

The present study aims at determining the effectiveness of different latest fungicides for management of brown leaf spot of rice caused by *B. oryzae* and to suggest the farmers to use recommended synthetic chemicals to prevent the disease. Moreover, this research may contribute to an improved knowledge of the best times to apply fungicides at various stages of crop growth.

Materials and methods

Isolation, purification and identification of pathogen. Sample collection on the basis of visual symptoms was done from different rice growing zones of District Faisalabad, Pakistan. Leaf samples were cut into small pieces about 3-5 mm size and surface sterilized with 1% sodium hypochlorite (NaOCl) solution, then thoroughly washed with distilled water and placed on blotter paper for drying purpose. After that sterilized petri plates were poured with autoclaved potato dextrose agar (PDA) medium and samples were positioned on the center of that plates with the help of sterilized forceps. All the plates were wrapped and incubated at 28°C temperature in an incubator. Mycelial growth was regularly observed.

By using single hyphal tip method, a minute portion of fungal colony was transferred to fresh PDA plates for purification of the pathogen. On synthetic media, *B. oryzae* has been observed to grow as a cottony mycelial growth that was seen as dark grey, black, or greenish in color. Microscopically, the spores were fucoid to cylindrical, light to dark brown, and curved with septate conidia [25].

In vitro evaluation of fungicides against Bipolaris oryzae. For management of *Bipolaris oryzae,* 12 fungicides i.e. Propiconazole, Thiophanate Methyl, Contaf Plus, Bavistin, Cabrio Top, Fossil, Amistar Top SC, Curzate M, Aliette, Polyram DF, Vidal and Score were evaluated to check their efficacy against the targeted pathogen. For this purpose, stock solutions for these fungicides were prepared separately to make different concentrations.

Preparation of stock solution. Stock solution was prepared by checking the active ingredients of concerned fungicide and dissolving the adequate amount of active ingredients in 100ml of sterilized distilled water (SDW) in glass bottles [26], stock solution was prepared [27].

Required amount of each fungicide was dissolved in SDW to make stock solution. Three different concentrations such as 50, 100 and 150ppm for each fungicide treatment were prepared by using 0.5, 1 and 1.5 ml of stock solution respectively.

Screening of fungicides under in vitro conditions against B. oryzae. 12 different fungicides were examined against *B. oryzae* by using poisoned food technique*,* all the fungicides were brought from local pesticide market of Faisalabad. 100 ppm concentration for each fungicide were used. Autoclaved fresh PDA media was amended with fungicide before pouring in petri plates. A 5 mm fungal plug from a pure culture of *B. oryzae* was placed in the center of each plate and incubated at 28 °C temperature. The un-amended plates were treated as control. All the treatments were replicated thrice under completely randomized design (CRD). Mycelial growth was calculated using digital Vernier caliper.

In vitro assessment of most effective fungicides against *B. oryzae*. Separate conical flasks were used for each fungicide. 100 ml PDA media was placed in each conical flask and autoclaved at 121℃ temperature and 15psi pressure for 15-20mins. Three concentrations i.e. 50, 100 and 150 ppm were prepared for each fungicide and mixed with PDA media. Certain amount of amended PDA media was poured in sterilized petri plates and inoculated with 5mm disc of pure culture of *B. oryzae*. Three replications of each treatment were used to minimize error. Control treatment were remained un-amended. After that, all plates were wrapped with parafilm and labeled with date, name of fungicide and concentration with the help of permanent marker. All plates were incubated carefully at 25°C± 2°C temperature. Data of mycelial growth was recorded for 3 days with 24 hours of interval by measuring diameter of colony growth with the help of digital Vernier caliper.

Exploitation of most effective synthetic fungicide and their combination against brown leaf spot of rice under greenhouse conditions

The moderately susceptible variety of rice (Shaheen basmati) was sown on raised seedbeds and these were irrigated twice-daily with tap water. Rice seedlings of 45 days age were transplanted into earthen pots containing 4 kg of sterilized soil. All recommended agronomical practices were followed to ensure a healthy crop. The plants were artificially inoculated with spore suspension previously prepared by using pure mycelial culture [28]. The pots were categorized in three different groups, the first group was treated with fungicide after ensuring that the crop was well-established, and two days later it was artificially inoculated with spore suspension using a hand sprayer. The spore suspension was applied to the second group two days just before the application of synthetic fungicides. The third group of plants was given a fungicide treatment after the confirmation of disease development. The control treatment also received the similar inoculation with spore suspension, but instead of being sprayed with fungicides, they were just given the sterile distilled water. All the treatments were replicated with three replications under completely randomized design (CRD). Weekly observation of disease incidence was ensured upto three week of intervals. The percentage of disease incidence data was calculated as given below

$$\text{Disease Incidence} = \frac{\text{Number of infected Plant}}{\text{Total number of plants}} \times 100$$

Results and discussion

Screening of twelve fungicides against B. oryzae under in vitro conditions. In first experiment, 12 chemicals including Thiophanate Methyl, Propiconazole, Contaf Plus, Bavistin, Cabrio Top, Fossil, Amistar Top SC, Curzate M, Aliette, Polyram DF, Vidal, and Score were screened out at 100 ppm concentration against *B. oryzae* under lab conditions. Among all investigated chemicals, Propiconazole, Thiophanate methyl, Contaf plus, and Bavistin were the only chemicals that showed significantly inhibitory response with least mycelial growth of 7.51, 9.41, 11.44, and 12.53 mm, respectively as compared to control plate (56.35 mm). Whereas, others showed minimum effectivity against pathogen such as Cabrio top

(14.55 mm), Fossil (15.52 mm), Amistar Top SC (14.83 mm), Curzate M (16.11 mm), Aliette (19.57 mm), Polyram DF (18.88 mm), Vidal (13.66 mm), Score (15.31 mm) (Figure 1). Therefore, based on above results, these four most effective chemicals were further assessed under *in vitro* conditions by applying different concentrations i.e. 50, 100 and 150ppm for further investigation.

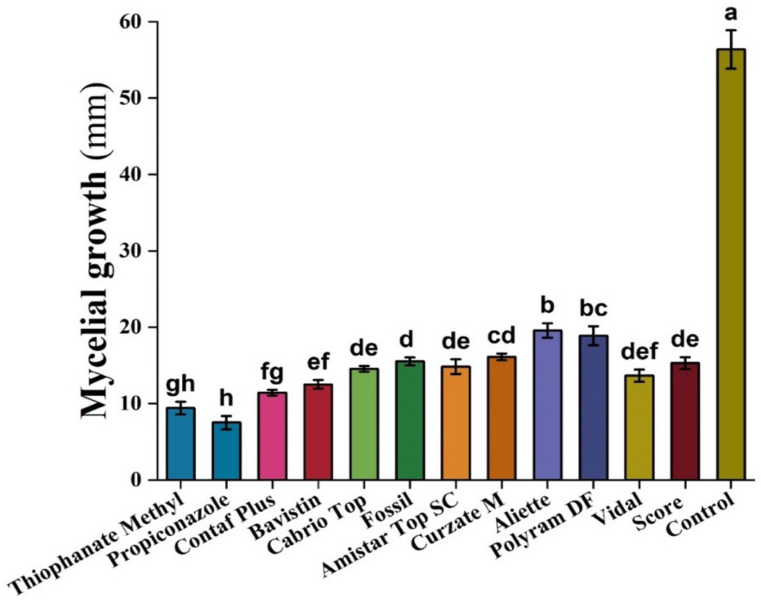

Figure 1 – Screening of different synthetic fungicides against *B. oryzae* under laboratory conditions

In vitro assessment of four most effective fungicides against B. oryzae. Four fungicides were evaluated against the *Bipolaris oryzae* under *in vitro* conditions. The mycelial growth was sufficiently inhibited by all treatments. Among all fungicides, Propiconazole expressed highly effective result in reducing the mycelial growth (5.32 mm) followed by Thiophanate Methyl, Contaf Plus and Bavistin, showed the mycelial growth 5.94, 6.31, and 9.25mm, respectively as compared to control (30.13 mm) (Figure 2). Relation between treatment and concentration exhibited that Propiconazole was the most effective fungicide tested against *B. oryzae,* with mycelial growth of 6.57, 5.19, and 4.20mm at 50, 100, and 150 ppm concentrations, respectively, while Bavistin, with concentrations of 50, 100, and 150 ppm, showed the least growth inhibition (10.77, 9.38, and 7.67 mm), respectively (Figure 3). Interaction between treatment and days showed that Propiconazole exhibited excellent result against *B. oryzae* under laboratory conditions after 24, 48, and 72 hours with the lowest mycelial growth of 3.52, 5.21 and 7.23 mm, followed by Thiophanate methyl (4.40, 5.75 and 7.67 mm), Contaf plus (4.01, 6.03, and 8.87 mm) and Bavistin (6.66, 9.16, and 11.93 mm) mm as compared to control (10.63, 29.77, and 50 mm) (Figure 4).

Assessment of fungicides against brown leaf spot of rice under greenhouse conditions using pre-inoculation treatment

Two most effective synthetic fungicides Propiconazole and Thiophanate Methyl were evaluated under greenhouse conditions. The combination of Propiconazole+ Thiophanate Methyl applied before inoculation of pathogen showed the highest efficacy against brown leaf spot of rice, with disease incidence of 17.43% followed by the solo application of Propiconazole and Thiophanate Methyl with 24.96% and 28.46% disease incidence as compared to control (55.48%) (Figure 5a). Interaction between treatment and days also revealed that, at 7, 14, and 21 days, the combination of Propiconazole+Thiophanate Methyl expressed lowest disease incidence (22.00, 17.33 and 12.95%), followed by single application of Propiconazole (28.19, 25.08, and 21.62%) and Thiophanate Methyl (31.18, 28.15 and 26.04%) (Figure 5b).

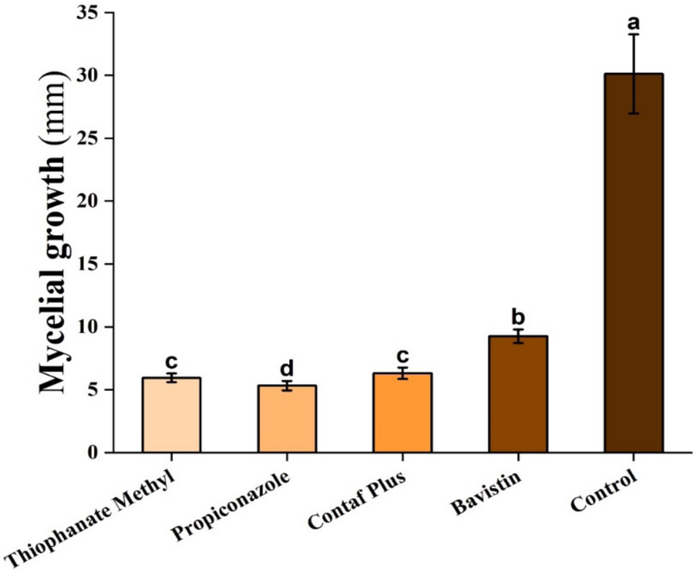

Figure 2 – Impact of treatments on mycelial growth of *B. oryzae* under *in vitro* conditions

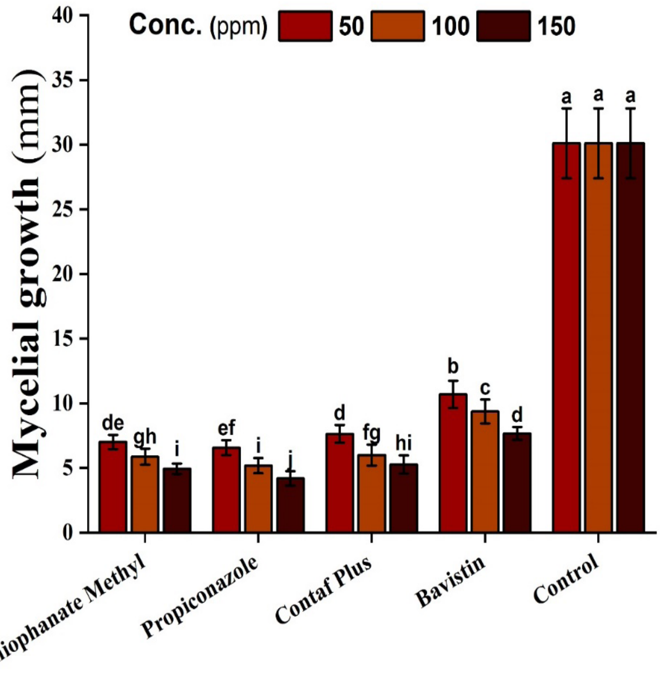

Figure 3 – Impact of an interaction between treatment and concentration on mycelial growth of *B. oryzae* under *in vitro* conditions

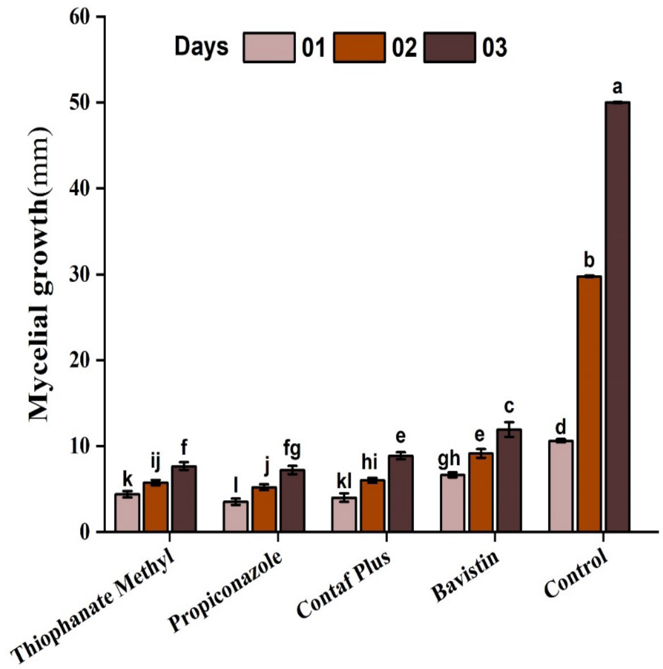

Figure 4 – Impact of interaction between treatment and days on growth of *B. oryzae* under *in vitro* conditions

Assessment of fungicides against brown leaf spot of rice under greenhouse conditions using post-inoculation treatment

According to the findings, least disease incidence (11.04%) was expressed by the application of Propiconazole+Thiophanate Methyl in post inoculation treatment, followed by the single application of Propiconazole (17.06%) and Thiophanate Methyl (20.82%) as compared to the untreated control (55.48%) (Figure 5c). Fungicides were applied 2 days after spore inoculation under greenhouse conditions. All treated plants exhibited less disease incidence before or after inoculation as compared to the untreated control. At two days after inoculation, rice plants treated with the combination of propiconazole and thiophanate methyl showed significantly excellent result with disease incidence of 14.27, 11.52, and 7.33%. The solo application of propiconazole and thiophanate methyl showed disease incidence of (20.00, 17.01, and 14.16%) and (23.28, 21.72, and 17.46%) respectively (Figure 5d).

Assessment of fungicides against brown leaf spot of rice under greenhouse conditions using after symptom appearance treatment

In third experiment, fungicidal treatment was applied after appearance of visible symptoms. Where, combination of Propiconazole and Thiophanate methyl expressed the lowest disease incidence percentage (15.40%) as compared solo application of propiconazole and thiophanate methyl (20.19 and 23.79%) respectively as compared to control treatment (55.48%) (Figure 5e). The application of combination of propiconazole+ thiophanate methyl was found highly effective up to three weeks with disease incidence of (19.41, 15.68 and 11.17%) followed by propiconazole (23.21, 20.20 and 17.16%) and thiophanate methyl (26.07, 23.95 and 21.36%) in comparison to untreated control (38.18, 56.50 and 71.77 %) (Figure 5f).

Brown leaf spot of rice, caused by the fungus *B. oryzae* is a potential threat to farmers causing significantly yield losses [10]. The use of resistant varieties, cultural practices, biological control and the application of synthetic fungicides are being adopted to manage this disease. Due to changes in climatic conditions, instability of pathogen is a major threat to rice crop. Cultural practices alone cannot effectively control disease. Biological control is inaccessible for larger fields. Therefore, farmers are interested towards the use of synthetic chemicals due to their quicker and effective response against disease by contributing higher yields and better quality.

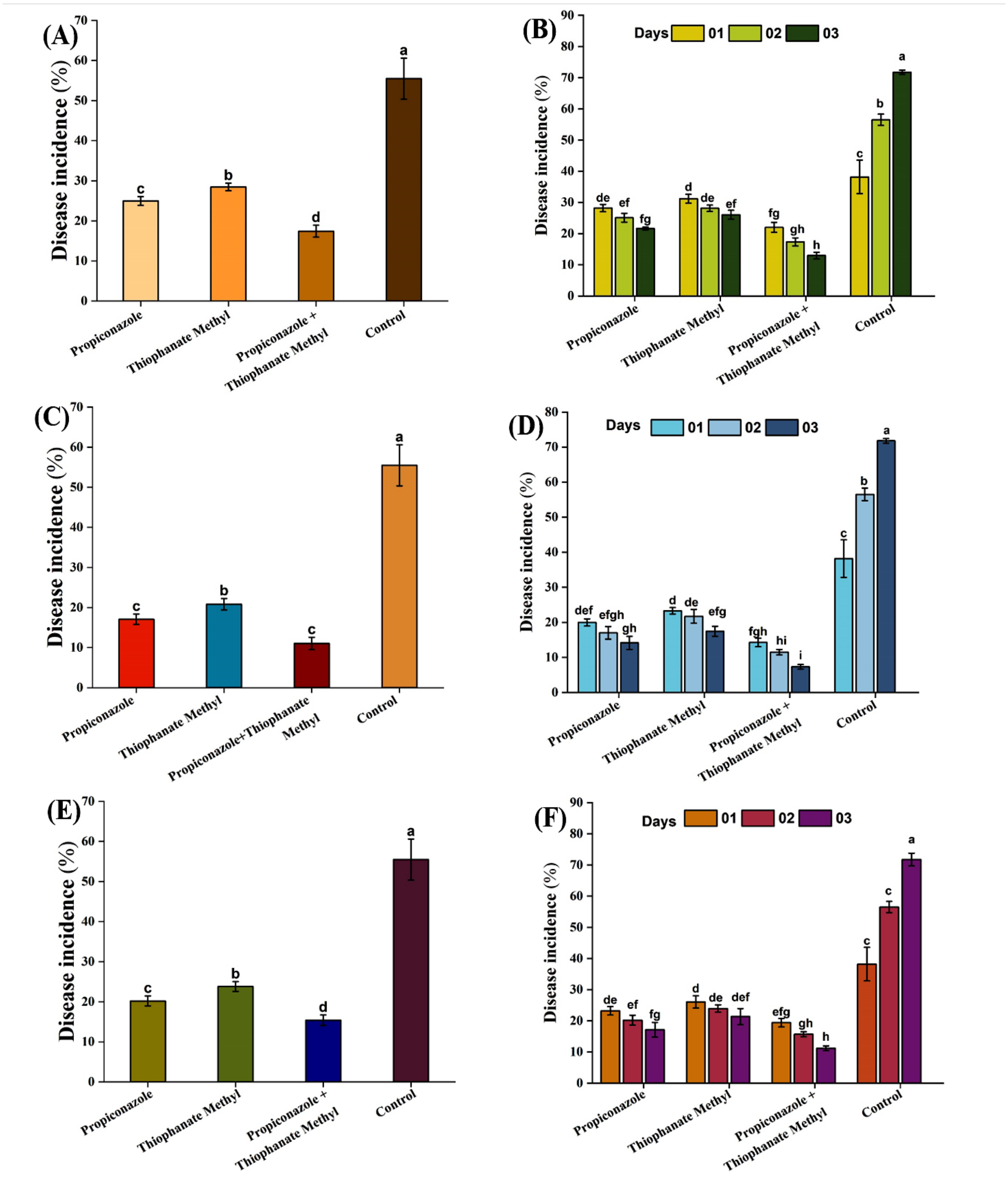

Figure 5 – Assessment of fungicides. a: Assessment of fungicides against brown leaf spot of rice under greenhouse conditions using pre-inoculation treatment, b: Interaction of treatment and days on disease incidence in pre-inoculation treatment, c: Assessment of fungicides against brown leaf spot of rice under greenhouse conditions using post-inoculation treatment, d: Interaction of treatment and days on disease incidence in post-inoculation treatment, e: Assessment of fungicides against brown leaf spot of rice under greenhouse conditions using after symptom appearance treatment, f: Interaction of treatment and days on disease incidence after symptom appearance

In our current study, we evaluated 12 different commercial fungicides against *Bipolaris oryzae*. All of them demonstrated an inhibitory response in reducing mycelial growth, but, surprisingly, propiconazole demonstrated the excellent response under laboratory conditions. The best performing fungicides propiconazole and thiophanate methyl were examined under greenhouse conditions. A highly significant response was seen when (propiconazole+ thiophanate methyl) were used in combination against brown leaf spot of rice. [24] studied the efficacy of 8 fungicides under *in-vitro* conditions in which three fungicides propiconazole, pyraclostrobin + epoxyconazole andtebuconazole + trifloxystrobin inhibited the mycelial growth up to 100% at 0.75ppm concentration. However, propiconazole was found to be effective at 0.1% concentration under field conditios. Our findings are also supported by the research of [29], in which they used the Khumal 9, a long-lasting rice variety in the Baglung district and applied seven fungicides. Among seven fungicides, Propiconazole, provides strongly inhibitory response in reducing disease severity and boosting economic yield. [21] evaluated four various fungicides, including Carbendazim 50WP, Carboxin 50 WP, Propiconazole 25 EC, and Hexaconazole 25EC against *B. oryzae.* At 500 ppm concentration, propiconazole was reported to be an effective fungicide that showed maximum inhibition of 96.58% of the fungal growth under lab conditions. According to [30], Azoxystrobin and propiconazole, at a concentration of 0.1%, decreased the severity of the brown spot disease in rice and increased yield. In addition, [31] reported that propiconazole 25 EC @ 0.1% was highly effective against diseases such as sheath blight, sheath rot, brown spot, and glume discoloration. The same results have been studied by [32], they evaluated several fungicides to check their efficacy against the brown leaf spot of rice. Among all tested fungicides, propiconazole was found to be highly effective against the *B. oyzae* with maximum inhibition of 97% at 250ppm concentration. They also observed that propiconazole @0.1% significantly reduces the disease incidence respectively. Four fungicides Mencozeb, Thiophanate Methyl, Iprovalicarb +Propineb and Propineb were evaluated against the *Helminthosporium oryzae* [33]. They reported that Mancozeb and Thiophanate methyl were found to be the most showing no linear colony growth (0.0 mm) at 150 and 200 ppm concentrations, respectively.

The result showed that 3rd experiment (after symptom appearance) provides satisfactory inhibitory response as compared to pre-inoculation treatment but less than that of post-inoculation treatment. Our study concluded that all three experiment either preventive (pre-inoculation treatment), curative (post-inoculation treatment) and after symptoms appearance significantly reduce the disease incidence but interestingly, post-inoculation treatment provides excellent result against brown leaf spot of rice as compared to other two experiment under greenhouse conditions.

Conclusion

We eventually came to the conclusion that propiconazole was excellent under lab conditions. However, the combination of propiconazole+thiophanate methyl were effective when applied under greenhouse conditions in all three experiment pre-inoculation, post-inoculation and after symptoms appearance respectively as compared to control. These fungicides have been proved to be effective for long-lasting control of brown leaf spot of rice, so farmers are strongly encouraged to apply and use these fungicides in large field conditions.

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