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In vitro inhibitory effect of fungicides on mycelial growth of *Rhizopus oryzae* (Went & Prins Geerl.)

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Abstract

Rhizopus oryzae is a member of Zygomycete group of fungi. It is known to cause disastrous diseases of plants, like root rot, stem rot, fruit rot and seed rot etc. Fruit rot of brinjal is very common in Tikamgarh District of Madhya Pradesh during transit, storage and marketing. Several fungicides are being used for the management of these diseases. In this study, seven fungicides viz, Brassicol, Captan, Dithane M-45, Fytolan, Parasan, Sulfex and Thiram were evaluated for their efficacy against *Rhizopus oryzae* causing fruit rot of brinjal under in vitro condition by food poisoning technique. Ten different concentration i.e., 0.025, 0.05, 0.1, 0.25, 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0% were used for assessment of their inhibitory activities against the pathogen through radial growth inhibition on Fernando's agar media.

Amongst the tested fungicides parasan was found to be the most effective and its 0.05% concentration could cause 100 pre cent inhibition of radial growth. The 0.1% concentration of Parasan was found to be fungicidal and killing the mycelial inoculum of pathogen. Next to Parasan, Thiram has proved to be best against *Rhizopus oryzae*. The growth was completely inhibited at its 0.5% concentration. Its 2.5% concentration was found to be fungicidal. Fytolan was also effective against *Rhizopus oryzae* and it could cause 100 per cent inhibition at 1.5% concentration. At 3.0% concentration is proved to be fungicidal. After Fytolan, Brassicol, captan and Dithane M-45 have been found to be the next effective fungicides against test pathogen. The 2.5% concentration of these three fungicides cause 100 per cent inhibition of growth and 3.0% concentration of these fungicides proved to be fungicidal. While on the other hand Sulfex was proved to be least effective as it did not show cent per cent inhibition even in the higher dose i.e., 3.0% concentration. At 3.0% concentration it proved to be fungistatic.

Keywords: *Rhizopus oryzae*, Mycelial growth, Fungicides, Fernando's agar medium

Introduction

The genus *Rhizopus* was first established in 1820 by the description of *Rhizopus nigricans* (Ehrenberg, 1820) and is known for the production of many extra and intra cellular enzymes.

Rhizopus oryzae is a complex of closely related heterothallic species that are common cosmopolitan saprotrophs in soil, dung and rotting vegetation (Zheng et al. 2007). It is one of the most economically important members of Zygomycete group of fungi and represents the first sequenced fungus from the early lineages of the fungal phylogenetic tree and thus the genome sequence sheds light on the evolution of the entire fungal kingdom (Ghosh and Ray, 2011).

Rhizopus oryzae is known for the formation of fermentation products like ethanol (Bakir et al., 2001; Zheng et al., 2009; Thongchul et al., 2010), lactic acid (Park et al., 2004; Thongchul et al., 2010), fumaric acid, (Thomos, 2008; Sood et al., 2014) and to a lesser extent malic acid.

Rhizopus oryzae strains are often used in Asia for food fermentation to manufacture alcoholic beverages, ragi or tempeh and the strains are generally regarded as safe. Nevertheless, *Rhizopus oryzae* is known as an opportunistic plant pathogen and has a high prevalence to cause various diseases such as soft rot of banana (kwon et al., 2012), soft rot of garlic bulbs (Mathur and Sankhla, 1964), root rot of sugar beet (Shrivastava and Mishra, 1972). Soft rot of ginger (Khanna and Chandra, 1975), fruit rot of mango (Laxminarayana and Reddy, 1977), fruit rot of brinjal (Ali and Abha Shukla, 1981; Chaurasia et al., 2013), fruit rot of apple (kwon et al., 2011), fruit rot of strawberry (kwon et al., 2014a), soft rot of watermelon (Kwon et al., 2014b), boll rot of cotton (Shamsi and Naher, 2014), stem disease of sunflower (Mathew et al., 2015), fruit rot of citrus medica (Naz et al., 2015), storage rot of carrot and radish (Khatoon et al., 2016) and storage rot of sweet potato (Khatoon et al., 2017). Out of these fruit rot of brinjal caused by *Rhizopus oryzae*, is one of the serious disease in Tikamgarh District of Madhya Pradesh. This disease occurred mostly during storage, Marketing and transportation. It was also frequently observed that the valuable fruits of brinjal are severely damaged by *Rhizopus oryzae* even in the field. Know a days it need to use most effective and reliable method that effectively protect brinjal fruits against this disease.

A number of fungicides are known to inhibit or kill the growth of several phytopathogenic fungi. The inhibition or killing of fungal growth may help in the control of plant diseases. The mode of action of different fungicides against the pathogen is quite different because every fungicide has its own mode of action and effects some are broad systematic while others are target specific. The use of fungicides for the control of plant diseases is a common practice all over the world. There are several reports available in which fungicides have been used to control the plant diseases caused by fungal pathogens (Patil et al., 2010; Gondal et al., 2012; Mailto et al., 2014; Hoque et al., 2014; Amrutha et al., 2014; Yadav et al., 2015; Anwar et al., 2015; Singh et al., 2015; Patra and Biswas, 2016). Recently Singh et al., (2000), Kopacki and Wagner (2006), Yaqub and Shahzad (2006), Naik et al., (2007), Soumik et al., (2010), Nisa et al., (2011), Sahi et al., (2012), Jalander and Gachande (2013), Parveen et al., (2013), Rahman et al., (2013), Dar et al., (2013), Das et al., (2014), Chaurasia et al., (2014), Shamsi et al.,(2016), Ghazanfar et al., (2016), Thejakumar and Devappa (2016), Bana et al., (2017) have studied the efficacy of different fungicides against various plant pathogen. Therefore, in the present investigation, inhibition of mycelial growth of *Rhizopus oryzae*, exposed to different concentrations of some fungicides were studied. The aim of the present study was to compare the effect of some selected fungicides on *Rhizopus oryzae* mycelial growth in vitro and identify the concentration of fungicide that have fungicidal properties.

Materials and Methods

Isolation of *Rhizopus oryzae*:

Rhizopus oryzae was isolated from diseased brinjal fruit on potato dextrose agar medium and identified (IMI No 223116). The freshly grown slant culture (at 30^0C) was subsequently used for further work or stored at 5^0C. The slants were sub-cultured routinely at an intervals of four-five week.

Fernando's agar medium:

For experimentation of pathogen, the Fernando's agar medium was used. In previous experiments, it was found most suitable one for the growth as well as sporulation of the test pathogen. The Fernando's agar medium was prepared by the following composition and autoclaved at 121^0C for 15 minutes:

$MgSO_4.7H_2O$	….	5.0 g
KH_2PO_4	….	6.8 g
Asparagine	….	5.0 g
Glucose	…..	15.0 g
Agar agar	…..	20.0 g
Distilled water	…..	1000 ml

Effect of fungicides on mycelial growth:

In vitro effect of fungicides on mycelial growth of *Rhizopus oryzae* was determined by food poisoning technique (Chaurasia et al., 2014). The list of fungicides used in the present study are given below:

1. **Brassicol** :
   75% pentachloronitro-benzene
2. **Captan** :
   N-trichloromethylmercapto-4-cyclohexene 1, 2-dicarboximide

3. **Dithane M**-45 :
   78% Manganese ethylene bisdithiocarbamate
4. **Fytolan** :
   88% copper as copper oxychloride
5. **Parasan** :
   Phenyl mercury acetate 1% Hg
6. **Sulfex** :
   70% sulphur
7. **Thiram** :
   75% tetramethylthiuram disulphide
   (wettable thiram)

The effect of above seven fungicides each with ten concentration i.e. , 0.025, 0.05, 0.1, 0.25, 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0% were evaluated against *Rhizopus oryzae*. Fernando's agar medium was prepared, equally distributed measuring 100 ml in 250 ml conical flask and sterilized in autoclave. Requisite quantity of each of the fungicide was added in sterilized melted (45^0C) Fernando's agar medium separately so as to obtained desired concentration. Conical flask containing pasioned Fernando's agar medium were shaken well to have even and uniform distribution of fungicide. Equal quantity (20ml) of melted poisoned fernando's agar medium was poured in each sterilized Petri plate and allowed to solidify. These Petri plates were inoculated by test fungus (*Rhizopus oryzae*) separately. Seven mm diameter disc of two days old fungus was cut with a sterilized cork borer, lifted and transferred aseptically in the center of a Petri plate containing the medium poisoned with test fungicide. Fernando's agar medium without fungicide served as control. Each treatment was replicated thrice. Treated Petri plates were incubated at 30^0C for a period of 25 hours. Colony diameter was recorded in mm and per cent of mycelial inhibition over control was calculated by the equation given by Chaurasia et al., 2014.

$$I = \frac{C - T}{C} \times 100$$

Where,
I=Per cent of inhibition
C =Average Growth of *Rhizopus oryzae* in control Petri plates
T=Average Growth of *Rhizopus oryzae* in each fungicide treated Petri plates.

Fungicidal and fungistatic action of fungicides:

To determine the fungicidal and fungistatic action of each fungicide, after 25 hours of incubation, the mycelial disc showing no growth was taken out from treated Petri plate, and washed in sterile distilled water with five changes in order to remove trace of fungicide sticking to fungal disc and transferred aseptically to fresh Fernando's agar medium. These Petri plates were then incubated at 30^0C for 25 hours and result on fungal growth was recorded.

Results and Discussion

Effect of fungicides on mycelial growth:

In vitro evaluation of fungicides provides useful preliminary information regarding its efficacy against a pathogen within the shortest period of time and therefore, serve as guide for further field testing. From the results, it is clear that the growth response of *Rhizopus oryzae* to different concentration of fungicides used varied greatly within the different treatments (Table 1 and Fig. 1). Generally higher concentrations of fungicides were more effective than the lower concentrations. In most cases, a gradual decline in pathogen growth noted with an increasing concentration of fungicides except in case of Parasan. In Parasan, *Rhizopus oryzae*_can able to grow (4.0 mm) only at 0.025 per cent concentration while its growth was completely checked (100%) at all other concentrations (i.e., 0.05 to 3.0%). Chaurasia et al., (2014) also reported that Parasan (0.25%) caused 100 per cent inhibition of mycelial growth of S*clerotium rolfsii*. Next to Parasan, Thiram was also found to be toxic but here 100 per cent inhibition in radial growth occurred at 0.5% concentration. Yadav et al., (2015) also found that Thiram was highly effective in inhibiting the growth of the *Alternaria alternata*. Next to Thiram, Fytolan (Copper oxychloride) has been found to be significantly effective and 100 per cent inhibition in radial growth was recorded above 1.0% i.e., at 1.5% concentration. Kantwa et al. (2014) also reported that copper oxychloride (Blitox -50) was most effective against in vitro mycelial growth of *Alternaria alternata*. Chaurasia et al., (2014) also reported significant response in growth inhibition of *Sclerotium rolfsii* at higher concentration of Fytolan.

In the present study, Brassicol, Captan and Dithane M-45 were also proved to be effective and 0.025 to 2.0% concentrations of these fungicides were sufficient to cause the 66.6 to 96.0 per cent inhibition in radial growth over control while above 2.0% concentrations (i.e., 2.5 to 3.0%) were proved to be toxic, therefore, cent per cent (i.e., 100 per cent) inhibition in radial growth was recorded. Similar findings with these fungicides were also reported by Iboton et al., (2000), Yaqub and Shazad (2006), Ponmurugan et al., (2006) , Begum et al., (2010), Chaurasia et al., (2014) and

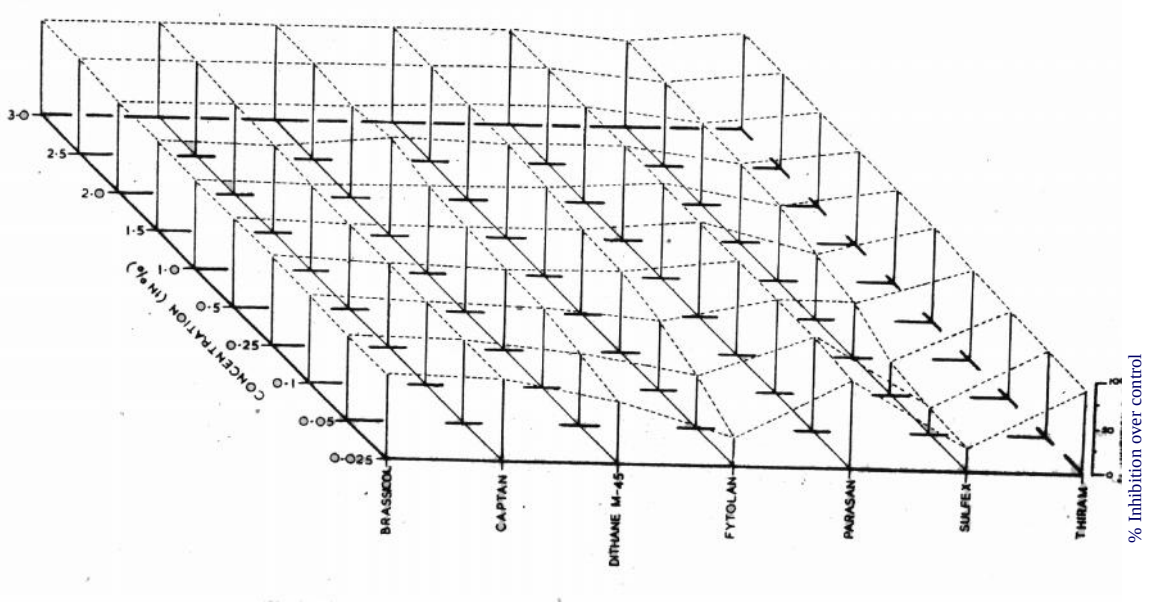
Ghazanfar et al., (2016) to other fungi. Sulfex was also effective but the inhibition in the radial growth of test pathogen gradually increased with the increase in the concentrations and cent per cent (i.e., 100per cent) inhibition could not be recorded upto 3.0% concentration. Therefore, it is clear that sulfex has poor inhibitory effect on the radial growth of pathogen as compared to other tested fungicides. This may be due to fact that the test pathogen *Rhizopus oryzae* might have developed some resistance towards this fungicide. Chaurasia et al. (2014) also reported similar response of Sulfex against brinjal foot rot pathogen *Sclerotium rolfsii*. The different levels of reductions of radial growth by the fungicides may probably be due to varying extent of interference of these chemicals with the metabolism of the pathogen involved. Deacon (1980) reported that in addition to genetic requirement, the metabolism of pathogen depends, among other factors, on the substrate composition which could have been the active principles in the fungicides that affect the qualitative state of the fungus.

Table-1 Effect of different Concentrations of the fungicides on the mycelial growth of *Rhizopus oryzae*

Fungicide	Radial Growth in mm									
	Concentration (%)									
	0.025	0.05	0.1	0.25	0.5	1.0	1.5	2.0	2.5	3.0
Brassicol	5.0 (93.3)	5.0 (93.3)	4.0 (94.6)	4.0 (94.6)	3.5 (95.3)	3.5 (95.3)	3.0 (96.0)	3.0 (96.0)	0.0 (100.0)	0.0 (100.0)
Captan	9.0 (88.0)	8.0 (89.3)	7.5 (90.0)	7.0 (90.6)	6.5 (91.3)	6.0 (92.0)	5.0 (93.3)	3.0 (96.0)	0.0 (100.0)	0.0 (100.0)
Dithane M-45	25.0 (66.6)	17.0 (77.3)	12.0 (84.0)	10.0 (86.6)	7.0 (90.6)	5.0 (93.3)	4.0 (94.6)	3.0 (96.0)	0.0 (100.0)	0.0 (100.0)
Fytolan	52.5 (30.0)	33.0 (56.0)	20.0 (73.3)	10.0 (86.6)	7.0 (90.6)	4.0 (94.6)	0.0 (100.0)	0.0 (100.0)	0.0 (100.0)	0.0 (100.0)
Parasan	4.0 (94.6)	0.0 (100.0)	0.0 (100.0)	0.0 (100.0)	0.0 (100.0)	0.0 (100.0)	0.0 (100.0)	0.0 (100.0)	0.0 (100.0)	0.0 (100.0)
Sulfex	55.2 (26.4)	53.5 (28.6)	48.0 (36.0)	30.7 (59.0)	23.2 (69.0)	15.0 (80.0)	12.0 (84.0)	10.0 (86.6)	8.6 (88.5)	6.6 (91.2)
Thiram	8.0 (89.3)	6.0 (92.0)	5.0 (93.3)	3.0 (96.0)	0.0 (100.0)	0.0 (100.0)	0.0 (100.0)	0.0 (100.0)	0.0 (100.0)	0.0 (100.0)
CONTROL (No fungicide)		75.0 (0.0)								

*Redial Growth: After deducting the inoculum disc of 7.0 mm diameter. The data given in the brackets show per cent inhibition over control.

Fig-1: Effect of different Concentrations of the fungicides on the mycelial Growth inhibition of *Rhizopus oryzae*



Fungicidal and fungistatic action of fungicides:

The findings of fungicidal and fungistatic action of various fungicides are presented in Table 2. The nature of fungicidal and fungistatic action of fungicide was recorded in those cases were no fungal growth was obtained. For this purpose after 25 hours of incubation, the mycelial inoculum disc from treated plate was picked up, washed with sterile distilled water and transferred to fresh Fernado's agar medium Petri plate. Then these Petri plates were incubated and recovery of fungus was noted.

Table-2 Fungicidal and fungistatic action of fungicides against *Rhizopus oryzae*

Fungicide	Concentration (%)									
	0.025	0.05	0.1	0.25	0.5	1.0	1.5	2.0	2.5	3.0
Brassicol	+	+	+	+	+	+	+	+	+	-
Captan	+	+	+	+	+	+	+	+	+	-
Dithane M-45	+	+	+	+	+	+	+	+	+	-
Fytolan	+	+	+	+	+	+	+	+	+	-
Parasan	+	+	-	-	-	-	-	-	-	-
Sulfex	+	+	+	+	+	+	+	+	+	+
Thiram	+	+	+	+	+	+	+	+	-	-

\+ Fungistatic
\- Fungicidal

Findings of the Study clearly revealed that Parasan was found to be fungicidal in action even at very low concentration i.e., at 0.1% concentration, owing to the fact that no fungal growth was observed around the mycelial inoculum disc, transferred to fresh Fernando's agar medium. It showed that the fungus was dead due to fungicidal effect of the Parasan. In case of Thiram, the mycelial inoculum did not survive at 2.5% concentration, therefore, 2.5% of Thiram was proved to be fungicidal in action and below this concentration it was fungistatic action. It proved that below 2.5% concentration of Thiram had fungistatic property to test pathogen.

Brassicol, Captan, Dithane M-45 and Fytolan have been found to be fungicidal in action at 3.0% concentration and thus, mycelial inoculum of test pathogen did not survive. Below this concentration of these fungicides were found to be fungistatic in action because the fungus again started to grow around mycelial inoculum disc when transferred on fresh Fernando's agar medium.

Therefore, it is clear that mycelial inoculum of *Rhizopus oryzae* has been killed within 25 hours when it came in contact with those fungicides which prove to be fungicidal in action.

Conclusion

From the results of the study, it can be concluded that Parasan was found to be significantly effective against *Rhizopus oryzae*. The very low concentration (i.e., 0.05%) of Parasan could cause 100 per cent inhibition of growth and its 0.1% concentration proved to be fungicidal. Next to Parasan, Thiram has been found to be equally competent as it gave 100 per cent inhibition of growth at 0.5% concentration. Its 2.5% concentration was found to be fungicidal. Besides above two Fytolan has been found to be the next effective fungicide against *Rhizopus oryzae*. The 1.5% concentration of Fytolan could cause 100 per cent inhibition of growth of test pathogen. Brassicol, Captan and Dithane M-45 were also found to be toxic which cause 100 per cent inhibition in radial growth at 2.5% concentration. These four fungicides i.e. Fytolan, Brassicol, Captan and Dithane M-45 were recorded as fungicidal in action at 3.0% concentration. Sulfex have proved to be poor in this respect. Sulfex was found to be fungistatic inaction, in all the taken concentration i.e. from 0.025 to 3.0% concentrations. In the light of present investigation, fungicide like Parasan (0.1%), Thiram (2.5%), Fytolan (3.0%), Brassicol (3.0%), Captan (3.0%) and Dithane M-45 (3.0%) may be of practical importance in control of fruit rot disease of brinjal caused by *Rhizopus oryzae* which however requires further field investigation.

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