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Original Article

Inhibitory effect of various fungicides on mycelial growth of *Alternaria alternata*; cause of Alternaria leaf spot disease on *Rosa Indica* L. in Pakistan

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Abstract

Rosa indica L. (family: Rosaceae) is an important and widely grown plant in the floriculture industry. Alternaria leaf spot disease of rose was observed in rose plantation at College of Agriculture, University of Sargodha, Sargodha. The study was carried out to isolate and purify the pathogen, which was identified as *Alternaria alternata.* Additionally, *in vitro* efficacy of five fungicides; success, copper oxychloride, metalaxyl+mancozeb, topsin M and kumulus against *A. alternata* were tested by using food poisoning technique. Three concentrations (100, 200 and 300 ppm) of each fungicide were used. Mycelial growth after 3rd, 5th and 7th day was measured. All the fungicides significantly inhibited the mycelial growth of *A. alternata.* Among those, metalaxyl+mancozeb was the most effective as compared to others. Maximum inhibition was observed after 3rd (89%), 5th (91.6%) and 7th (93.3) day by metalaxyl+mancozeb followed by success 43.3%, 41.0% and 29.6% respectively. After 3rd and 5th day copper oxychloride was least effective with 19.6% and 9.6% mycelial inhibition respectively while after 7th day the minimum 4.3% mycelial inhibition was observed by topsin M instead of copper oxychloride. Therefore, *A. alternata* is responsible for alternaria leaf spot disease of rose and metalaxyl+mancozeb was found to be the most effective fungicide against *A.alternata in vitro*.

Keywords: Rose, Mycelial growth, *Alternaria alternata*, Management

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Introduction

Rose (*Rosa indica* L.), a perennial woody shrub belonging to family *Rosaceae*, is widely distributed in the northern hemisphere. About two hundred species of rose has been divided into four subgenera (Wissemann, 2003). It is well known for its aromatic, cosmetic and medicinal value that makes it "Queen of Flowers" (Leghari et al., 2016). Rose is being cultivated in more than 145 countries (Nagar et al.,

2007). In Pakistan, climatic conditions are favorable for the cultivation of the rose and other ornamental plants (Leghari et al., 2016). Major rose cultivating areas in Pakistan are Pattoki, Chunian, Kasur, Lahore, Multan, Rawalpindi, Karachi, Hyderabad, Peshawar, Mansehra, Harripur and Quetta (Usman et al., 2014). Economically important cultivating verities of rose in Pakistan are; Bara bara, De culenry, mamorila day, decent peace, American beauty, rough royal, honey perfume, head liner and cendrila (Ramzan et al., 2014).

Previously several fungal pathogens have been reported to cause diseases in rose plant such as Cercospora leaf spot (Hagan et al., 2005), leaf spot of rose caused by *Passalora rosicola* (Feres et al., 2017), black spot of roses (Khatun et al., 2009), downy mildew (Ghosh and Shamsi, 2014), brown canker (Islam et al., 2010) and powdery mildew (Li et al., 2003). Among them, leaf spot caused by *A. alternata* (Fr.) Keissler, is serious threat for rose plantation. It has been reported that *A. alternata* attacks on 380 host species of plants and about 20% of agricultural spoilage is caused by *Alternaria* spp., with severe losses of about 80% of the crop (Bashir et al., 2014). *A. alternata* causes leaf spots which are initially small and circular with light brown color. When the spots get older these become larger and irregular surrounded with yellow hallow and the concentric rings may appear in the center (Sankar et al., 2012). Rao (1965) reported leaf spot disease caused by *A. alternata* on rose plants in India for the first time. Now it has been observed on rose plants in Pakistan as emerging threat to this crop.

Recently, *A. alternata* has been reported for the first time in Pakistan to cause black spot disease of rose (Abbas et al., 2017). Whereas, previous published literature showed that Black spot of rose and Alternaria leaf spot are two different diseases. According to published literature black leaf spot of rose is caused by *Diplocarpon rosae* (Jeliazkova et al., 2012; Yasin et al., 2016; Neu et al., 2017). Therefore, an initial confusion was arisen related to disease on the basis of symptoms and available literature. Fungicides are the quick, effective and commercially available way to control fungal diseases. Different fungicides such as copper oxychloride, mancozeb, metalaxyl+mancozeb, czrbendazim, topsin M, chlorothalonil and ridomil have been used for the effective management of *A. alternata* (Arain et al., 2012; Hassan et al., 2014; Kalieswari et al., 2016). In Pakistan, limited literature is available regarding management of Alternaria leaf spot disease on rose plants. This study was carried out with the objective to identify and purify the pathogen of leafspot disease on rose and to evaluate the efficacy of five fungicides with different concentrations against pathogen.

Material and Methods

Samples collection

Rose leaves and petals having typical symptoms were collected from the Rose garden (32°07'56.3"N 72°41'12.5"E) at College of Agriculture, University of Sargodha. Small brown spots were observed on leaves and petals surrounded by yellow lesions. On leaves, later on spots may collapse and cover whole leaves. Old and lower leaves show chlorosis and drop off (Figure 1).

Fig. 1: Symptoms of *Alternaria* leaf spot disease (A) Leaves (B) Petal

Isolation of pathogen

The pathogen was isolated by using tissue planting technique (Ghosh and Shamsi, 2014). After washing with tap water, infected part of leaves along with some healthy part was converted into small pieces of 2-4 mm. The specimens were dipped into 2% sodium hypochlorite for 3-5 minutes followed by washing with distilled water. After drying, five pieces were kept in Petri plates having potato dextrose agar (PDA) medium. Plates were kept in incubator for 72 hours at 25 ± 1 ºC temperature.

Purification and identification of pathogen

After colony development, a single spore isolation technique was applied to obtain pure colony of *A. alternat*a. Loops were taken from pure colonies of *A. alternata* from old Petri plate and inoculated in new plates on PDA. These plates were incubated at 25 ± 1ºC temperature (Ghosh and Shamsi, 2014). Macro and microscopic morphological characters such as colony color, texture, spore size, shape and septation

were used for pathogen identification (Abeer et al., 2014)

Pathogenicity test

Healthy rose plants were inoculated with *A. alternata* spore suspension by using spray inoculation method (Rahman et al., 2012). The spore suspension was prepared and sprayed on healthy plants. For control treatment, plants were sprayed with distilled water (Figure 3).

Application of fungicide

Five fungicides viz., kumulus 80% WG, copper oxychloride 50% WP, metalaxyl+mancozeb 72% WP, topsin M 70% WP and success 72% WP were evaluated against *A. alternata* by using food poison technique (Hassan et al., 2014). Three concentrations; 100, 200 and 300 ppm of each fungicide were tested. Fungicides list with active ingredients and manufacturer has been tabulated (Table 1).

Preparation of fungicidal concentration

Initially 1000 ppm stock solution of each fungicide was prepared. From stock solution required concentration of each fungicide was prepared by using the formula (Gul et al., 2015)

$$C_1V_1=C_2V_2$$

C_1= Concentration of stock solution,
V_1= Volume of stock solution to be used
C_2= Required concentration
V_2= Volume of required concentration

Food poisoning technique

A Total of 15 ml from each concentration was mixed with 85 ml autoclaved PDA and poured into 90 mm Petri plates. Un-amended PDA was used as control. A 5 mm plug from 5 days old colony of *A. alternata* was placed in the middle of Petri dish. Three replicates for each dose were used. Plates were incubated at 25 ± 1 ºC and observed for colony growth after 24 hours interval. Mycelial growth rate was measured at 3, 5 and 7 days after treatment. Percent inhibition was measured by the following formula (Neelakanth et al., 2017);

$$\text{Percent inhibition} = \frac{C - T}{C} \times 100$$

Whereas, C = Diameter of colony in control, T = Diameter of colony in treatment

Table 1: Detail of fungicides used to test the effectiveness against *Alternaria alternate*

Fungicide	Active ingredient	Manufact-urer
Metalaxyl+Mancozeb 72% WP	Metalaxyl 08% w/w Mancozeb 64% w/w	Green Zone
Copper Oxychloride 50% WP	Copper oxychloride 50% w/w	Capricorn
Success 72% WP	Chlorothalonil 64% w/w	Arysta Life
Kumulus 80% WG	Sulphur 80% w/w	FMC
Topsin-M	Thiophanate Methyl 70% w/w	Arysta Life

Data Analysis

Data was analyzed using analysis of variance through factorial arrangements under completely randomized design (CRD). The means were separated through Tukey HSD all-pairwise comparison test. The analysis was performed using SPSS 20.0 software.

Results

Identification of pathogen

A. alternata was identified morphologically on the basis of macroscopic as well as microscopic characters. Grey to a black color colony of *A. alternata* with fluffy growth was observed on PDA. Microscopic observation showed that light brown color conidia produced in two to five spores' chain or singly. Conidia were ellipsoid or obclavate to obpyriform with the short conical beak or beakless with 0-2 longitudinal and 2-4 transverse septa (Figure 2).

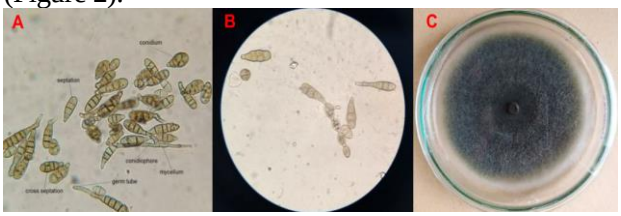

Fig. 2: Morphological characters of *Alternaria alternata* (A) Conidia, (B) Conidia in chain form and (C) Colony growth on PDA

Fig. 3: (A) Plant inoculated with distilled water and (B) with spore's suspension

Table 2: Effect of fungicides on percent inhibition (mean±SE) of *Alternaria alternata* after 3 days of exposure

Fungicides	Concentration		
	100 ppm	200 ppm	300 ppm
Success	34.667 ± 1.667[b]	39.000 ± 3.001[b]	43.333 ± 1.334[b]
Copper oxychloride	15.667 ± 1.333[c]	17.000± 2.309 [c]	19.667± 1.354[d]
Kumulus,	22.333 ± 5.333[bc]	30.333 ± 2.664[bc]	33.000 ± 1.023[c]
Topsin M	26.333 ± 1.333[bc]	32.000 ± 5.132[b]	22.333 ± 2.667[d]
Metalaxyl+ Mencozeb	52.667 ± 2.667[a]	68.333 ± 1.333[a]	89.000 ± 3.003[a]
F-value	24.3	36.7	202
P-value	*< 0.001*	*<0.001*	*<0.001*

P< 0.001 shows significance, means sharing similar letters within columns are not significantly different at *P > 0.05*

Efficacy of fungicides

The results showed that fungicides significantly suppressed the mycelia growth of targeted pathogen at 3rd, 5th and 7th day of exposure. Percent inhibition was higher at the 3rd day of exposure and gradually decreased at 5th and 7th day in all tested fungicides except in case of copper oxychloride in which the inhibition rate was more at 7th day as compared to 5th day. The inhibition of *A. alternata* mycelia were found higher after application of metalaxyal+mancozeb (70%, 67.6%, and 62.3%) at 3rd, 5th, and 7th days respectively as compared to other fungicides. The minimum inhibition of 8-18% was found due to the application of copper oxychloride (Figure 5).

The mycelia growth was significantly affected at 100 ppm, 200 ppm and 300 ppm concentrations of fungicides at 3rd, 5th and 7th day of exposure. All the fungicides reduced the mycelia growth with increasing the concentrations of fungicides except in case of topsin M. The inhibition rate was higher (89.00%) after application of metalaxyal+manncozeb at 300 ppm concentration after 3 days. However, topsin M and copper oxychloride showed minimum inhibition of mycelia growth (22.33% and 19.67% respectively) at higher concentration (Table 2).

After 5 days of fungicide exposure, the higher inhibition rate (91.67%) was found with metalaxyal+manncozeb treatment as compared to others. Similar results were found after 7th day, in which the efficacy of metalaxyal+manncozeb was higher (Figure 4, Table 3). The copper oxychloride and topsin M was least effective fungicides against *A. alternata* (Table 3). Similarly, the inhibition rate was found lower due to the application of copper oxychloride and topsin M at 7th days (Table 4).

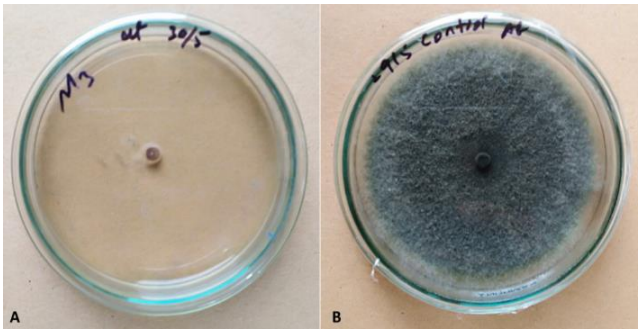

Figure 4. (A) *Alternaria alternata* treated with metalaxyal+mancozeb at 300ppm concentration after 7 days of exposure (B) Control

Table 3: Effect of fungicides on percent inhibition (mean±SE) of *Alternaria alternata* after 5 days of exposure

Concentration			
Fungicides	**100 ppm**	**200 ppm**	**300 ppm**
Success	32.667 ± 0.883[b]	38.667 ± 0.881[b]	41.000 ± 1.000[b]
Copper oxychloride	7.333 ± 0.548[d]	8.000 ± 1.000[e]	9.667 ± 0.333[e]
Kumulus,	15.333 ± 0.884[c]	19.667 ± 1.453[c]	27.000 ± 1.000[c]
Topsin M	9.667 ± 0.667[d]	14.000 ± 0.284[d]	14.667 ± 0.667[d]
Metalaxyl+Mencozeb	50.333 ± 1.667[a]	60.667 ± 0.333[a]	91.667 ± 1.667[a]
F-value	513	577	1813
P-value	*<0.001*	*<0.001*	*<0.001*

P< 0.001 shows significance, means sharing similar letters within columns are not significantly different at *P > 0.05*

Table 4: Effect of fungicides on percent inhibition (mean±SE) of *Alternaria alternata* after 7 days of exposure

	Concentration		
Fungicides	**100 ppm**	**200 ppm**	**300 ppm**
Success	23.333 ± 0.637[b]	26.000 ± 1.000[b]	29.667 ± 0.637[b]
Copper oxychloride	9.333 ± 0.347[c]	10.000 ± 0.5774[c]	10.667 ± 0.347[d]
Kumulus,	18.667 ± 1.353[b]	11.667 ± 0.667[c]	15.000 ± 1.037[c]
Topsin M	3.667 ± 0.372[d]	5.333 ± 0.348[d]	4.333 ± 0.234[e]
Metalaxyl+Mencozeb	42.667 ± 1.473[a]	51.000 ± 1.394[a]	93.333 ± 1.384[a]
F-value	204	912	1911
P-value	*<0.001*	*<0.001*	*<0.001*

P< 0.001 shows significance, means sharing similar letters within columns are not significantly different at *P > 0.05*

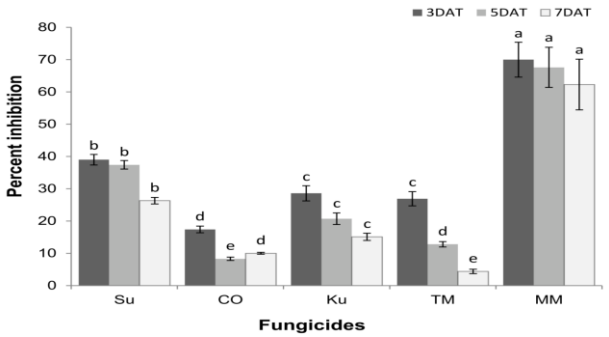

Fig. 5: Effect of fungicides on percent inhibition (mean±SE) of *Alternaria alternata* after different days of exposure

Means sharing similar letters are not significantly different at *P > 0.05*, Su = success, CO = copper oxychloride, Ku = kumulus, MM = metalaxyl+mencozeb, TM = topsin M and DAT = days after treatment

Discussion

Alternaria is a common pathogen found worldwide and causes well-defined symptoms on the foliage of different host plants. Previously, researchers have reported that *A. alternata* causes leaf spot of roses in Bangladesh and India (Rao, 1965; Sankar et al., 2012; Ghosh and Shamsi, 2014). In present study, *A. alternata* was identified on the basis of morphological characters and findings are in line with (Chen et al. 2018; Jankar et al., 2018). Recently, Abbas et al. (2017) have reported *A. alternata* as pathogen of black spot of roses in Pakistan; however, their findings are in contradiction with Jeliazkova et al. (2012) who reported *Diplocarpon rosae* Wolf as the causal organism of black spot disease on rose plants. Furthermore, many researchers have also identified *D. rosae* causing black spot disease through morphological and molecular techniques (Gachomo et al., 2006; Neu et al., 2017). Our micro and macroscopic study revealed the symptoms appeared in the pathogenicity test were similar to *Alternaria* leaf spot. Our results were in accordance to Rao (1965) and Nadziakiewicz et al. (2012) but in contradiction to Abbas et al (2017).

In Pakistan, leaf spot is one of the major diseases of most economically important crops such as broccoli, ber, potato, tomato, okra, Aloe vera (Bajwa et al., 2010; Arain et al., 2012; Shahid et al., 2017; Mehmood et al., 2018; Javaid et al., 2018). Different control practices; cultural, biological, and chemical have been used previously for the effective management of this disease. Alternaria leaf spot of rose, being an emerging disease in Pakistan, limited study has been carried out on management of this disease especially in rose. Five fungicides were tested for *in vitro* management of Alternaria leaf spot found on rose plants. Results of our study showed that the metalaxyl+mancozeb found to be the most effective fungicide in suppressing the mycelial development up to 93.3% of pathogen at higher concentration. The active ingredient metalaxyl affects the protein synthesis as well as the growth and reproduction of the fungi. While mancozeb is toxic to fungi when exposed to air and affect the functioning of fungal enzymes (Fitsum et al., 2014).

Fungicides are lethal to pathogens in numerous ways such as mycelium may stop growing, alteration in the metabolic process and spores may fail to germinate (Navi et al., 2016). Our results are similar to other researchers who reported that metalaxyl+mancozeb, chlorothalonil, and topsin M are significantly effective against leaf spot diseases (Chandel et al., 2010; Arain

et al., 2012; Kalieswari et al., 2016). Similarly, Kalieswari et al. (2016) evaluated different fungicides against *A. alternata* and reported that metalaxyal+mancozeb (0.05%) and chlorothalonil (0.2%) inhibited the mycelial development of *A. alternata*. Taware et al. (2014) tested eleven different fungicides through food poisoning technique against *Alternaria* sp. and reported metalaxyal+mancozeb as the most effective fungicide. Recently (Biswas et al., 2018) evaluated the different fungicides, bio agents and plant extracts against *A. brassicae*. Among the tested fungicides mancozeb gave maximum percent inhibition and its effect increased with increasing concentration.

Conclusion

It is concluded that alternaria leaf spot disease is caused by *Alternaria alternata* which is different from black spot of rose caused by *Diplocarpon rosae* in the published literature. Among the fungicides tested by using food poison technique, metalaxyl+mancozeb is the most effective against mycelial growth of *A. alternata*. Additionally, it is suggested that this fungicide should be tested under the field conditions along with environmental parameters which may affect the efficacy of this fungicide. Therefore, potential use of the fungicide should be investigated in this regard.

Contribution of Authors

Asim M: Conceived Idea, Designed Research Methodology, Manuscript Writing and Manuscript final Writing and approval
Iftikhar Y: Conceived Idea, Designed Research Methodology, Manuscript final Writing and approval
Arshad M: Data Interpretation and Statistical Analysis
Bashir S: Literature Search and Data Collection
Raza M: Designed Research Methodology and Data collection
Bilal S: Literature search and Data Interpretation
Bakhtawar F: Literature Review and Manuscript Writing

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