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RESEARCH ARTICLE

Inhibitory effect of Polyram DF and *Capsicum annum* on leaf spot of rose caused by *Curvularia lunata in vitro* and *in planta*

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Abstract

Rose plants are affected by several diseases caused by fungi, nematode, bacteria, viruses, and other pests. Among all of these, *Curvularia lunata* causes significant losses to Roses. Present study was focused on *In-vitro* and *In-vivo* management of the "Curvularia leaf spot of Rose" caused by *Curvularia lunata* by using different fungicides and phyto-extracts. Diseased samples were collected from floriculture area of University of Agriculture, Faisalabad for isolation of pathogen. Five fungicides i.e., Cabrio-Top, Curzate-M, Aliette, Polyram-DF and Recado @ (50ppm, 100ppm and 150ppm) and five plant extracts i.e., *Allium cepa*, *Capsicum annuum*, *Aloe vera*, *Mentha* and *Calotropis gigantean* with three concentrations @ (5%, 10% and 15%) were evaluated under lab conditions through poisoned food technique by using Complete Randomized Design (CRD), where *C. annuum* gave best results (9.129mm) followed by *Calotropis gigantea* (13.003mm), and Polyram-DF was effective (2.218mm) followed by Curzate-M (6.542mm). Best performing fungicides and plant-extracts were subjected to *In-vivo* management trials. Under green-house conditions, combination of *Capsicum annuum* + *Calotropis gigantean* and Polyram-DF + Curzate-M were shown least disease incidence (14.517 and 3.224%). LSD was used for comparing variations between treatments at 5% probability. The results of these experiments were to aid in the evaluation of fungicides and Phyto-extracts, which are the most effective chemicals and phyto-extracts against leaf Spot disease of Rose.

Keywords

Cabrio top; Curzate-M; Aloe Vera; Mentha; Poisoned food technique

Introduction

Rosa indica L. is the king of flowers, well-known and popular cut flower in the world as well as Pakistan, serving as a symbol of love, elegance, inspiration, and aesthetic pleasure (1). *Rosa* species are native to Netherland (2), and can be found from the Artic to the subtropics in the Northern hemisphere (cooler and temperate) zones (3). Roses contains antimicrobial agents, vitamins (A, C, D, E, and B3), minerals and nutrients (Calcium, Potassium and Iron) which are beneficial for human health, and used as stress reliever, mind relaxer and eye-wash agent etc. Moreover, they are used in the preparation of many byproducts like jams, jellies, kulfi, roohafzah drink, ice cream, flavors (rose oils), sauces, vinegar, pharmaceuticals and cosmetics (4). Globally, cut-roses were grown on an area of 60,447 hectares

with Netherland's share of 42.32 % (5). Pakistan yields nearly 10-12 thousand tons of flowers over an estimated area of 2865.17 hectares (6). Approximately 80 million potted plants and 220 million plants of garden roses are imported per annum (7).

Rose is affected by a number of pests, pathogen, soil and seed borne disease i.e., black mold, blights, cankers, mildews, rust, verticillium wilt, anthracnose and leaf spots (8). Among all diseases, leaf spot caused by a soil and seed borne fungal pathogen (*Curvularia lunata*) is one of the most serious menace to rose (9). This pathogen has a wide host range and responsible for many diseases like root rot, leaf spot, stem blight, leaf blight, and necrotic rot in different cereals, oil seeds, fruits, vegetables and legume crops (10, 11). This pathogen produces very small and rounded tan lesions (often, lesions are brown, bordered and surrounded by a yellow halo) on leaves surface which develops into leaf spots of 3-4 mm diameter. Lesions starts from the tips or edges of lamina and founds mostly on younger leaves. Later on, lesions extend and coalesced and cause leaf drop. The pathogen shows white colonies which becomes black or golden brown (with regular to irregular margins), obovoidal or broadly clavate brown conidia (avg. size 24.7 um), 3-septations (third cell is larger, broader and darker than others), with smooth-walls. The cells are normally curved but seldom straight having basal cells (rounded apical and obconical). The apical region of unbranched conidiophores was septate, straight, and flexuous, with flattened and dark brown scars (12). This pathogen grows well on PDA (potato dextrose agar) at 25-30^{0}C, and Blue light (450-495 nm) is favorable for survival of pathogen in Oat Meal Agar (13).

Due to its wide host range, mode of spread and survival, leaf spot disease is very difficult to control. So that, use of resistant cultivars is the only economical and successful strategy against the disease. No doubt, rose plants are hardy and resistant to many of the disease but under favorable environmental conditions, if disease appears in epidemic form then growers have no choice except the use of synthetic chemicals. For this reason, different fungicides including Metalaxyl + Mencozeb (14), Mancozeb + Carbendazim, Mancozeb, and Imidacloprid + Metalaxyl-M + Tebuconazole were found effective against *C. lunata* causing "Curvularia Leaf Spot of Rose" (15). In current studies, five synthetic chemicals (fungicides) were evaluated at three concentrations to control this disease under *In-Vitro* and *In-Vivo* conditions.

Although, synthetic chemicals showed quick response against leaf spot but they are dangerous for human, animals, environment, and for agricultural produce. Due to their toxic and residual effects, scientists are shifting towards the phyto-extract as an alternative because of their antimicrobial and antioxidant activities which are non-toxic to human beings. Feeling the gravity of scenario (16) conducted a research to evaluate different plant-extracts against *C. lunata* with different concentrations and concluded that the potential of Garlic extract could be used for the effective management of leaf spot of rose. Similar study was conducted by (17) they used different plant-extracts to check the antifungal potential of *Syzygium aromaticum* against *C. lunata*. Rose is an aromatic and loving flowering plant used to grow in different landscapes, it is severely affected by fungal pathogen *c urvularia lunata*, and it is need of time to manage the disease by using quick acted fungicides and ecofriendly phytoextracts, so the study was designed to check the efficacy of phyto-extracts and fungicides with different concentrations against *Curvularia lunata* causing leaf spot disease of rose.

Materials and Methods

Isolation, identification and purification of Curvularia lunata

For the isolation of *C. lunata*, diseased samples of rose were collected from different nurseries of district Faisalabad. Small pieces of infected rose leaves were made by cutting them (2-3 mm), and were sterilized with 1% sodium hypochlorite (NaOCl) solution for about 30 seconds and two times in sterile distilled water for about one minute. Then these samples were placed on Petri plates containing PDA with the help of sterilized forceps. Inoculated Petri plates were incubated (Heraeus) at temperature of 25± 2° C for 48-72 hours for the fungal growth. After 48 hours, the fungal growth was appeared and the culture was purified by using "single hyphal tip technique" (18). The identification of the *C. lunata* was done on the basis of morphology (shape, margins, septation, size, and color of the colony, conidia and mycelium) and compared with available literature (19).

Pathogenicity Test

A pipet tip (4mm diameter) was used to make culture plugs (7 day old) of the same size. Freshly detached rose leaves were wounded with the help of sterilized needle, then pathogen was inoculated (4mm disk), and these inoculated samples were placed in a dish by wrapping with cling film and were kept at room temperature. The symptoms were checked on daily basis for one week by keeping record of the observations. Re-isolation of the targeted pathogen was done to fulfil the Koch's postulates (09).

In-Vitro management of Curvularia lunata through chemicals and phyto-extracts

For the management of pathogen, five chemicals (Polyram-DF 70%, Recado 32.5 %SC, Cabrio-Top 60%WDG, Aliette 80%WP, and Curzate-M 70% WP) (Table 1) and plant extracts (Onion, Chili, Aloevera, Mint and Aak) (Table 2) were evaluated with three concentrations and replications by using "poisoned food technique" (20,21) with 24 hours of interval up to 4 readings. The concentration used for chemical was 50, 100 and 150 ppm, and for phyto-extracts 5, 10 and 15%. The stock solution of chemicals were based on the active ingredients present in the concerned product (22). The plant parts were blended separately in an electric blender machine, their juices were extracted by adding 250 ml of distilled water and 50 g of blended material. Then all the juices were sieved by using Muslin cloth and required amount of 250 ml was set in a flask.

Table 1. List of the fungicides used along with active ingredients and mode of action

Trade Name	Company	Active Ingredients	Mode of Action	References
Polyram-DF 70%	BASF	Metiram	Inhibit spore germination	(23)
Recado 32.5 %SC	Mera Mustaqbil	Azoxystrobin 200g/l + Diphenoconazole 125 g/l	Inhibition of respiration	(24)
CabrioTop 60%WDG	FMC	Metiram 550gm/kg + Pyraclostrobin 50gm/kg	Inhibit spore germination and electron transfer in the mitochondrial respiratory chain	(23, 25)
Aliette 80%WP	Bayer	Fostyl-Aluminum 80% w/w	Un-known	(26)
Curzate-M 70%WP	Arysta	Cymoksanil 8% w/w + Mancozeb 64% w/w	Multisite action and enzymes activity interference	(26,27)

Table 2. List of the Plant-Extracts used for the management of *Curvularia lunata*

Common Name	Scientific Name	Plant Part	Antifungal Agent	Mode of Action	References
Onion	*Allium cepa*	Bulb	Sulfur and phenolic compounds	Activates defense systems	(28,29)
Chili	*Capsicum annuum*	Fruits	Capsaicin	Disrupt membranes and ATP production (Energy poisoning)	(30)
Aloe vera	*Aloe vera*	Leaves	Saponins, aloe-emodin, aloin	Cell leakage, degradation of membrane and proteins	(31,32)
Mint	*Mentha*	Leaves	Menthyl Acetate, Menthol	Membrane potential across CW, disrupt ATP assembly and mitochondrial membrane of fungi	(33,34)
Aak	*Calotropis gigantea*	Leaves	Terpenoids, Saponins, Flavonoids, Alkaloids	Irritate CW, protein coagulation, prevent multiplication, diffusion of cell fluid	(35,36)

1g of detergent was added and the prepared solution was considered as standard (100%) and left for 16 hours at normal temperature. Three concentrations were prepared by mixing 5ml, 10ml and 15ml stock solution in 100ml PDA media.

Establishment of diseased plants

Fungal spore suspension was prepared with help of Hemocytometer for the inoculation of the rose plants. 5 mL distilled water was added into the Petri plate containing 7-10 days old pure culture of *Curvularia lunata* and the spores were harvested with the help of sterilized razor. The suspension was sieved with 4 layers of muslin cloth and the filtrate was kept in a beaker with 250 mL of distilled water in it. 1 mL spore suspension was placed on the Hemocytometer and the number of spores were calculated 3 times under Stereomicroscope and adjusted through Hemocytometer in the beaker at 1×10^6 spores/mL of water. Two consecutive sprays of suspension of *C. lunata* was made with the interval of ten days on the healthy rose plants and allowed for the development of disease (37).

In-vivo management of Curvularia lunata through phyto-extracts and chemicals

For *In-vivo* management of *C. lunata*, plants were grown in the Plant Pathology Research Area, University of Agriculture, Faisalabad. The two most effective fungicides (Polyram-DF, Curzate-M) and their combinations were sprayed after 7 days interval (3 weeks) on diseased plants to evaluate their antifungal activities at three concentrations (50, 100 and 150 ppm). Similarly, two most effective phyto-extracts (Chili, Aak) and their combination were sprayed on rose plants up to 3 weeks with 7 days interval at 3 concentration (5, 10 and 15%). The disease incidence was recorded for further analysis by the following formula (38)

$$\text{Disease incidence} = \frac{\text{No. of infected plant}}{\text{No. of Total plants assessed}} \times 100$$

Statistical Analysis

All the experiments for *In-vitro* management of the curvularia leaf spot disease of rose through various fungicides and phyto-extracts were conducted under Complete Randomized Design (CRD), and *In-vivo* trials were conducted under Randomized Complete Block Design (RCBD). Recorded data was subjected to analysis of variance (ANOVA) at 0.05% level of significance and for statistical analysis by using Least Significance Difference (LSD) and "Statistics 8.1" software at $p\leq0.05$ (39).

Results

Isolation and identification of the causal agent

Curvularia lunata was isolated on PDA (prepared with fresh potatoes) medium from the diseased leave parts of rose (*Rosa indica* L.). Observed colonies were cottony white which changed color (black or golden brown) with passage of time. Conidia were obovoidal, brown to black with regular to irregular margins, showing average size of 24.7 um, smooth-walls and 3-septation having larger, normally curved and seldom straight cells. There was septation and flexuous scars on the apical region of conidiophores with flattened and dark brown color.

In-vitro management of Curvularia lunata through different fungicides

Five fungicides namely Polyram-DF 70%, Recado 32.5 %SC, Cabrio-Top 60%WDG, Aliette 80%WP and Curzate-M 70% WP were investigated. The prepared stock solutions of each fungicide were amended in the PDA medium for three dosages (50ppm, 100ppm, 150ppm). Meanwhile, culture plugs were prepared with the help of sterilized pipet tip (4mm dia.) and these culture plugs were placed in the center of each Petri plate (90mm) having fungicide amendment with different concentrations. The control plates were only amended with sterilized distilled water and

allowed for the establishment of the fungal colony. Data was recorded for upto four days with 24 hours of interval. Mean values showed that among all the treatments, Polyram-DF exhibited the greatest mycelial growth retardation (2.218mm) followed by Curzate-M (6.542mm), Recado (8.053mm), Cabrio-Top (9.088mm), and Aliette (15.461mm). Aliette was the least effective in controlling the fungal growth of the *Curvularia lunata* at all dosages (Fig. 1A). According to an interaction between treatments, concentrations and days, Polyram-DF application resulted in the highest control (6.6550mm, 0.0000mm and 0.0000mm) at all concentrations (50, 100, 150 ppm), respectively (Fig. 1B), after the 1st (1.390mm), 2nd (1.923mm), 3rd (2.561mm) and 4th (2.999mm) days (Fig. 1C) as compared to all treatments including control. On the other hand, Polyram-DF was followed by Curzate-M showing 7.3083mm, 6.9575mm and 5.3608mm at respective concentrations, while 2.878mm, 5.772mm and 9.838mm of the fungal growth on respective days.

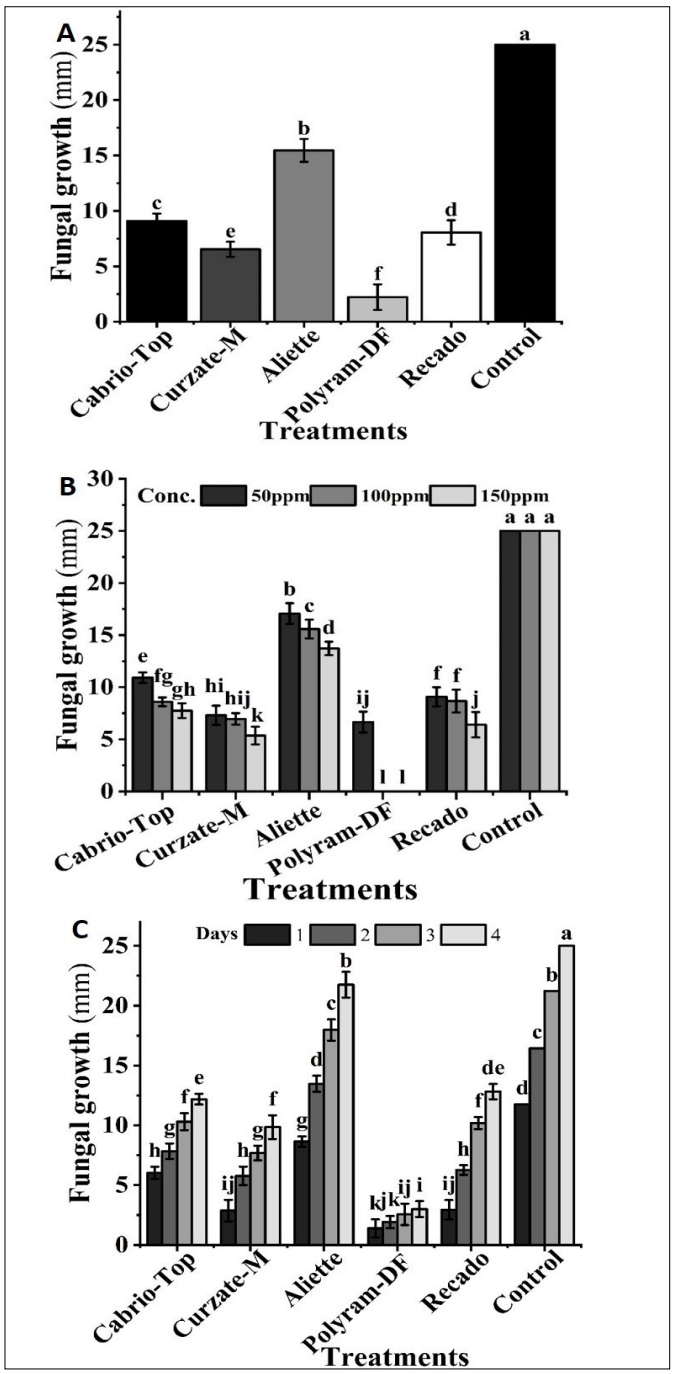
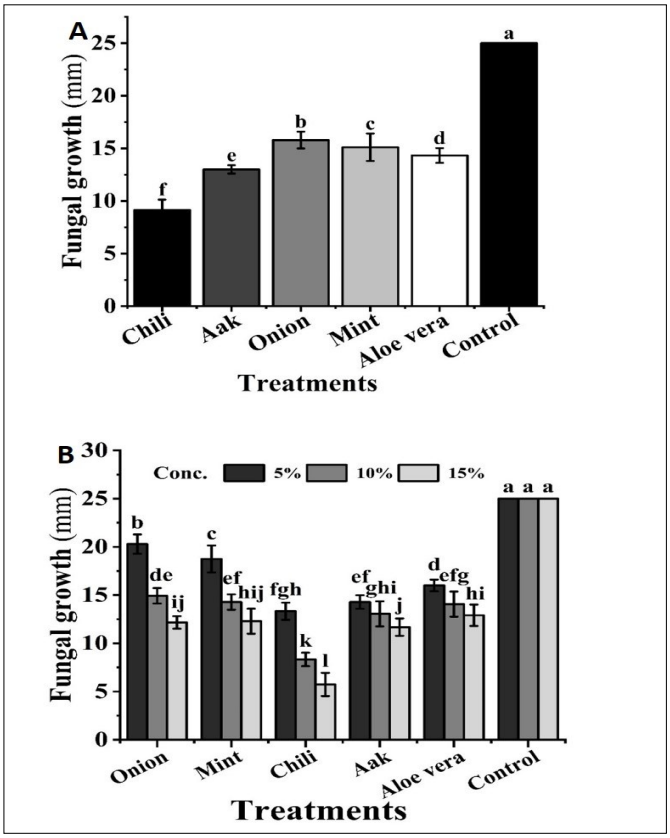


Figure 1. Impact of fungicides **(A)**, their interaction between treatment and days **(B)**, and treatments and concentrations **(C)** against mycelial growth of *Curvularia lunata*

In-vitro management of Curvularia lunata through different phyto-extracts

In accordance with chemical treatments, five plants namely *Allium cepa, Capsicum annuum, Aloe vera, Mentha* and *Calotropis gigantea* were used for the evaluation of their fungal activities. Different plant part like fruits, leaves and bulb were obtained for the preparation of the phyto-extract's stock solutions. All the treatments were evaluated with three concentrations (5%, 10% and 15%) by using "poisoned food technique" and data was recorded for four days with 24 hours of interval. Mean comparison among all the phyto-extracts indicated that Chili (*Capsicum annuum*) showed the highest mycelial growth retardation (9.129mm) followed by Aak (13.003mm), Aloe vera (14.322mm), Mint (15.104mm), and Onion (15.791mm), which was the least effective in controlling the fungus (Fig. 2A). Chili application exhibited the highest control at high concentration (5.730mm), while (8.330mm) and poor results (13.328mm) at medium and low concentrations, respectively (Fig. 2B). Fungal growth was showing an increasing trend (4.334mm, 7.101mm, 10.711mm and 14.370mm) on the respective days with 24 hours of time interval. On the other hand, Aak was least effective (14.280mm) at low concentration, medium effect was on moderate (13.057mm) concentration while most effective (11.673mm) at high concentration as compared to control (Fig. 2B). The interaction effect of treatment and days revealed that there was an increasing trend of fungal growth. As the days increased the growth rate also increased showing (8.048mm), (10.908mm), (14.511mm), and (18.547mm) values at regular time interval of 24 hours up to four days, respectively (Fig. 2C).



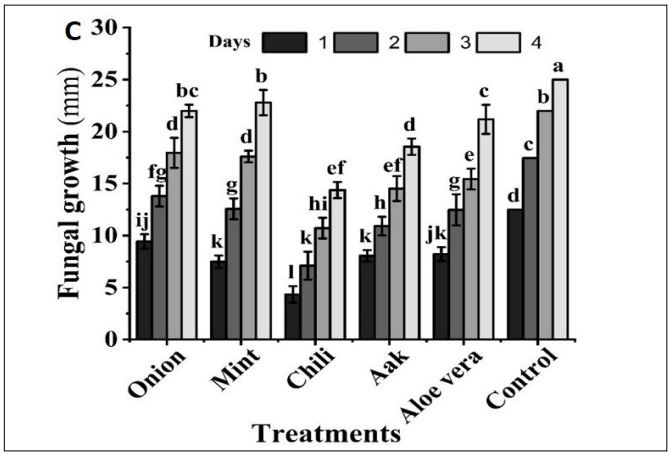


Figure 2. Impact of phytoextracts **(A)**, their interaction between treatments and days **(B)**, and treatments and concentrations **(C)** against mycelial growth of *Curvularia lunata*

In-vivo management of curvularia leaf spot of rose through the application of phyto-extracts and chemicals

The *In-vitro* experiments were performed against *C. lunata* to assess the individual effect of different chemical-based fungicides and phyto-extracts, which disclosed that all the treatments were effective against the isolated pathogen with different mode of action and ranges. No doubt, all the treatments were effective but two best performing fungicides (Polyram-DF, Curzate-M) and phyto-extracts (Chili, Aak) along with their combinations were applied to diseased plants to check their efficacy in terms of disease incidence (D.I) at different concentrations {(0.50%, 1.00%, 1.50%) and (5%, 10%, 15%)}, respectively. The diseased plants were sprayed with Chili, Aak, and their combination with each other, but the control was only sprayed with sterile distilled water. Output entries revealed that the individual effect of two treatments were lower as compared to the combination of treatments and control. The disease incidence was minimum with the combination of two treatments (14.517%), followed by Chili (18.814%) and Aak (24.860%) (Fig. 3A). Although, every treatment was best in controlling the disease at all dosages but the recorded disease incidence was minimum at their high level of concentrations. Combination of treatments were leading (10.214%), followed by Chili (16.140%), and Aak (20.197%) was less effective in that respect where combi-product were leading (Fig. 3B). The disease incidence was recorded up to three weeks, where the recorded disease incidence of combi-product was 11.473%, 14.491% and

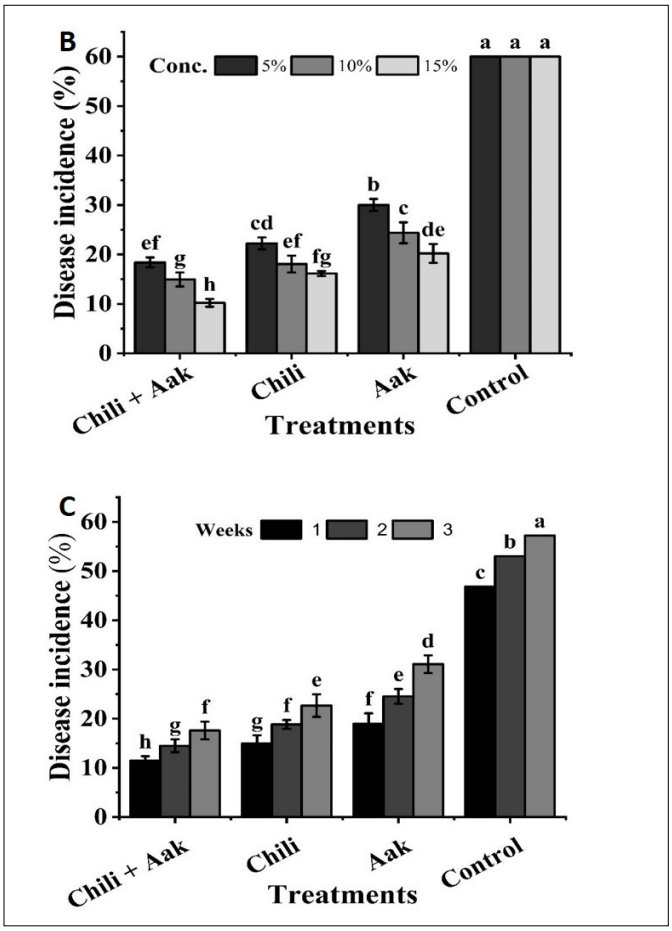
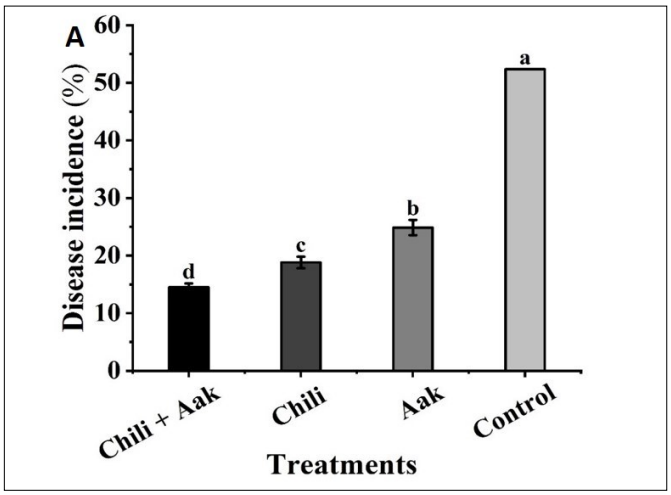
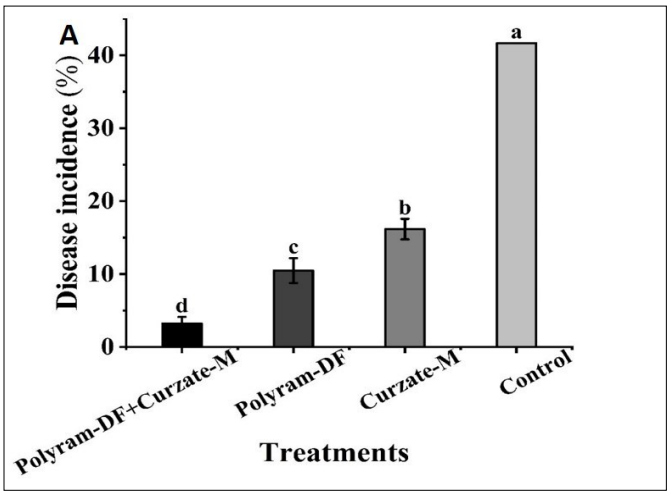


Figure 3. Impact of phytoextracts **(A)**, their interaction between treatment and days **(B)**, and treatments and concentrations **(C)** against Curvularia leaf spot of rose

17.586%, Chili (14.983%, 18.834% and 22.669%) and Aak (18.980%, 24.533% and 31.066), respectively (Fig. 3C). On the other hand, overall effect of chemical-based fungicides was 3.224% (Combination of treatments), 10.474% (Polyram-DF) and 16.163% (Curzate-M) (Fig. 4A), the interaction between fungicidal treatments and weeks showed that the least disease incidence was expressed by combination of Polyram-DF+Curzate-M (5.202%) at 3rd week (Fig. 4B), while the interaction between treatment and concentration showed minimum disease incidence percentage (1.868%)by application of Combination treatment at highest concentration (1.5%) (Fig. 4C). These results also indicated that they are more efficient over plant-extracts.





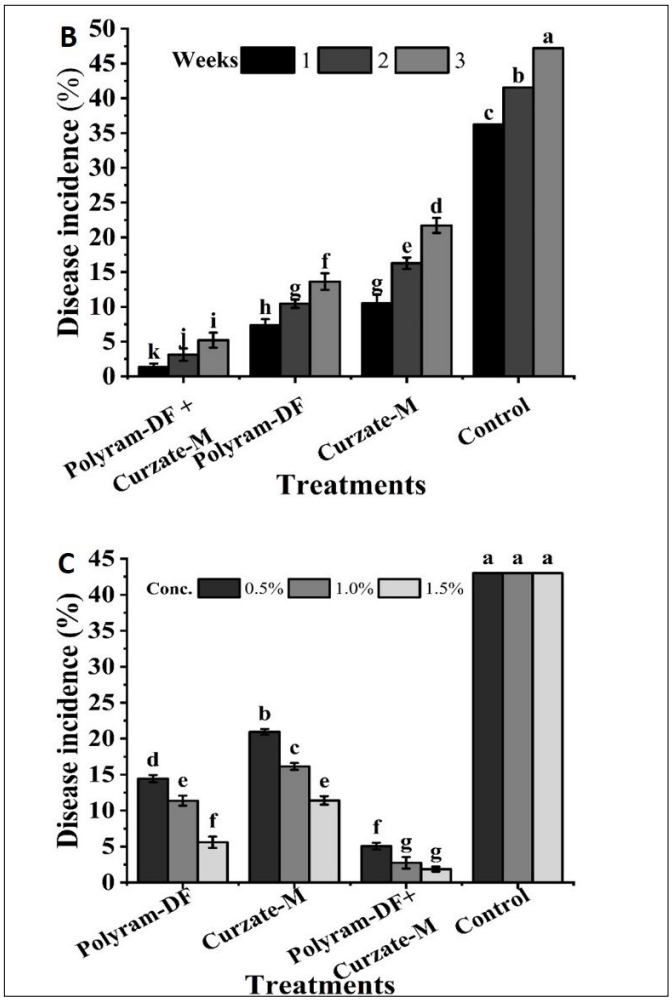


Figure 4. Impact of fungicides **(A)**, their interaction between treatments and days **(B)**, and treatments and concentrations **(C)** against Curvularia leaf spot of rose

Discussion

Roses are called as the king as well as queen of flowers which indicates that both majesty and beauty are its inherent qualities and hence used as symbol of love, peace, honor, war and as medicine. *Curvularia lunata,* causal agent of "Curvularia leaf spot of rose" is a serious threat to the rose production in Pakistan as well as worldwide. *Curvularia* is a widespread fungal pathogen which cause different types of diseases in different crops including roses. Under favorable environmental conditions, it contributed 15-80% losses in the per farm yield of rose (40). Feeling the gravity of the scenario, the current studies was designed to tackle this problem by using synthetic chemicals and phyto-extracts for the management of leaf spot disease of rose caused by curvularia.

Use of chemical formulations is a best way to control disease if appeared in epidemic form due to its quick action, easy availability and cost effective. The findings of this study demonstrate the significant variation in *C. lunata*'s mycelial growth in response to each fungicide. With the rise in each fungicide's concentration, there was a noticeable decrease in fungal growth. Five different fungicides completely inhibited the mycelial growth of *C. lunata* at their various tested concentrations, with the highest sensitivity being recorded for Polyram-DF and Curzate-M, while Recado and Cabrio-Top exhibited an intermediate, and Aliette showed the least effectiveness. Current findings are interconnected with Pei *et al*., who tested different fungicides *In-vitro* and reported that Prochloraz EW45 % and Mancozeb WP 80 % showed the best inhibitory activities (97.8% at 1.0 mg/L) against the *C. lunata*, while Thiophanate-Methyl 70% failed to affect the growth of this pathogen (41). However, *In-vitro* experiments of Abubakar and Likita,stated that Mancozeb, Mancozeb+ Carbendazim, and Imidacloprid + Metalaxyl-M + Tebuconazole were the best performing fungicides, which showed little or no mycelial growth during 2–14 days after inoculation (15). Similarly, Mancozeb 75%WP were 100% effective in controlling the fungal growth of *C. lunata* in the findings of Adaangadi *et al.*, (42). Bisht *et al.*, (43) reported that Carboxin and Mancozeb were recorded as best to control fungal growth with 25 ppm and 200 ppm, respectively. As well in recent studies, Curzate-M (Cymoksanil 8% + Mncozeb 64%) was the second top acting fungicide at all dosage. Further more, it's high dosage (150ppm) showed considerable results (6.542mm) which is much more effective and reliable. In contrary, Aliette 80% (Fostyl-Aluminum) was least effective in controlling the fungal growth of *C. lunata* which is supported by the experiments of Abrar Ul Hassan *et al*., (14), who stated that Metalaxyl+Mencozeb reduced fungal growth by 26–67% was the efficient fungicide over control while Thiophenate Methyl and Fosetyl-Al were ineffective against the pathogen.

Numerous plant species are said to possess antifungal qualities (44). Low phytotoxic, safe, eco-friendly, quickly biodegradable, and translucent natural substances can be found in plants (45) and seen as a replacement for plant pesticides. Therefore, five plant extracts (*Allium cepa, Capsicum annuum, Aloe vera, Mentha* and *Calotropis gigantean*) were assessed in the current study against "Curvularia leaf spot of Rose" under *In-vitro* and *In-vivo* conditions. Recent investigations disclosed that among all phyto-extracts, Chili showed the highest mycelial growth retardation (9.129mm) followed by Aak, Aloe vera, Mint, and Onion. Current results are supported by Abd-El-Khair *et al*., where nine Egyptian plant species including Chili fruits, Onion and Peppermint were evaluated, among all Chili extract showed significant suppressive effect against fungal pathogen *In-vitro* and least disease incidence under field conditions (46). Similarly, our study is in resemblance with findings of Al-Samarrai *et al*., where Chili was found effective with 100% inhibition of mycelial growth @ 3000ppm (47). Correspondingly, Pal *et al*., reported the efficacy of Aak against fungal pathogen (48). In present research, *Aloe vera* exhibited the intermediate results against *C. lunata* which is supported by the investigations of (49), where phyto-extracts of *Aloe vera*was found effective against different fungal pathogens.*In-vitro* response of *Allium cepa* demonstrated that it was the least effective against *C. lunata* at various concentrations (5, 10 and 15%) which is in agreement with findings of (45), who evaluated four plant-extracts at three concentrations (15, 25, and 50 %) and reported that *A. indica,* followed by *Cassia fistula, Allium sativa*, and *Allium cepa* was the most effective plant

extract against *Sclerotium rolfsii Sacc.* Similarly, comparative efficacy of Neem, *Calotropis*, Garlic, Turmeric, *Asafoetida* and *Datura* against *C. lunata* at different concentration levels were assessed. Garlic extract witnessed significant results with 100% inhibition at every concentration, Neem extract inhibited 100% at the concentration level of 15 and 20 percent, while *Calotropis* were least effective (21). Besides, in current studies, *Calotropis gigantea* was the second-best plant-extract, which performed well at all dosages (5, 10, and 15%). Likewise, *Aloe vera* exhibited intermediate results in controlling the fungal pathogen at low concentration (5%). As the concentration increased, the fungal growth of pathogen decreased, which is in agreement with the findings of Guohui *et al*., who reported that Aloesin and Aloe gel suppressed growth of *C. lunata* at different concentrations (50).

Moreover, *In-vivo* experiments revealed that application of Chili, Aak, Polyram-DF and Curzate-M were effective in controlling the disease incidence of "Curvularia Leaf spot of Rose" caused by *C. lunata* over control, (18.814%, 24.860%, 10.474% and 16.163%) respectively. Current experiment is supported by (45), where Systhane was the leading fungicide followed by Cymoxanil+Mancozeb and Matalaxyl+Mancozeb. On the other hand, *A. indica* and *Cassia fistula* showed greatest efficacy under field conditions.

Conclusion

Furthermore, in current experiments, combination of treatments was more effective in minimizing disease incidence caused by *C. lunata* as compared to their individual response. In conclusion, the output of the current investigations would suggest that the use of phyto-extract (Chili and Aak) and synthetic fungicides (Polyram-DF and Curzate-M) holds potential control against Curvularia leaf spot disease of rose. Further research should be done to explore the active ingredients in phytoextracts and their potential against the fungal pathogens to manage the disease in ecofriendly manner.

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Authors contributions

MHQ executed the field research and wrote manuscript, NA and MA conceived the idea and supervised the work, AR reviewed and edited the manuscript, GAK and HA Helped in statistical analysis of the data, whereas HZ, HMUS and HKR helped in conducting experiments and writing the manuscript.

Compliance with ethical standards

Conflict of interest: The Authors declare that there is no conflict of interest.

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