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In vitro and *in vivo* evaluation of different measures to control Ascochyta blight in chickpea

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Abstract

Ascochyta blight, an infection caused by *Ascochyta rabiei* is a destructive disease in many chickpea growing regions and it caused significant yield losses. To minimize the impact of *Ascochyta* blight, 5 fungicides viz., Aliette, Cabrio Top, Thiovit Jet, Cymoxanil and Difenoconazole, 5 plants extracts namely *Azadirachta indica*, *Azadirachta azedarach*, *Datura stramonium*, *Chenopodium album* and *Allium sativum* L. and 2 strains T-22 and E58 of bio-control agents (BCAs) *Trichoderma viride* and *Aspergillus flavus* were evaluated on the growth of *A. rabiei* under *in vitro* conditions by using the food poison technique. The colony growth of *Ascochyta rabiei* was inhibited at all concentrations of fungicides @ 0.07, 0.15, 0.21%, plants extracts @ 4, 6 and9% and bio-control agents @ 10^5, 10^6 and 10^7 conidia ml^{-1} respectively. Among all applied treatments, maximum inhibition colony growth of pathogen was recorded in the case of Aliette (83.4%), followed by Cabrio Top (74.3%), *Azadirachta indica* (50.3%) and *Trichoderma viride* (60.3%) at their high concentrations. Field trials showed that Aliette and Cabario Top significantly reduced the disease severity to 10 % and 24% respectively, followed by *Azadirachta indica* and *Allium sativum* which reduced the disease severity to 40% and 50% respectively. Bio-control agent *Trichoderma viride* proved less effective in controlling Ascochyta blight severity under field conditions. The present study showed that systemic and sulphur containing fungicides, plant extracts and BCAs have the potential to control Ascochyta blight in both *in vitro* and *in vivo* conditions.

Keywords

Ascochyta blight, biocontrol, chickpea, fungicides, Plant extracts

Introduction

Chickpea (*Cicer arietinum* L.) is a highly nitrogen-fixing, climate-resilient, and nutritious crop, capable to provide significant economic benefits and nutrients for increasing world populations (1). This crop is an important source of fibers, vitamins, minerals (especially zinc, iron, copper, calcium, phosphorus and potassium), proteins (especially arginine, leucine and lysine) and energy (2). Despite these advantages, the crop is influenced by many biotic and abiotic factors *viz.,* drought, cold and diseases (3).

The registry of plant diseases controlled by the Fuentesauco-

Chickpea ("Garbanzo de Fuentesauco" in Spain) from 2003 to 2020 exhibited that, according to the reports released by the Regional Diagnostic Centre in the Regional Government of Castilla Leon (Spanish), in more than 80% cases, the pathogen found is the *Ascochyta rabiei.* The pathogen causes the Ascochyta blight. As the pathogen is soil and seed-borne in nature, it can survive up to 13 years in infected seed and 4 years in the soil (4) and can infect all aboveground parts of the plants (5). The lesions, pod infections, tissue death, and stem breakage along with the collapse and girdling of twigs are the main symptoms of the disease. The disease occurs in severe epidemics, in regions where humid conditions and cool temperatures prevail for a longer period (6).

The main control strategy for the effective management of pathogen is based on the application of fungicides, but the massive use of chemicals is uneconomical where chickpea production is low (7, 8). The use of many fungicides *viz.,* captan, thiabendazole, propiconazole, penconazole, zineb, maneb, chlorothalonil and antracol has been reported all over the world to avoid the secondary inoculum of *Ascochyta rabiei* (7). Recently, applications of many plant extracts such as *Tagetes erectus*, *Magnolia grandiflora* and *Aloe vera* have been tested and proved most effective against several fungal pathogens (9, 10). Plant extracts have secondary metabolites which contain high antimicrobial activity (11). Similarly, many bio-control agents (BCAs) such as *Acremonium implicatum*, *Trichoderma viride* and *Chaetomium globosum* have been reported for the effective control of Ascochyta blight (12, 13).

The BCAs are widely used under field conditions due to their naturally growing ability in different climatic regions and diverse mechanisms of action (14). Some species such as *Trichoderma harzianum* are capable of mycoparasitic on the *Rhizoctonia solani* under natural environmental conditions. The fungi compete for the nutrients and space in the root zone of plants and also release the chemical compounds which reduce the pathogenic growth of *Rhizoctonia solani,* a phenomenon known as antibiosis (15). The *Trichoderma* species enhance the plant tolerance against biotic stress i.e. salinity and drought and promote the crop production and plant growth (16). In addition, BCAs root colonization includes the capability to adhere to and penetrate the roots and withstand the toxic compounds released by the plants in response to invasions (17). The main objective of the present study was to evaluate the effects of different fungicides, plant extracts and BCAs against *A. rabiei* causing Ascochyta blight in chickpea under field conditions.

Materials and Methods

First trials were conducted under lab conditions to evaluate the fungicides, BCAs and plant extracts with their best concentrations.

Isolation and identification of Ascochyta rabiei

The *Ascostya rabiei* infected pods of the genotype CM-2000 were collected from the Field Area of Plant Pathology Research Institute, Ayub Agriculture Research Institute (AARI) Faisalabad and placed in the refrigerator at 4-7 °C temperature for the isolation and purification of the pathogen. A chickpea seed meal agar media (CSMA) containing 20 g^{-1} of each glucose, agar and chickpea seed meal, was used for isolation of *A. rabiei* (18). The infected pods were heated on the flame burner with the help of forceps in such a way that only the outer surface could be sterilized and an inner surface of the pods remains unaffected. The infected seeds were brought out from the sterilized pods and placed (5 seeds) in each petri-plate containing autoclave chickpea seed meal agar media. The petri-plates were incubated at 20 ± 2 °C for two weeks (18). When pathogen colonies formed on the CSMA media, they were isolated and pure culture was made by using the single spore culture technique (19).

In vitro evaluation of chemicals, plant extracts and bio-control agents against A. rabiei

Five fungicides *viz.,* Aliette (Fosetyl Al 80% WP), Cabrio Top (Metiram 55% + Pyraclostrobin 5% WG), Thiovit Jet (800 g/kg Sulphur), Cymoxanil 8% (Methoxyimino) and Difenoconazole (1H-1,2,4-Triazole) and five plant extracts namely neem (*Azadirachta indica*), Bakayan (*Melia azedarach*), Datura (*Datura stramonium*), White goosefoot (*Chenopodium album*) and Garlic (*Allium sativum* L.) at 0.07, 0.15, 0.21% and 4, 6, 9% concentrations respectively, were used for colony growth inhibition of *Ascochyta rabiei* by using food poison technique (20). For the preparation of required concentrations of fungicides, 70, 120 and 170 mg amount from each fungicide was weighed and mixed in 100 ml purified water. To make the plant extracts concentrations, cloves/leaves of all plants were collected and grinded in the electric grinder after surface sterilized with the 1% sodium hypochlorite solution. The grinded material was soaked in the distilled water to obtain the 25% W/V concentrations of plant extracts. Then, a concentrated aqueous solution was filtered through the filter papers and muslin cloth. The solutions were stored at room temperature (4 °C) and used within 3 -5 days to ensure the antifungal potential. The required doses of plant extracts were made in sterilized water (21). Following that, petri-plates were prepared by soaking the plant extracts and fungicides in the chickpea seed meal agar (CSMA) media in the laminar airflow chamber to ensure the aseptic conditions (22). The control petri-plates were placed with only CSMA media without the application of any fungicides or plant extracts. The disks of 6-8 mm isolated *A. rabiei* culture were taken and punched inside the center of each control petri-plates and those containing plants and fungicides extracts. The plates were incubated at 20 ± 2 °C temperature for 7 days until complete fungal appeared in the control plates. In this study, Completely Randomized Design (CRD) design was used and each treatment was replicated thrice. The mycelium growth inhibition percentage was observed by using the following method (23).

$$Inhibition\ (\%) = \frac{\text{Growth in control} - \text{Growth in fungul treatment}}{\text{Growth in Control}} \times 100$$

For *in-vitro* bioassay of bio-control agents, two strains T-22

and E58 of BCAs *T. viride* and *A. flavus* were used against *Ascochyta rabiei* respectively. Strain T-22 was purchased from the Department of Biochemistry, the University of Agriculture Faisalabad and strain E58 was purchased from the Fungal Culture Bank of the University of Punjab-Lahore, Pakistan. The spore concentrations of these BCAs were made @ 10^5, 10^6 and 10^7 conidia ml^{-1} by using the hemocytometer, and their efficacy was determined through a dual culture assay (24). The BCAs strains T-22 and E58 were inoculated on the potato dextrose agar and then incubated at 24±2 °C temperature. To perform the dual culture assay, 7 mm desks of *A. rabiei* culture was taken and placed on the one side of petri-plates and the other side of plates required concentration of BCAs were put with a sterilized syringe. The plates were incubated at 20 ± 2 °C for 14 days until full growth of the pathogen was recorded in the control. In the current study, completely randomized design (CRD) was used with three replications of each treatment. The % growth inhibition of *A. rabiei* was calculated by using the standard formula (25).

Preparation of mass culture of Ascochyta rabiei

The chickpea seeds were surface sterilized by consecutive shaking in the 5% sodium hypochlorite and 70% ethanol solutions for 8 min each and then washed three times in the distilled water. These seeds at 500 gm bag^{-1} were transferred to the polythene bags and sterilized by the autoclave at 121 °C and 138 KPa for 15 min twice in 24 hours and inoculated with 4-5, 8 mm agar plugs from the 14 days old culture of *A. rabiei* having maximum sporulation of 1 ×10^6 conidia/ml. To avoid bacterial contamination, streptomycin at 50 mg was mixed with these seeds. The culture was incubated for two weeks at 20±2 °C for further sporulation of *A. rabiei* (26).

In vivo evaluation of chemicals, plant extracts and bio-control agents against A. rabiei

The trials were located in the Research Area of Plant Pathology, Ayub Agriculture Research Institute Faisalabad and were carried out from 2020 to 2021. During both seasons, the chickpea varieties, CM-2000 and Pb-1, which are highly susceptible to *A. rabiei* infection were planted in the small plots of 4.9 m x 1.8 m following Randomized Complete Block Design (RCBD) with three replications. In each block, there were 8 rows of each variety, 7 for treatments and one as a control. The inoculum of *A. rabiei* was applied to all plots with a concentration of 5 × 10^5 spores/ml to increase disease epidemics on all plots. The eight treatments including three fungicides as T_1 = Aliette (0.21%), T_2= Cabario Top (0.21%), T_3= Thiovit Jet (0.21%); three plant extracts as T_4= *Azadirachta indica* (9%), T_5= *Allium sativum* (9%), T_6 = *Datura stramonium* L. (9%); one BCA as T_7 = *Trichoderma viride* (10^7 conidia/ml^{-1}) and one control (distilled water) were applied on plots after every seven days interval (27). The inoculated plots resulted from different levels of disease severity due to different efficacy of the fungicides, plant extracts and bio-control agents. The disease severity index (DSI) % was calculated at crop maturity for 10 plants per replicates by using the standard methods (28).

$$DSI = \frac{Sum\ of\ all\ rating}{Number\ of\ plants\ examined} \times \frac{100}{Maximum\ disease\ rating}$$

Data for disease severity was recorded based on disease symptoms using the disease rating scale 1-10, where 1 represents plants with small lesions or no infections and 10 indicates the plants that had completely died (29).

Statistical analysis

The data were subjected to analysis of variance (ANOVA) at a 5% level of significance using software Minitab ver.17. Fisher's Least Significant Difference (LSD) test was used for statistical comparison among treatments (30).

Results and Discussion

The *in vitro* evaluation of fungicides at concentrations *viz.,* 0.07%, 0.15% and 0.21% against *A. rabiei* reported a significant reduction in the pathogen colony growth as compared to control (0.02%). In the case of Aliette, 83.4% pathogen inhibition colony growth was recorded. The cabrio Top inhibited the colony growth to 74.3% at 0.21%, 72.3% at 0.15% and 70.2% at 0.07%, respectively. Difenoconazole proved significantly less effective to inhibit the colony growth of pathogen at all concentrations as compared to other treatments (Fig. 1). Regarding the evaluation of plant extracts, *A. indica* and *M. azedarach* proved most effective

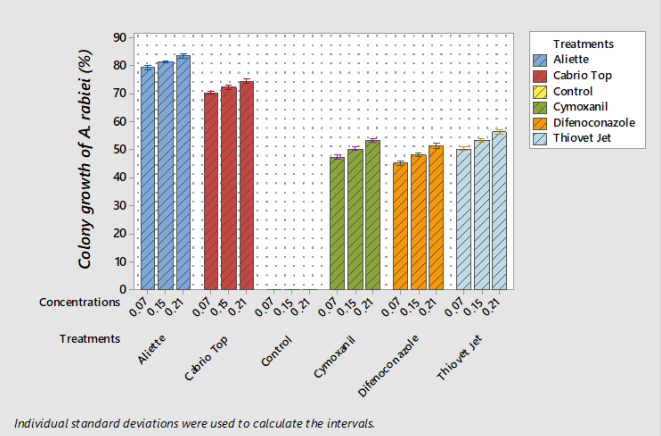

Fig. 1. Evaluation of different concentrations of five fungicides against colony growth of *A. rabiei.*

against colony growth of *A. rabiei* as compared to other plant extracts treatments. *A. indica* at concentrations 4%, 6% and 9% inhibited colony of pathogen to 40.2%, 45.2% and 50.3% respectively. Similarly, *M. azedarach* inhibited the colony growth of the pathogen to 34.2% and this inhibition was significantly less with the application of *D. stramonium* (22.2%), *C. album* (20.4%) and *A. sativum* L. (16.3%) at their maximum concentration 9% as compared to control (Fig. 2).

The efficacy of BCAs against *A. rabiei* showed significantly more inhibition of the colony growth of the pathogen at higher spore concentration as compared to low concentrations. *T. viride* and *A. flavus* at maximum spore concentration 10^7 conidia ml^{-1} showed significantly maximum inhibition in the colony growth of the pathogen to 60.3% and 40%, respectively. The BCA *A. implicatum* at spore concentrations 10^5, 10^6, 10^7 conidia ml^{-1} indicated an

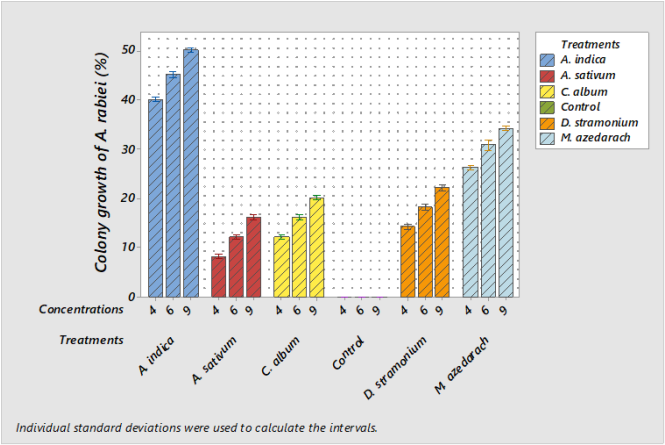

Fig. 2. Evaluation of different concentrations of five plant extracts against colony growth of *A. rabiei.*

8%, 21% and 30% reduction in the colony growth of the pathogen over control (Fig. 3).

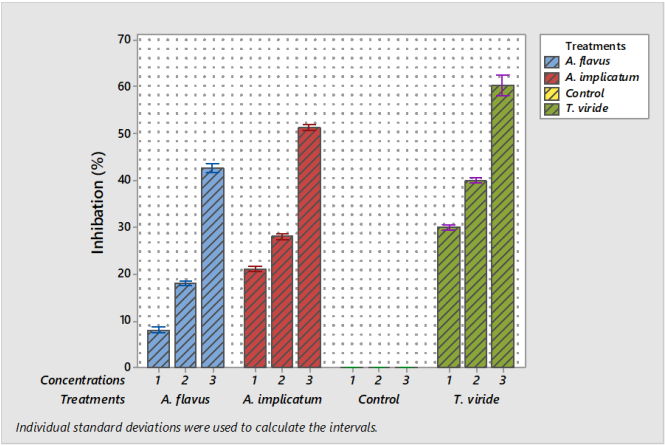

Fig. 3. Evaluation of different concentrations of two bio-control agents against colony growth of *A. rabiei.*

In vivo evaluation of all applied treatments showed a significant reduction in diseases severity such as Aliette (18%), Cabrio (23.4%) and Thiovit$_{Jet}$ (24.5%) followed by plant extracts and biological control agents (Table 1). A

Table 1. Field evaluation of fungicides, plant extracts, and biocontrol agents for controlling chickpea blight disease severity (%)

	Treatments	Mean value of chickpea blight disease severity (%)
T_1	Aliette @ 0.21%	18.7±0.438^{g}
T_2	Cabario@ 0.21%	23.4±1.13^{f}
T_3	Thiovit$_{Jet}$ @0.21%	24.5±1.34^{f}
T_4	*A. indica A. Juss.*@ 9%	52.4±0.643^{e}
T_5	*Allium sativum* L. @9%	55.5±0.693^{d}
T_6	*Datura stramonium* L@.9%	58.7±0.695^{c}
T_7	*T. veridi* @ 1x 10^7	61.5±0.698^{b}
T_8	Control	68.2±0.353^{a}
L.S.D.		1

*Means with similar letters are not significantly different at $P \leq 0.05$

three-way interaction demonstrated that when the number of sprays increased, disease severity decreased (Table 2). Maximum reduction in chickpea blight disease severity was recorded with the application of the first, second and third spray of fungicides on both chickpea varieties such as CM-2000 and Pb-1. Whereas, the effects of both bio-control agents and plant extracts in controlling chickpea blight were statistically not as par in both varieties (Table 2). Fungicides proved most effective in reducing disease severity on both varieties followed by plant extracts as compared to control. *T. viride* statistically proved less effective to control chickpea blight disease severity under field conditions (Table 2).

Table 2. Interaction effects of treatments, years and sprays of fungicides, plant extracts and biocontrol agents to control chickpea blight disease under field conditions

Treatments	CM-2000			Pb-1		
	Spray1	Spray2	Spray3	Spray1	Spray2	Spray3
Aliette at 0.21%	28±1.15^{h}	23±1.14^{h}	13±0.88^{g}	23±.15^{h}	15±1.11^{h}	10±1.13^{h}
Cabario Top at 0.21%	36±1.15^{g}	38±1.13^{g}	21±1.15^{f}	38±1.13^{g}	32±1.34^{g}	24±1.15^{g}
Thiovit Jet@at 0.21%	41±0.66^{f}	31±1.76^{f}	29±6.23^{e}	45±1.76^{f}	40±1.78^{f}	34±1.14^{f}
A. indica at 9%	58±1.15^{e}	50±.667^{e}	41±1.15^{d}	54±1.15^{e}	46±0.67^{e}	40±1.13e
Allium sativum at 9%	64±1.15^{d}	54±1.15^{d}	49±3.53^{c}	62±1.34^{d}	57±1.22^{d}	50±1.11^{d}
Datura stramonium at.9%	64±1.14^{c}	58±0.67^{c}	50±1.15^{c}	70±1.36^{c}	64±3.45^{c}	56±1.17^{c}
T. veridi at 1x 10^7	68±1.15^{b}	62±1.14^{b}	54±1.13^{b}	76±1.14^{b}	66±1.15^{b}	60±0.00^{b}
Control	78±1.14^{a}	74±1.13^{a}	68±1.12^{a}	82±1.12^{a}	74±1.36^{a}	68±1.15^{a}
L.S.D	5	5	3	5	4	5
L.S.D		4			5	

*Means with similar letters in a row are not significantly different at $P \leq 0.05$.

Through *in vitro* and *in vivo* confrontation of the fungicides, plant extracts and BCAs against *A. rabiei*, we were able to prove that these were able to inhibit the growth of pathogens due to different mechanisms of action that can act in antibiosis, mycoparasitism or competition for nutrients and space (31). Significant control of disease severity by Alitte under field conditions is due to the systemic nature that allows it to destroy the pathogen in established infection (18**,** 29**,** 32, 33). During the present investigation, maximum disease severity was controlled due to three foliar applications of Alitte. Thiovet Jet also provided the successful control of disease severity, and this attributed to its good movement ability into newly developed tissues of the host and multiple site mode of action (34).

Disease control by plant extract i.e. *A. indica* was due to its ability to induce systemic acquired resistance in chickpea varieties against *A. rabiei* (35). Plant extracts have many antifungal compounds which inhibit the growth of pathogens effectively. It was reported that *M. azedarach* contained many compounds such as *B*-amyrin, *B*-sitosterol, 3, 5 dimethoxybenzoic acid, maesol, ursolic acid and bezoic acid which were highly toxic to *A. rabiei* (10). Similarly, several compounds have been isolated from *A. indica* such as limonin, limonoids, nomilin and

obacunone which inhibit the mycelium growth of fungi (36). Secondary metabolites have been also isolated from plant extracts that proved most effective against fungi and insects (37).

It was reported that *T. viride* was capable of parasitizing the plant pathogenic fungi *viz., A. rabiei* and producing specific secondary metabolites that were involved in suppressing the growth of the pathogen (38). Some BCAs i.e. *T. harzianum*, *T. koningii* and *T. atroviridi* have been already evaluated against many fungal pathogens. In the specific case of *A. rabiei,* this study is the first to describe how *T. viride* acts as an effective BCA under field conditions. However, only one study has been reported that demonstrate the effectiveness of *T. hamatum* against lentil wilt pathogen i.e., *F. oxysporum* f. sp. *lentis* under field conditions (39).

Diseases controlled by BCA such as *T. viride* have been widely studied due to their rapid growth in the rhizosphere, due to its production of secondary metabolites *viz.,* atrichodermones A-C or its mycoparasitism ability for example on the *Colletotrichum lindemuthianum*, Sclerotium cepivorum, *B. cinerea* and *rhizoctonia solani* (40). In the case of foliar applications, BCAs reduced the disease severity caused by the blight pathogen at the affectation of plant vascular bundles, stress and vitality as well as at biomass level and that was related to the lower level of plant tissue colonization by a pathogen (40). Moreover, activation of plant defense systemic response associated with the application *T. viride* had also been observed in many previous studies (40, 41).

Conclusion

This study concludes that Ascochyta blight severity in susceptible varieties increased progressively from seedling to maturity. A successful management approach should therefore target to prevent the Ascoshyta blight in the field or control disease immediately after infection regardless of the growth stage of crop. Application of systemic and sulphur containing fungicides such as Aliette, Cabario Top and Thiovit Jet proved the best choices for preventing the Ascochyta blight of chickpea under both in *vitro* and *in vivo* conditions. Additionally, plant extracts i.e. *M. azedarach, A. indica* and bio-control agent *viz., T. viride* were also associated with the mild reduction in disease through activation of plant defense responses against *A. rabie*.

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Authors contributions

All the authors jointly conceptualized the experimental strategy and financial support. They supported in field data collection, laboratory works, compilation, analyses, manuscript writing, submission, correction etc.

Compliance with ethical standards

Conflict of interest: The author(s) declare(s) that there is no conflict of interests regarding the publication of this article.

Ethical issues: None

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