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Research Article

Integrated Management of Ascochyta Blight on Chickpea Germplasm in Pakistan

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ABSTRACT
Chickpea, an important pulses product of Pakistan, ranks 3rd among legumes in the world. The most important fungal disease agent of chickpea *Ascochyta rabiei* is anthracnose, which causes 50 - 70% yield losses in chickpeas. In this study, 10 chickpea genotypes inoculated with *A. rabiei* were screened. Under artificial inoculum pressure, agronomic and physiological data were recorded. To manage this disease, the commercial preparation of the biocontrol antagonist *Trichoderma harzianum* and some fungicides were then applied to these genotypes. The resistivity of pathogen spores to *T. harzianum* antagonist and fungicides was tested in vitro. While the chemical fungicides performed equal inhibition with the 1st and 2nd levels, the 3rd and 4th levels of inhibition differed from each other. The biological antagonist commercial *T. harzianum* was found to be effective in anthracnose disease. control.

Keywords: Antagonist**,** *Ascochyta rabiei,* Chemical management, Chickpea Biomass, Chickpea screening, cell membrane stability.

Pakistan'da Nohut Germplazmında Ascochyta Blight'ın Entegre Mücadelesi

ÖZ
Pakistan'nın önemli bir bakliyat ürünü olan nohut, dünyada baklagiller arasında 3. Sırada yer almaktadır. Nohut'un en önemli fungal hastalık etmeni *Ascochyta rabiei* nohutta %50 - 70 arasında verim kayıplarına neden olan antraknozdur. Bu çalışmada *A. rabiei* ile inoküle edilmiş 10 nohut genotipinde tarama yapılmıştır. Yapay inokulum ile agronomik ve fizyolojik veriler elde edilmiştir. Bu hastalıkla mücadele için, daha sonra bu genotiplere biyokontrol antagonist *Trichoderma harzianum* ticari preparatı ve bazı fungisitler uygulanmıştır. *İn vitro* koşullarda patojen sporlarının *T. harzianum* antagonisti ve fungistlere karşı dirençleri test edilmiştir. Kimyasal fungisitler 1. ve 2. seviye ile eşit inhibisyon gerçekleştirirken 3. ve 4. seviye inhibisyonda biribirinden farklılık göstermiştir. Biyolojik antagonist ticari *T. harzianum* preparatı hastalık kontrolünde etkili bulunmuştur.

Anahtar Kelimeler: Antagonist, *Ascochyta rabiei,* Hücre zarı dayanıklılığı, Kimyasal mücadele, Nohut biyokütlesi

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Introduction

Chickpea (*Cicer arietinum* L.) is a self-pollinated field crop that belongs to the family *fabeace*. It is a diploid crop with 12 chromosomes. It is an important legume crop, rich in protein, and an important part of a diet for the vegetarian population of the world. It can grow on a large agro-ecological range of climate that's why grown in more than 50 countries of the world. (FAOSTAT, 2017; Tadesse et al., 2017). It can grow in fertile to non-fertile soil ranges. Chickpea is attacked by Ascochyta blight disease which is caused by *Ascochyta rabiei* and is considered as a major limiting factor of yield worldwide (Jamil et al. 2010). The disease can cause 40-70% losses if optimum conditions last for 48 hours. (Malik and Bashir, 1984). Due to epiphytotic occurrence, it causes complete crop loss. (Pande et al., 2005). Symptoms appear on the stem, leave, and pods of the plant which are concentric lesions orange to brown. Blight can be managed by several means like the use of resistant varieties, cultural practices include removal of diseased plants from the field avoiding cultivation in the already infected field, and crop rotation. (Lubian et al., 2019). In Pakistan major part of south Punjab, consist of a desert range where agriculture is based on chickpea cultivation so crop rotation is not applicable in this region. (GOP, 2017). Different biocontrol agents and plant extracts are used to manage the disease. (Hernandez-Terrones et al., 2007; Khajista et al., 2011). Chemical fungicides are also used to control blight disease in chickpeas. (Pande et al., 2005). Biocontrol agents like *T. viride, C. globosum,* and *A. implicatum* were reported effective against *Ascochyta rabiei* under *In-vitro* conditions. (Bisen et al., 2020). Shafique et al. (2011) evaluated the fungi toxic potential of *Tagetes erectus* L. against *A. rabiei* the cause of gram blight disease. At various concentrations pathogen exposed (1, 2, 3, and 4% w/v) of aqueous and methanol extracts of shoot and flower of *T. erectus* using food poisoning technique. Concentrations of both shoot and flower extracts significantly suppressed the growth target pathogen. Reduction of colony diameter was 4-35% and 55-73% of *A. rabiei* due to different concentrations of flower and shoot extracts of *T. erectus* and 12-50% and 4-42% due to different compositions of the methanolic shoot and flower extracts of *T. erectus* respectively.

In this research, *A. rabiei* was isolated screened on different cultivars along with a management strategy using different chemical and biocontrol agents.

Methodology

Isolation and purification of the fungal pathogen

The pathogen usually infects seedlings and is soil and trash borne in nature. Infected plant tissues like stem, leaves, and pods were cut into 2cm pieces and then sterilized in a 2% aqueous solution of hydrogen peroxide. The samples were placed on media which were incubated for 20 days at 25°C in the incubator. (Walter, 2009). Purification of the pathogen was done by transferring mycelium using the hyphal tip method and was identified morphologically by using available literature especially based on plate colour, colony pattern, presence of conidia, spore shape, size, and structure by slides preparation for microscopy (Keogh et al., 1980; Barnett and Hunter, 1972).

1. Collection and sowing of germplasm

Chickpea was sown in sick plots (fungal inoculum were given in soil and covered the soil with polythene sheet for 48 hours) present in the experimental area of the Department of the Plant Pathology University of Agriculture Faisalabad. Seeds of 10 genotypes were sown in plots in four replications. Germination percentage was recorded in plots. *A. rabiei* was grown on chickpea media. After 21 days, spores were harvested by adding chilled water to the Petri plate followed by sieving through four layers of sterile muslin cloth. The spore of *A. rabiei* (Figure 1) was counted under the light microscope by using a hemocytometer. The inoculum concentration was adjusted to 10^4 spores/ml. Artificial inoculum of 10^4 spores/ml was given near the roots and sprayed on plants in such a way that it is disturbed equally and similar treatment for

all plants. Different parameters in the controlled and inoculated plants were recorded like seed germination, germination percentage of seed, number to flowering, no of pods per variety flowering colour, and seed weight.

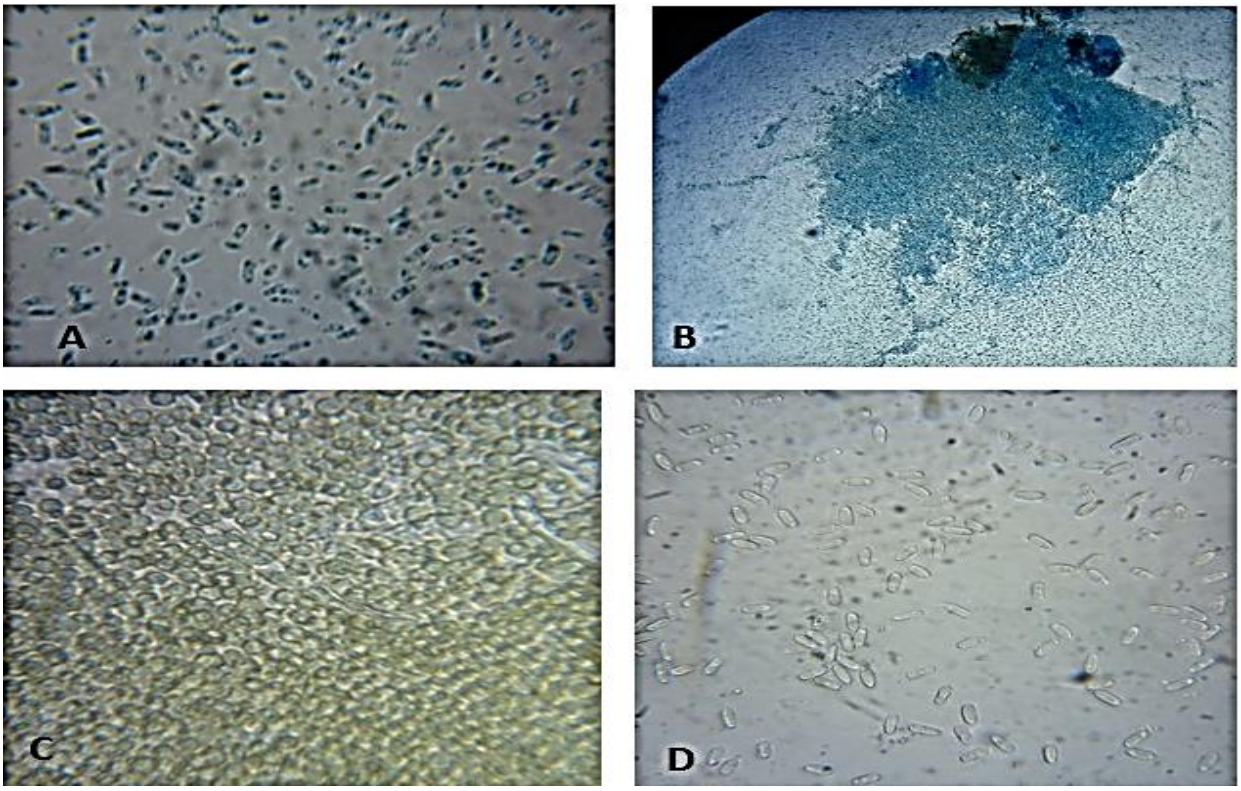

Figure 1: Shows (a) *A. rabiei* (b) fruiting body of *A. rabiei* (c) *Trichoderma harzianum* (d) *Beauvaria bassiana* isolate based on visual testing and microscopy.

2. Application of biocontrol agents

After one week of inoculation of the pathogen in plots biocontrol agents which are *B. bassiana* and *T. harzianum* was applied. They were grown on PDA media at 25± 2ºC for 2 weeks, 10 ml of chilled double distilled water was added to the Petri plate containing cultures and scratch with needle gently and then filter with 4 folding of muslin cloth. Then these spore suspensions were applied to plants in such a way that *T. harzianum* + *B. bassiana* were applied to the first replication. *T. harzianum* was applied to the 2nd replication and *B. bassiana* was applied to the 3rd replication and 4th replication was kept as control.

3. Physiological parameters

After inoculation of biocontrol agents, different physiological parameters and biomass (Excised leaf water loss, Relative water contents, Relative dry weight of leaf, Cell membrane stability, Root/shoot ratio, Grain yield) of an individual plant. Such as Ali et al. (2011).

Excised leaf water loss

Chickpea plants were harvested, plants were washed under running tap water to remove soil from roots. Labelled plants according to their variety and treatment and weight each plant. This was the fresh weight of chickpea plants. Place plants into a hot dry oven for 6 hours at 28ºC. After that weigh plants one by one and again place them in a hot dry oven for 24 hours at 70ºC. Weighted the dried plants, after collecting all readings by using the formula given below find out excised leaf water loss. It was done according to the method followed by Ali et al. (2009b).

ELWL = [(Fresh weight -Weight after 6 h)/ (Fresh weight - Dry weight)] ×100

Relative water content

Chickpea plants were harvested, plants were washed under running tap water to remove soil from roots. Labelled plants according to their variety and treatment and weight each plant. This was the fresh weight of chickpea plants. Place plants into a tub containing water for 24 hours in the dark so that plants get strongly turgid. Weighted plant and again place in a hot dry oven for 48 hours at 78ºC. Again weighted the dried plants, after collecting all readings by using the formula given below to find out the relative water content.

RWC (%) = (Fresh weight – Dry weight)/ (Turgor weight – Dry weight) × 100

Cell membrane stability

Take fresh leaflets from the chickpea plants of each treatment. Wash each leaflet with deionized double distilled water (D3water) and place in a test tube containing 20 ml of D3 water and take a reading of sample by EC meter (electron conductivity) and place in an autoclave at 121ºC and 15psi pressure after this take the reading again with EC meter than using formula and calculation find out Cell membrane stability.

CMS (%) = [(1-(L1/L2)] × 100

The relative dry weight of leaf

This was calculated by taking the weight of ELWL dry weight and turgid weight and dry weight from relative water content. The relative dry weight of the leaf finds out by putting value in the formula given below. Ali et al. (2009b).

RDW = Dry weight / (Turgor weight - Dry weight)

Disease assessment and disease rating scale

As the crop was grown under natural inoculum pressure, the crop plants were observed for assessment of the disease. At various growth stages like flowering and pod formation stages, disease development was monitored and recorded. A disease rating scale was adapted as was already reported in the literature. This disease usually appears at flowering and pods stages. It was monitored at both stages to collect valid information as reported (Farooq et *al.,* 2005). Disease grading was done in the field and micro plots.

The disease was recorded visually and rated by using the following scale given by

- Highly Resistant = Less than 1% of plant wilted.
- Resistant = 1-10% of plants wilted.
- Moderately Resistant = 11-20% of plants wilted.
- Susceptible = 21-50% of plants wilted.
- Highly Susceptible = 51% or more of plants wilted. (Iqbal *et al.,* 2005)

Disease severity:

Disease severity was calculated by the following formula given by (Mehrotra and Aggarwal, 2003).

$$\text{Disease Severity} = \frac{Area\ of\ plant\ tissue\ infected}{Total\ area\ (farm)}$$

$$\text{Disease Incidence \%} = \frac{Diseased\ plants}{total\ no\ of\ plants} * 100$$

4. *In-vitro* management of *A. rabiei* pathogen of chickpea:

In-vitro management of *A. rabiei* was done by using various chemicals. For this purpose, commercially available chemicals were used in different concentrations. In this experiment, 12 chemicals were tested against single isolates of *A. rabiei.* Seven concentrations were made with 3 replications each in 96 well plates. Spores of *A. rabiei* were harvested from a pure culture grown on chickpea media in the Petri plate. For *in-vitro* testing 96 well plates were used. Liquid media @ 100µl was mixed with 50µl of spore suspension @ 10^4 spores in this volume. A liquid suspension of fungicides @ 50µl was added to each well according to the concentration of the plate. Seven different levels (using serial dilution) of fungicides were used in this experiment. Instead of chemicals, water was used in the positive control. In the second control spores and distilled water of the same volume were used as control. A total volume of 200 µl in each well was maintained. The chemical-treated spores containing plates were kept at the same temperature for incubation. After 24 hours the growth of spores and their germination was measured based on optical density at the

wavelength of 630 nm by using a spectrophotometer at the department of Center of Agriculture Biotechnology and Biochemistry (Abbas et al., 2013).

Results and discussion

Chickpea plants were artificially infected with *A. rabiei* and then after the appearance of symptoms plants were treated with biocontrol agents. All the physiological and biomass data were collected and analyzed by using statistical tools with a 0.05 level of significance. The ANOVA table below represents the variance of root length, shoot length, number of pods per plant, and weight of grain from one plant. Along with treatment, ten different varieties were used therefore, the experiment contain two factors which were treatments with varieties. Table 1 represents that root length, shoot length number of pods per plant, and weight of grain from one plant gives a higher level of significance with treatment and varieties individually but in case of interaction all were non-significant except root length which favours the experiment as *A. rabiei* doesn't affect root system of the plant

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Table 1: ANOVA table of physiological trait of treated chickpea plants. The significance level for the trait was taken 5 % for comparison of mean value among selected genotypes

Source	Mean SS of root length	Mean SS of shoot length	Mean SS of No. of pods per plant	Mean SS of the weight of grain per plant
Treatment	65.8692**	551.104 **	150.000**	260.962**
Variety	22.2993**	764.00 **	260.759**	87.2867**
Treatment*variety	7.9729**	7.681 N/A	4.946 N/A	3.683 N/A

MSs: Mean sum of the square, **: Highly significant, *: Significant, N/A: Non-significant

Different physiological parameters were observed with disease attack, varieties, and biological treatments along with control. Results are presented in the form of graphs, cell membrane stability graphs show that treatments showing similar lettering were statistically non-significant. *T. harzianum* enhances the cell strength of all the cultivars under observation in contrast with control while least was observed in the combination of biocontrol agents. Likewise, the same pattern was somehow followed within other parameters. In relative water, the content was maximum with biocontrol combination as well as *B. bassiana* in contrast with *T. harzianum* and control. Excised leaf water loss was maximum observed in the control treatment and least were observed with *T. harzianum.* Maximum chlorophyll content was observed with *T. harzianum* in all varieties and the least were observed with control treatment. All the treatments showing different lettering were statistically significant.

In-vitro management was done with 12 fungicides by measuring the OD of the 96 well plates. Results are shown in the form of graphs as well an ANOVA table is given below. Graphical representation in figure 5 showed the inhibition percentage with different levels of each fungicide along with control after 96 hours of the experiment. As fungicides were in higher concentration in the first 2 levels that's the reason for maximum inhibition by all fungicides with those levels. So it was observed that mic 50 is common for all fungicides at 3rd and 4th levels. ANOVA Table 2 and 3 showed inhibition of *A. rabiei* with level and time with 0.05% level of significance. Results with levels and time individually showed a higher level of significance while in interaction with each other it showed non-significant results for all the used fungicides.

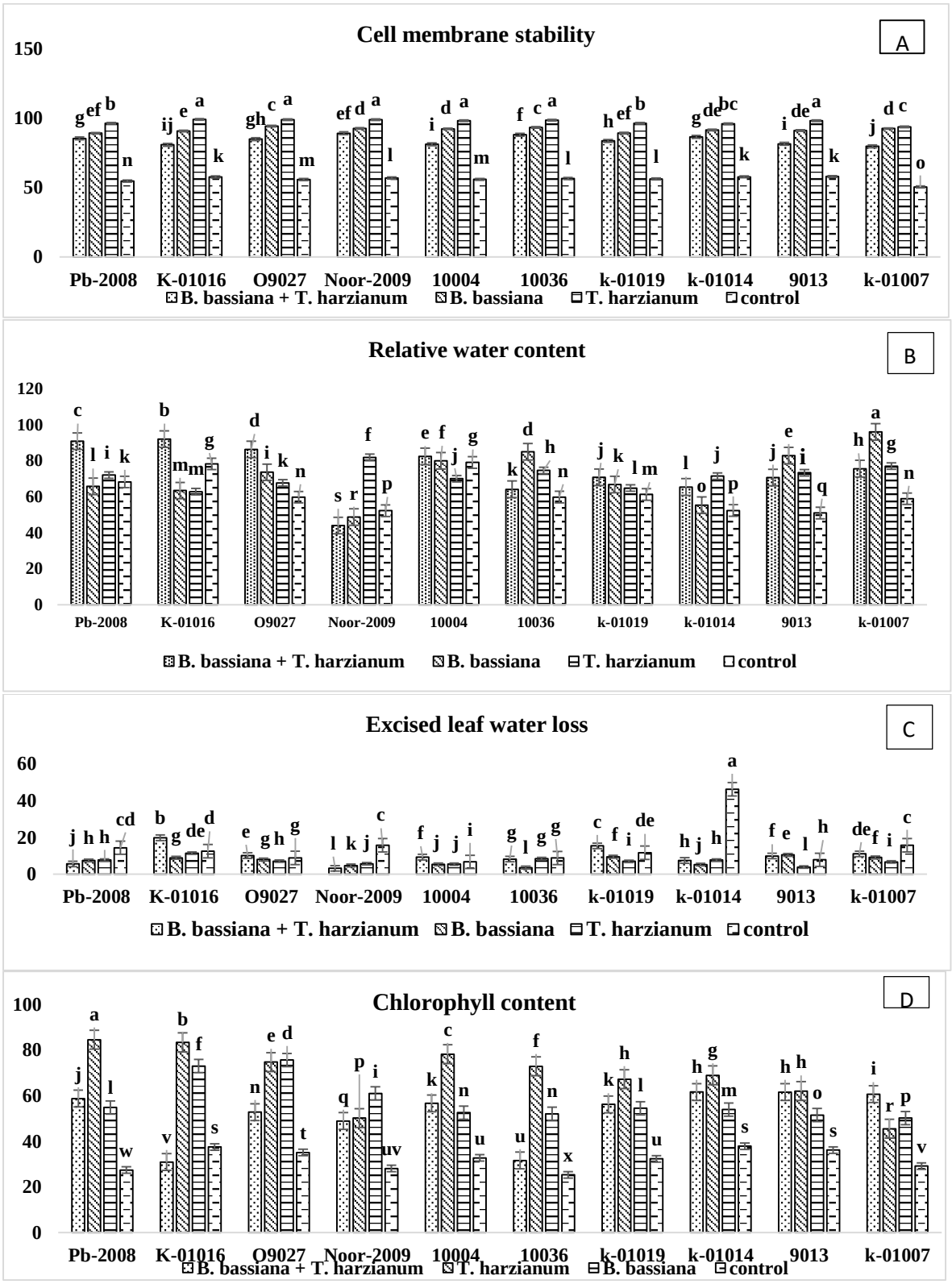

Figure: 2 shows cell membrane stability (A), relative water content (B), excise leaf water content (C), and chlorophyll content after inoculation of the pathogen (D) and *B. bassiana* and *T. harzianum* individually and in combinations along with control.

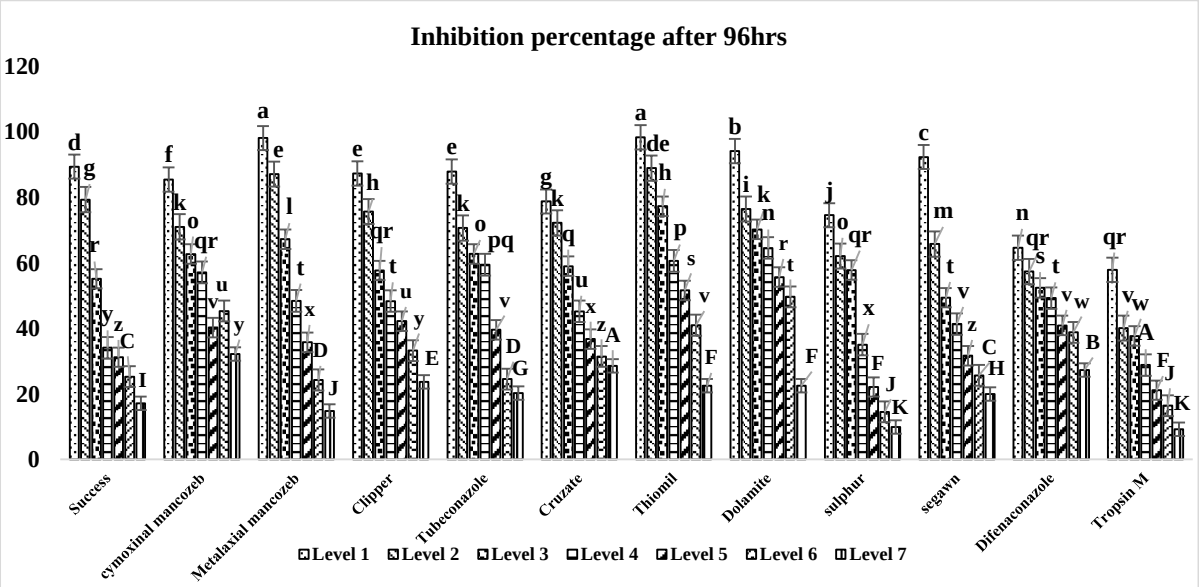

Figure 3. Presenting inhibition percentage with 7 levels of different fungicides.

Table 2: ANOVA table of chemical inhibition on *A. rabiei*. ANOVA table for the significance level for inhibition was taken 5 % for comparison of mean value among themselves

Source	MSs Tropsin M	MSs Thiomil	MSs Sucess	MSs Segawin	MSs Sulphur	MSs Curzate
Level	0.41706 **	0.07838**	0.05913**	0.19429**	0.44689**	0.39258**
Time	0.03614**	0.00211**	0.08367**	0.05240**	1.19480**	0.19039**
Level*Time	0.08148 N/A	0.00539 N/A	0.01302 N/A	0.24915 N/A	0.30035 N/A	0.01424 N/A

MSs: Mean sum of the square, **: Highly significant, *: Significant, N/A: Non-significant

Table 3: ANOVA table of chemical inhibition on *A. rabiei*. ANOVA table for the significance level for inhibition was taken 5 % for comparison of mean value among themselves

Source	MSs Metalaxial mancozeb	MSs Clipper	MSs Tubeconazole	MSs Cymoxial mencozeb	MSs Difenaconazole	MSs Domalite
Level	0.24766**	0.20629**	0.03941**	0.69129**	0.18111**	0.42452**
Time	0.40352**	0.60638**	0.00011**	0.47521**	0.10575**	0.43815**
Level*Time	0.01951 N/A	0.02976 N/A	0.00824 N/A	0.09217 N/A	0.08823 N/A	0.00640 N/A

MSs: Mean sum of the square, **: Highly significant, *: Significant, N/A: Non-significant

Figure 4 below represent the spore and mycelia of *A. rabiei* after treatment with the chemical. Slides were prepared from the broth media present in 96 well plates to visualize the chemical mode of the spores and mycelia of the fungus and it has been clear that all the spores and mycelium are de-shaped in the slides and mycelium become the mass of debris in the end along with control. Slides were stained with lactophenol and observed under 40X to capture images.

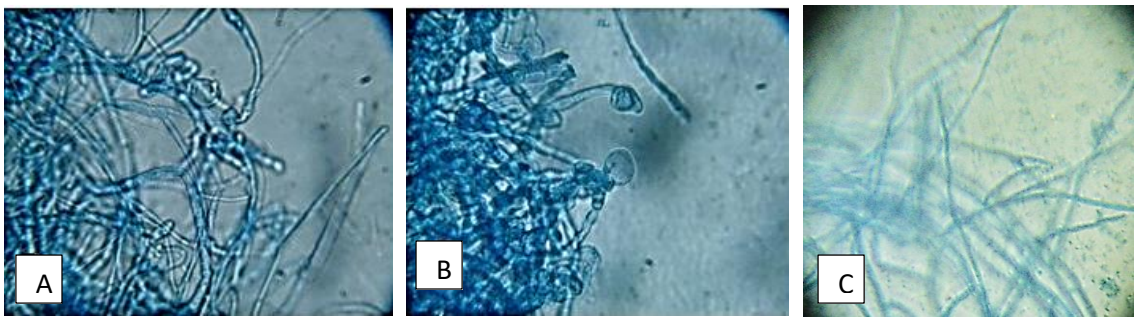

Figure 4: Mycelium growth of *A. rabiei* with A and B are chemical treatment along with C as control.

Discussion

Ascochyta blight caused by *Ascochyta rabiei* in chickpea. Its distribution varied depending upon environmental factors and the amount of inoculum in the field. *A. rabiei* is controlled by using several chemical fungicides and biocontrol agents worldwide. But the use of the chemical is not economical as well as not eco-friendly approach, it also makes pathogen resistant (Pande et al., 2005).

Research conducted for testing several chemicals against *A. rabiei*, it was noted that chlorothalonil, zineb, captan, antracol, propiconazole, penconazole, and thiabendazole is effective and controlling the spread of Ascochyta blight (Ahmad et al., 2021). Likewise, biocontrol agents, *T. viride*, *Chaetomium globosum*, and *Acremonium implicatum* under *In-vitro* conditions proven effective against *A. rabiei* (Bisen et al. 2020). Chickpea blight is controlled by Aliette fungicide under *in vitro* conditions and causes significant inhibition which supports the present research. Chongo et al. (2003a) reported that the application of chemicals at the right time is very important to reduce the losses caused by *A. rabiei*. The use of chlorothalonil at two different stages reduce the incidence up to 8% which was 45% for the control treatment. Gan et al. (2006) concluded that foliar application along with integrated management is very effective for disease management in chickpea, which supports our research that plants with the proper application of fungicide or correct time for biocontrol helped in disease reduction. The use of protective fungicides helps to keep away disease pathogens from coming in contact. Choice of good and effective fungicide is very important. A mixture of foliar and protective fungicides was used for the experiment to find the effective one. Demirci et al. (2003) tested chlorothalonil, azoxystrobin under in-vitro and *In-vivo* conditions and found that these two fungicides do not perform well on the plate but under field conditions, they perform very well against *A. rabiei*. Shtienberg et al. (2000) concluded that protective fungicides like zineb, Bordeaux mixture, captan are very important in disease reduction but not effective enough on susceptible cultivars. In recent years number of new fungicides had been reported as effective against *A. rabiei*. Effective fungicides against *A. rabiei* are boscalid, pyraclostrobin, difenoconazole, azoxystrobin, tebuconazole, mancozeb which support our research because several fungicides are part of current research (Gan et al., 2006).

MacLeod and Galloway (2002) Mancozeb is used in Australia, Canada, and Israel for the control of chickpea blight. In the present research, mancozeb performs well for blight fungus. MacLeod et al. (2002) also found that carbendazim which is now banned, difenoconazole, and tebuconazole was tested in India, the Western part of Asia, Australia, and

North of Africa, and these fungicides have proven effective.

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