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Biological control of tomato leaf spot disease caused by *Alternaria alternata* using *Chaetomium globosum* and some other saprophytic fungi

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Abstract. This study was conducted in the laboratories of the Plant Protection Department/College of Agriculture/University of Basra between 2016 and 2017.The aim of this study was to evaluate the efficiency of *Chaetomium globosum* and some other saprophytic fungi to control of tomato leaf spot caused by *Alternaria alternata*. The result of this study showed that *C.globosum, Aspergillus niger*, and *Penicillium* spp had a high antagonistic activity against the pathogenic fungus *A.alternata* in dual culture plates. The results of the plastic house experiment showed that disease severity was decreased in tomato plants treated with bio-agent as disease severity run to 21.66% compared to 45% in control treatment (plants treated with *A.alternata*). Disease severity in other treatments ranged between 23.2-27 %. Infection rate(r)ranged between 0.07-0.09 in bioagent treatment compared to 0.1 in control treatment. It is also showed that *C.globosum* had a positive effect on plant yield as it reached 332.3g/plant compared to 154.4 g/plant in control treatment.

1. Introduction

Tomato plants is subjected to many plant pathogen such as *Alternaria alternata* fungus that is considered as one of the most important fungus causing leaf spot, stem necrosis and black mold on tomato fruit [1]. Many fungicides are used in controlling of the tomato leaf spot disease caused by this pathogen. However, the fungicides cannot be adopted as a long-term solution due to the high coast, risk to employers exposed to fungicides residues, developing resistant phytopathogenic strains for some fungicides and their harmful effects on human health in particular and the environment in general .There is, therefore, an urgent need for alternative means to control plant diseases including the leaf spot disease. In general, biological control by antagonistic fungi such as *Chaetomium globosum* is one of desirable options which can be applied in diseases management. This biocontrol fungus produces more than 200 compounds such as toxins, antibiotics and inhibitory enzymes that have effective biological activities against plant pathogens [2]. Thus, *Chaetomium* spp. have been developed as bio-fungicide to control anthracnose disease of Coffee [3].

As well as, many studies have approved that *C.globosum* induced systemic resistance on wide range of plants against different pathogenic fungi, bacteria and virus [3,4]. Therefore, as a result of the importance of tomato leaf spot disease caused by *A.alernata* and the need for alternative control method, this study was conducted for the purpose of evaluating the efficiency of *C.globosum* and other saprophytic fungi to control of the pathogen of tomato leaf spot.

2. Material and Methods

2.1. Isolation and identification of Alternaria alternata

A.alternata was isolated from tomato leaves infected with leaf spot disease collected from greenhouses of Agricultural College at University of Basra. The fungus was identified on the basis of the morphological and microscope characteristics [5].

2.2. *A source of fungi used as Bio-agent*

The *C.globosum* fungus was obtained kindly from Dr.Abdul Nibi A.Matrood (plant protection Dept./ College of Agriculture/University of Basra). *Penecillium* sp. and *Aspergillus niger* were isolated from soil samples collected from greenhouses of College of Agriculture /University of Basrah by dilution plates methods and the fungi were identified according to previous description [6].

2.3. Molecular identification of C.globosum and A.alternata

C.globosum and *A.alternata* fungi were grown on PDA media at 25 °C. After three days, the mycelium was scraped, and DNA extraction was done using commercial kits (Promega Genomic DNA Purification kit ,GD 100,USA).The quality and quantity of extracted DNA was confirmed using Nanodrop (Themo-Scientific Nanodrop 2000. A Polymerase reaction amplification was performed using ITS1-ITS4 primer set (Table 1) to amplify the ITS-rDNA region.

Table 1. The primers used in the amplification of ITS-rDNA region

Primers	Primer direction	Primer sequence
22-ITS 4El	Forward	5`-TCCGTAGGTGAACCTGCGG-3`
	Reverse	5`-GCTGCGTTCTTCATCGTGC-3`
24-ITS1El	Forward	5`-GCATCGTGAAGAACGCAGC-3`
	Reverse	5`-TCCTCCGCTTATTGATATGC-3`

The PCR conditions were 5 min of initiation at 90 °C followed, 35 cycles of denaturation at 58 °C for 1 min, 2 min of extension at 72 °C and a final extension at 72 °C for 5 min. The PCR products were sent to Macrogen Inc .(Macrogen Korea: 10F,254 Beotkkot-ro, geumcheon-qu,Seoul,08511,Republic of South Korea) for sequencing. Molecular identification was done employing the BLAST software.

2.4. Antagonism test between biocontrol agents and A.alternata

A protocol [3] was applied to investigate the antagonistic ability between the bio-agent used in this study and the pathogenic fungus *A.alternata*. Petri dishes (9 cm in diameter) containing PDA were divided into four equal parts. Then the center of the dishes were inoculated with a diameter of 0.5 cm from the colony edge of the seven-day-old of *A.alernata* while the center of each part was inoculated with a diameter of 0.5 cm from the colony edge of the 7-day- old colony of *C.globosum*, *A. niger* and Penicillium spp in distance of 3 cm from the pathogen disc (the centre of the dish). Then the dishes were incubated for 7-days at 25 °C, the control treatment included dishes containing *A.alternata* alone. The experiment was replicated four times for each treatment. The antagonistic activity of the bio-agents was calculated according to the following equation:

C=A-B

C: The antagonistic activity, A: The original distance between the bioagent and the pathogenic fungus (3 cm), B: The growth of pathogenic fungus toward bio-fungus. Three categories of antagonistic ability were regarded in this experiment.

- High antagonistic ability if C value is more or equal to 2 cm (+++).
- Moderate activity if C value 1-1.9 cm (++).
- Weak activity if C value less or equal to 0.9 cm (+).

2.5. The Pathogenicity test of A.alternata

Four-week old of tomato seedlings (cultivar wijdan) was cultivated in plastic pots, three seedlings per pot. Each pot contains a mixture of peat moss and soil with ratio 1: 2. After two weeks from the date of cultivation, the plants were sprayed with the fungal suspension of *A.alternata* with concentration 10^6 spores /ml using hand sprayer V. 500 ml. The concentration of spores was adjusted by Haemocytometer. The plants were covered with plastic bags within the first three days after spraying with the fungal suspension to ensure maintain humidity. After three days, the plastic bags were removed and the plants were left for 2 weeks. The infection was measured according to a scale depending on the number of spots /leaf, as 0=no spot; 1=1-3spots/leaf; 2=4-6 spots/leaf; 3=7-9 spots/leaf ; 4= more than 9 spots /leaf. The experiment was conducted with three replicates. The disease severity was measured according to the following [19].

$$\text{Disease Severity} = \frac{\text{Sum (No. of leaves in each degree x degree)}}{\text{No. of leaves calculated x maximum scale degree}} \text{ X } 100$$

2.6. The effect of biocontrol agent on tomato leaf spot disease (pot experiment)

The experiment was conducted in the fields of College of Agriculture /University of Basrah in a greenhouse. The soil was first tilled and then levelled into three rows. Tomato seedling was planted for each row with 25 cm distance between plants. All cultures processes were made for the plants as needed. Tomato plants were sprayed with the A.alternata spore suspension (10^6) at beginning of the flowering stage. After 3 days, the plants were sprayed with *C.globosum* , *A.niger* and *Penicillium* sp. spore suspension. After one month of inoculation, disease severity was measured within three-time spans with average on month between each record and another. The experiment was conducted with three replicate for each following treatment:

(a)The pathogenic fungus *A.alternata* only control 1; (b)The pathogenic fungus + *C.globosum*; (c) The pathogenic fungus +*A.niger* ; (d) The pathogenic fungus +*Penicillium* sp.; (e) The Pathogenic fungus + Hexaconazol 5%EC (1 ml/L distilled water) and (f) Control 2 (plants sprayed with distilled water only).

Disease severity (D.S) were measured according to previous scale and equation. The D.S was elevated three times interval and the infection rate (r) was calculated according to the following equation [24].

$$r = \frac{2.3}{t_1 - t_1} \log 10 \ \frac{x_2(1 - x_1)}{x_1(1 - x_2)}$$

r=infection rate; 2.3= Coefficient; T1=The time of first measure; T2=The time of second measure; X1=Disease severity of the first measure; X2=Disease severity of the second measure.

Some of the plant growth and Yield parameters were also calculated.

3. Results and discussion

3.1. Alternaria alternata and C.globosum identification

The *A.alternata* fungus was isolated from tomato leaves showing leaf spot symptoms. The upper surface of the fungus colonies was olivaceous tend to black color and they were slow-growing colonies in PDA culture. When they were subjected microscopically with the magnification power (40X), the conidiophores individually bearing a chain of tiny spores characterized by having three long septa as well as transverse septa, Mostly, spores are neckless or they may have a short neck and these characteristics agree with [5].

Polymerase chain reaction (PCR) technique was used to confirm the identification of *A.alternata* and *C.globosum* by amplification of the ITS region of ribosomal DNA. Then, nucleotides sequences of such regions were compared to those preserved at GenBank sequence database. The comparison confirmed that the investigated isolates were *A.alternta* and *C.globosum* (Table 2) [9].

Table 2. Molecular identification of *A.alternata* and *C.globosum*

Isolate name	Compatible isolate	% of identities	Score	% of compatibility
A.alternata	KY78030.1	%99	941	%100
C.globosum	GQ365152.1	%99	955	%99

3.2. Isolation and identification of Aspergillus niger and Penicillium sp.
A.niger and *Penicillium* sp. were isolated from soil samples and identified by morphological features according to previous descriptions [6,10].

3.3. The pathogenicity test of Alternaria alternate
The results of the *A. alternata* pathogenicity test showed that Disease severity was up to 55.5% on Tomato plants inoculated with *A.alernata* spores suspension. The infection starts with brown-yellowish spots surrounded by a yellow halo. This result is in agreements with previous study [11] as *A.alternata* is considered to be one of pathogens causing leaf spot diseases on tomato and other vegetable crops.

3.4. The effect of bioagent on the growth of A.alternata
The results of this experiment showed that bio-fungus used in this study had a high antagonistic activity against *A.alternata*, with the inhibition zone 2.34, 2.46, and 2.85 for *C.globosu*, *A.niger*, and *Penicillium* sp. respectively (Figure 1). The fungus *C.globosum* was characterized by its ability to produce several active compounds in inhibition of the growth of many fungi of the plant, as well as its ability to compete for food and place and combine different mechanisms. There are many studies state that *C.globosum* had the efficiency to inhibit the growth of fungi such as *Fusarium oxysporum* [13], and *Pestalotia macrotich* [12]. In another study [4] 22 strains of *C.globosum* and *C.cuperum* provided protection for a long time against spotting and rotting fungi. Likewise, *C.globosum* was found to produce many active compounds that have a significant role to inhibit many fungi such as *Cochliobols sativa* and *Drechslere sorokiniana* [13; 15].The results of this study also showed that *A.niger* and *Penicillium* sp. had high antagonistic capability against *A.alternata*. Many previous studies were indicated that *A. niger* and *Penicillium* spp. have an ability to inhibit the growth of many pathogenic fungi such as *A.alternata* [13,16].

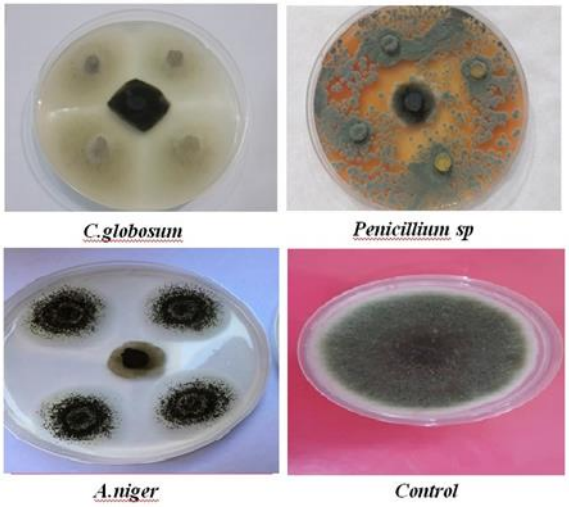

Figure 1. Antagonistic activity of Bio-agent against *A. alternata*

3.5. Effect of C.globosum and some saprophytic fungi on A. alternata leaf spot disease

The results of this experiment showed that all bioagents used as spore suspension reduced tomato leaf spot disease severity caused by *A.alternata* (Table 3). However, it seems that *C.globosum* was more effective than *A. niger* and *Penicillium* sp. The D.S of *A.alternata* +*C.globosum* treatment reached 21.6% compared with 49%in control treatment (*A.alternata* alone) while the D.S in the remaining treatment was ranged between 23.3 and 25 %. There was a slight increase in D.S in all treatments of second and third records as D.S was 24.3 and 28.33% compared with 55.9 and 56.66% for control treatment.. The reduction of D.S in *C.globosum* treatment may be attributed to the capability of this fungus to produce some compound which is classified as toxins or antibiotics such as chaetoviridins A,C, and Chaetoglobcins [4] It was also found [9] that the isolate g-6 of *C.globosum* produce Chaetomin antibiotics which has an anti-enzymatic activity against enzymes produced by *Phytophthora infestans*. Furthermore, it was discovered that *C.globosum* stimulating plants to produce Pathogenesis Related Proteins (PRP) [17]. It is quite well-known that the specific toxin is regarded as the most important factor in the pathogenicity of some fungi such as *A.alternata*. Accordingly, breaking such toxins and eliminate their toxicity via bio fungi may constituent one of the potential mechanisms used to reduce such diseases. It was identified [18] that the most important mechanism of bacteria *Pantoea dispersa* (*Erwinia hericola*) to reduce the infection of sugar cane blight caused by *Xanthomonas albilneans* was by breaking the albicidin toxin excreted by pathogenic bacteria. Moreover, it was showed that *Penicillium* spp. reduced leaf spot disease severity due to producing enzymes and antibiotics that are capable to inhibit growth and spore germination of pathogenic fungus *A. alternata* [19; 2].

Table 3. Effect of Bio-agents in tomato leaf spot disease severity

Treatments	% of Disease severity		
	First record	Second record	Third record
Control 1 (*A.alternata* only)	45	55.92	56.66
A.alternata + *C.globosum*	21.66	24.33	28.33
A.alternata + *A.niger*	24.16	28.33	33.33
A.alternata + *Penicillium* sp.	23.33	30.83	34.16
A.alternata + Hexaconazole	27	30	28.33
Control 2	0	15.8	18.33
L.S.D.=0.05	9.5	7.2	14.4

3.6. Effect of the bioagents studied in infection rate(r)value

Results of this experiment (Table 4) showed that plants treated with bio-agent (*C.globosum*, *A.niger*, and *Penicillium* sp.) and Hexaconazol fungicide leaded to reduce infection rate value compared with control treatment as r value was 0.102, 0.07, 0.08, 0.096 and 0.085 in control treatment, *A. alternata*+*C.globosum* , *A. alternata* +A.niger , *A. alternata* +*Penicillium* sp.,and *A. alternata* +Hexaconaz respectively. The ability of bio-agent in reduce of infection rate(r) may be attributed to some factors among which is the fact that bio-agent may reduce the pathogenic fungus growth ,reduce its capability to grow inside the infected tissues or induce the plant resistance which hinder the disease development ,due to the fact that such factors secrete some active biochemical compound such as Chaetomin and Cahetoglobsin as well as glucanases enzymes[19; 3] . The previous study mentioned that *A.niger* , *Penicillium* produced some active compounds like Nominine ,Paspaline, and Aspernomine which reduce the negative impact of *A.alternata* and some other pathogenic fungi[20]. Other studies have shown that capability of *Penicillium dangeardi* in reduce potato wilt caused by *Verticillium dehliae* may be attributed to the fact that glucose existed in root exudates were oxidized

by glucose oxidase enzymes execrated by *Pencillium* fungus and this process lead to generating hydrogen peroxide(H_2O_2) which is toxic to pathogenic fungus [21].

Table 4. Effect of Bio-agent on infection rate (r)

Treatment	infection rate (r)
Control 1(*A.alternata* only)	0.102
A.alternata + *C.globosum*	0.077
A.alternata + *A.niger*	0.082
A.alternata + *Penicillium* sp.	0.096
A.alternata +hexaconazol	0.085
L.D.S 0.05	0.0771

3.7. Effect of Bio-agent on some yield parameters

Results of this experiment (Table 5) displayed that leaf spot disease caused by *A.alternata* reduced yield parameters of tomato plants such as the number of inflorescence per plant and plant yield as example the number of inflorescences reduced to 12.43/plant in control1 treatment(plant treated with *A.alternata* only) compared to 14.3 in control 2 treatment (untreated plants). It is clear that the application of all bio-agent improves such parameters as inflorescence /plant was 19.5 and 18.4/plant in *A.alternata* +*C.globosum* and *A.alternata* +*Penicillium* sp. respectively. Results of this experiment were with an agreement with the previous study mentioned that *C.globosum*, *A.niger* and *Penicillium* sp. increased the yield of tomato plants [4; 22; 23].

Table 5. Effect of Bio-agent on some yield parameters

Treatment	Inflorescence No./Plant	Yield gm/plant
Control 1	12.43	154.43
A.alternata + *C.globosum*	19.5	332.37
A.alternata + *A.niger*	18	289.48
A.alternata + *Penicillim* sp.	18.4	254.40
A.alternata + Hexaconazol	13.06	221.11
Control 2	14.33	216.64
L.S.D 0.05	4.794	59.67

4. Conclusion

Results of this experiment showed the effectiveness of *C.globosum* in reducing tomato leaf spot disease caused by *A.alternata* suggesting that it can be used as an alternative to chemical control.

5. Acknowledgment

We thank Dr. Labeed Al-saad for his help in the Molecular identification of fungi and Dr. Abd-Alneebi Abd-Al-ameer for providing the *Chaetomium globosum* fungus.

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