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ANTAGONISM CAPABILITY IN VITRO OF *TRICHODERMA HARZIANUM* AGAINST *ALTERNARIA ALTERNATA* ON *CERATONIA SILIQUA*

Zahra Ibrahim El-Gali*

Department of Plant Protection, Faculty of Agriculture, Omer Al-Mukhtar University, El-Beida, Libya.

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***Correspondence for Author**

Zahra Ibrahim El-Gali

Department of Plant Protection, Faculty of Agriculture, Omer Al-Mukhtar University, El-Beida, Libya.

ABSTRACT

The *in vitro* studies on the antagonism of *Trichoderma harzianum* on pathogenic fungal *Alternaria alternata* isolated from carob leaves showing symptoms of brown spot disease grown at north-east Libya, was carried out to test the ability of the antagonist fungus in inhibiting the growth of the plant pathogen using dual culture assay, *Trichoderma* conidial suspension and *Trichoderma* filtrate. Direct confrontation of the colonies of *T. harzianum* with this of the pathogen results in an inhibition and growth arrest at a distance of the parasite. Inhibition and The mycelia of *Alternaria* showed abnormal morphology under direct influence of *Trichoderma.* The results of the activity assay of filtrate of the antagonistic fungus at different concentrations on the radial mycelial growth and percent radial mycelial growth inhibition of pathogenic fungus at all concentrations of the antagonist filtrate. Further evaluation of *Trichoderma* bio-control potential in field condition was recommended.

KEYWORDS*: Alternaria alternata, Ceratonia siliqua, Trichoderma harzianum*, Biocontrol, Antagonism.

INTRODUCTION

Alternaria genus contains a great number of species plus of sixty, parasites or saprophytes. It associated with a wide variety of substrates including seeds, plants, agricultural products, animals, soil, and the atmosphere (Rotem, 1994; El-Gali et al, 2014; El-Gali, 2014a; El-Gali, 2014b). Some species are commonly correlated with deterioration of painted wall surfaces

(Elumalai et al, 2014). The brown spot disease plays a significant role among some economically important diseases on carob trees in Libya. Its causing agent is the pathogenic fungus *Alternaria alternata* (El-Gali, 2014c). The specific symptom (brown spots) causes biochemical changes and reduce in green area that impair the tree, and result in decline on carob production.

The chemical pesticides cause a significant damage to the public health, environment and groundwater pollution; it is uneconomical, so that scientists went recently to the biological control. In bibliographical study for more than 200 research carried out by Mausam and al (2007) confirmed that the *Trichoderma* sp. plays an important role in biological control, and it represents 60% of all other biofungicides include bacteria, nematode and virus, and was used as a pesticide, and herbicide, and use of this fungus contributes to the improvement of plant growth.

Trichoderma spp. are widespread in the soil as saprophytic fungi highly competitive to plant pathogens. Among *Trichoderma* isolates, the most studied are *T. harzianum* (Chaur-Tsuen and Chien-Yih 2002), *T. reesei* (El-Naggar et al. 2008), *T. atroviride* (Brunner et al. 2005) and *T. viride* (Mishra et al. 2011). The biological control activity of the *Trichoderma* strains against fungal phytopathogens has been tested and described in several research papers (Meszka and Bielenin 2009, Joshi et al. 2010, Lone et al. 2012). *Trichoderma* isolates have been shown to be successful in controlling soilborne diseases in the greenhouse and under field conditions. Some of the *Trichoderma* strains are currently available as components of commercial bioproducts: KRL-AG2 (*T. harzianum*) controls a wide range of soil-borne diseases (Spiegel and Chet 1998), Trichodex (*T. harzianum*) is used against *B. cinerea, Sclerotinia sclerotiorum, Cladosporium fulvum* diseases in greenhouse grown tomato and cucumber, and in vineyards (Freeman et al. 2004), Binab T (*T. harzianum* and *T. polysporum*) controls wound decay and wood rot (Mehrotra and Aggarwal 2003), Supresivit (*T. harzianum*) inhibits the growth of *Phytophthora* spp. and *Pythium ultimum* and might stimulate the growth of plants (Brožová 2004).

Several mechanisms are important in antagonistic interactions, including mycoparasitism and competition for substrates and sites of infection (Benitz et al. 2004; Reena et al., 2013). The fungal strains for biological control against plant pathogens must have an activity that is manifested by the ability to use the same *Trichoderma* community resources that pathogenic fungi, *Trichoderma*, but uses this mode of action primarily to occupy the premises before the

arrival of events. (Gaetan LeFloch et al. 2006). The use of *Trichoderma* with all mechanisms of biological control will help suppress this pathogen. In this investigation, one antagonistic local isolate of *Trichoderma harzianum*, was evaluated against *A. alternata* was isolated from carob tree leaves.

MATERIALS AND METHODS

Fungal material

In this study was used the strain of *T. harzianum* which isolated from atmosphere and *A. alternata* isolated from carob leaves naturally infected with this pathogens. Both fungi identified at Department of Plant Pathology, Faculty of Agriculture, El-Beida- Libya and used *In Vitro*.

Efficacy of *Trichoderma* against certain pathogen

Dual culture technique

In vitro antifungal activity of *T. harianum* against *A. alternata* was tested by employing dual culture technique (Rao, 2003) . I used the method of direct confrontation; this method was consisted to put on the same Petri dish containing 15ml of PDA medium, two agar pellets (5 mm diameter) one strain of *T. harzianum* and other agent pathogen were positioned along a diametrical axis 3cm away. The control was presented only by the pathogen; incubation was performed at 23°C±2for six days in the dark.

Inhibition of radial growth of fungi and encroachment over pathogens by *Trichoderma* were recorded and compared with the control. The evolution of mycelial growth was performed after 2, 4 and 6 days by measuring the diameter of the colony of the pathogen and the antagonist. The valuation of inhibition by *Trichoderma harianum* is estimated by calculating the percentage inhibition of mycelia growth by the following formula as proposed by Datta et al (2004): %I = [(C-T)/C] x100).

Where:

I= percentage inhibition of pathogen by antagonist.

C= radial growth in control.

T= radial growth in the treatment.

The degree of antagonistic activity was estimated as follows (Sookchaoy et al. 2009): 4 – very high antagonistic activity (R > 75), 3 – high antagonistic activity (R = 61-75), 2 – moderate antagonistic activity (R = 51-60), 1 – low antagonistic activity (R < 51).

***Trichoderma* suspension:** A conidial suspension of the test Isolate of *Trichoderma* was prepared from a 7-day old culture of the isolate on PDA. The plate (9cm diameter) was flooded with 10ml of sterilized distilled water and shaken for a few minutes. The resulting suspension was filtered through muslin cloth. After filtering the suspensions through double layer of cheese towel, the conidial concentration was determined using a haemocytometer. The spore concentration of the filtrate was adjusted to 10^6 conidia/ml using sterilized distilled water.

Conidia suspensions (1ml) of *T. harzianum*, prepared as described above, was poured into a sterilized Petri dish followed by 15ml of PDA. One ml of distilled water instead of the spore suspension was used in the control. Four mm diameter discs were obtained from an actively growing region of a 7-day old *A. alternata* culture on PDA and the disc was transferred aseptically to the center of each *Trichoderma* amended PDA medium. The treatments were replicated five times. The Petri dishes were incubated at 25± 2°C. Growth of *A. alternata* was determined at 2, 4 and 6 days after inoculations by measuring the diameter of the culture diametrically. The percentage growth inhibition (I) was calculated using the above formula.

***Trichoderma* filtrate:** One hundred milliliters (ml) of potato dextrose broth (PDB) were dispensed into 250 ml Erlenmeyer flasks and inoculated with 5 mm diameter disc from edge of 7 days old culture of the *T. harzianum* and set up was shaken at 100 rpm for 15 days at 25±2^{o}C on Shaker. After the optimum period, the culture was filtered through Whatman No.1 and sterilized by millipore membrane filtration of 0.24 Lm and stored at 4^{o}C for further use.

The sterilized filtrate were amended in PDA to make three concentrations (10%, 20% and 30%) in Petri plates. 5mm wide mycelial discs of the pathogen were placed at the center of solidified agar plates and incubated at optimum temperature for 7 days. Plates devoid of culture filtrates served as control. Radial growth of *A. alternata* was measured and its inhibition percentage of mycelial growth was calculated using the formula above.

Statistical analysis: Complete randomized design was used and the treatments were replicated five times. The same set was repeated three times. The data on effect of the treatments on the growth of pathogens was analyzed by analysis of variance (ANOVA), and treatment means were compared by using Duncan's multiple range test (DMRT) and least significant difference test (LSD) at P = 0.05.

RESULTS

Effect of direct confrontation of the tested bioagent against *A. alternata*

Experiment was conducted in order to study the behavior of *T. harzianum* against the tested fungal. Results revealed that *T. harzianum* has controlled the pathogen *A. alternata* being mycoparasite on it.

Antibiosis and myco-parasitism are the well-known mechanisms involved in biocontrol of pathogens by *Trichoderma*; competition for nutrition, space and dominance being equally important and mutually inclusive phenomenon. The complete course of interaction between *Trichoderma* and *Alternaria* as observed on the dual culture plates can be divided into two phases. The initial phase marked by interaction with mycelia contact in which diffusible metabolites from both the organisms decide the fate of interaction. The intermediate phase in which *Trichoderma* may be able to overcome the inhibitory effect of *Alternaria*. In the intermediate phase. And, the final phase where *Trichoderma* parasitizes *Alternaria* (Fig 1-b). Experimental observation showed that the borderlines where *Trichoderma* and *Alternaria* encountered each other offensive as well as defensive mechanisms were activated. *A. alternata* showed brown-grey specifically where it encountered metabolites of *Trichoderma* and *Trichoderma* showed heavy sporulation where it encountered metabolites of *A. alternata* (Fig 1-c). No inhibition zone was observed and their mycelium quickly invade the colony of the tested pathogen

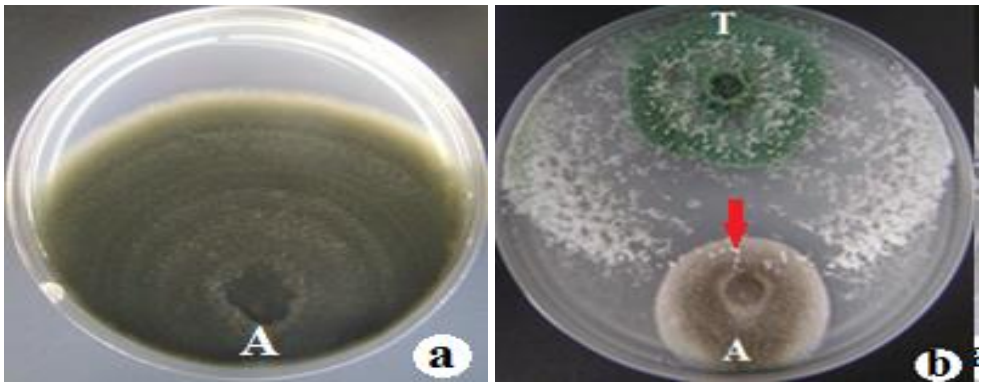

Fig. 1. Antagonistic activity of isolate *T. harianum* against *A. alternata* (on the left: pure culture of *A. alternata*, on the right: the dual culture of *A. alternata* and *Trichoderma isolate*)

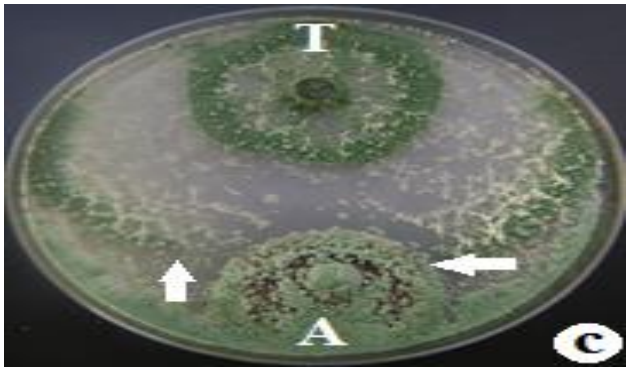

Fig. 1. Overwhelming growth of *T. harzianum* isolate on *A. alternata* which indicates parasitic interaction. Heavy sporulation (at arrow) by *Trichoderma* is seen on and in vicinity of *Alternaria* colony.

Microscopic slide observations show that the different interactions hyphal displayed that the antagonistic fungus affected the pathogenic fungus with several biological forms: the first, *T. harzianum* showed parasitic behavior against *Alternaria* (Fig 2-a, b) by coiling round the host hyphae and degrading it. The dual cultures plates showed initial rapid growth of the host which stopped at the point of contact with the parasite. *T. harzianum* over grew the pathogen resulting into complete degradation of the latter and sporulation of the former over the entire plate. The second, Parasitism phenomenon (Mycoparasitism): it was found that the hyphae of *Trichoderma harzianum* has formed Haustoria on the cell wall of *A. alternata* hyphae and were penetrated within them. Figure (2-c). The third, Decomposition phenomenon (Lyses): the antagonistic fungus was analyzed the mycelia and spores of *A. alternata*, figure (2-d)

Mycelial growth effect: Highly significant effect ($P < 0.01$) was observed in the study of mycelial growth of *Alternaria* colony faced with *Trichoderma* strains which showed reduction in growth compared to the control (Table 1).The results showed that decreased the liner growth of *A. alternata*. It recorded 3 cm in dual culture plates compared with 8.9 cm in control plate at 6 days from incubation. The degree of antagonistic activity was recorded 3 (on 1- 4 scale). The inhibition ratio was 51.4% at 2 days, then increase to 66.3% at 6 days (Fig. 3).

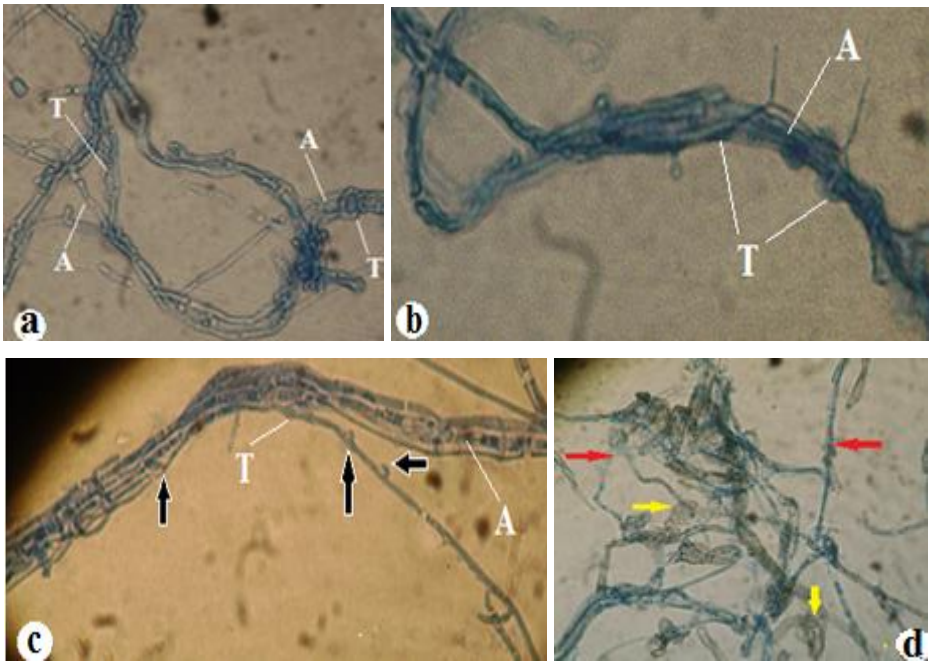

Fig. 2. a, b: Micrograph of light microscope showing coiling of *T. harzianum* over *A. alternata,* c: haustoria formation (black arrows) and penetration within the large hyphae of *Alternaria* (A) by the smaller hyphae of *Trichoderma* (T) and d: Lyses the mycelia (red arrows) and spores (yellow arrows) of *A. alternate*

Table 1: Inhibition of the growth of *A. alternata* by *T. harzianum* and its and its antagonistic activity against the pathogen in dual culture tests

Treatments	Linear growth (cm) Incubation (days)			antagonistic activity (on 1-4 scale)*	*Trichoderma* colonization
	2	4	6		
A. alternata	3.5	5.6	8.9	-	-
A alternata + *T. harzianum*	1.7	2.9	3.0	3	Complete colonization over the medium surface
LSD at 0.05	Treatments: 0.0162, Days: 0.198, Interaction: 0.356.				
Each value is a mean of 5 replicates. *1 = low antagonistic activity (R < 51), 2 = moderate antagonistic activity (R = 51-60), 3 = high antagonistic activity (R = 61-75), 4 = very high antagonistic activity (R > 75)					

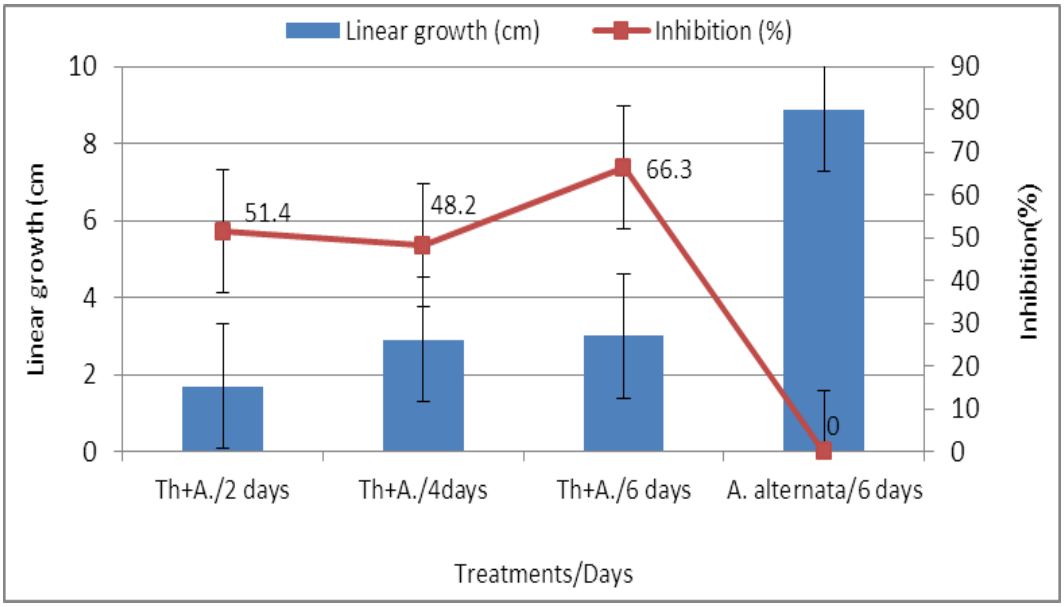

Figure 3. Effect of *T. harzianum* on the growth of *A. alternata*. The Bars indicated to standard deviation.

Effect of conidial suspension of the tested bioagent against *A. alternata*

Effect of *Trichoderma* suspension on cultural growth of *A. alternata* was illustrated in Figure (4). The results indicated to inhibition in colony growth and don't spores (4-a) under effect of antagonistic conidial suspension compared with control (4-c).

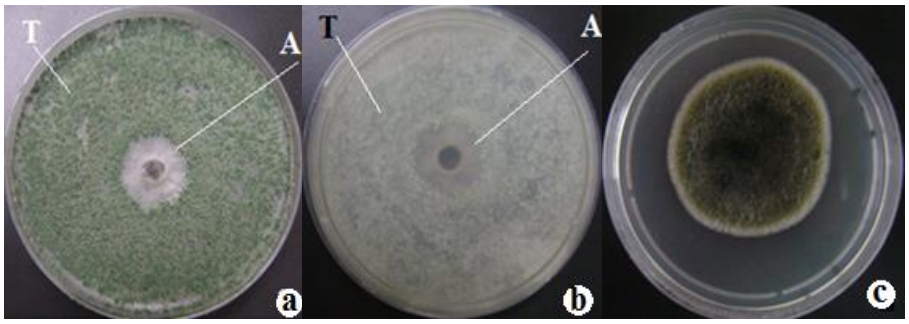

Fig. 4. Growth of *A. alternata* under effect of *Trichoderma* conidial suspension. a. upper side, b. lower side, c. Control.

Microscopic observations of the pathogen after the interval of incubation, showed deformations of hyphae. The mycelia of *Alternaria* showed abnormal morphology such as cell wall appeared thick and dark, hyphal cell shrinking cell shortening and septa (Fig. 5-a), then cutting in *Alternaria* mycelia (Fig. 5-b). The mycelia of *Alternaria* in control did not show such abnormalities (Fig. 5-c)

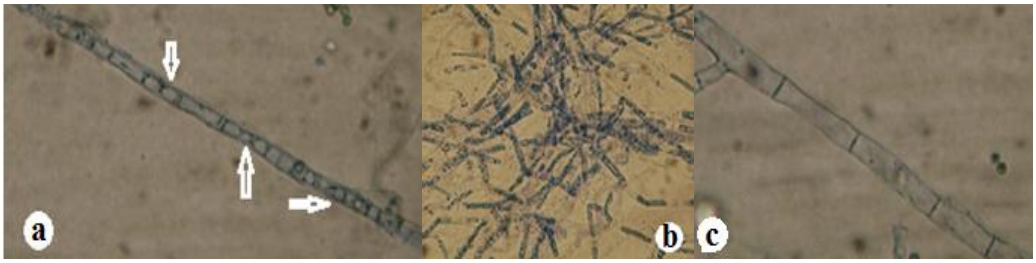

Fig. 5. Morphological characteristics between *T. harzianum* and *A. alternata*: hyphal shrinking cell shortening and septa thickening (a, b) of *Alternaria*, in the presence of *Trichoderma.* c: Control

Data in Table (2) and Figure (6) showed that the colony diameter was stopped at 1.4 cm after 2 days from incubation in plates treatment compared with control that increased in diameter with increase days from 2 to 6. Also the percentage of inhibition was increased with increased in incubation period from 2 to 6 days. It recorded 30% at 2 days, then increased to 74.5% and reach to 83.3% at 4 and 6 days respectively.

Table 2: Effect of *Trichoderma* suspension (1ml/15ml PDA) on *A. alternata* growth.

Treatments	Incubation period (days)					
	2		4		6	
	Colony diameter (cm)	Inhibition (%)	Colony diameter (cm)	Inhibition (%)	Colony diameter (cm)	Inhibition (%)
A alternata	2	0	5.5	0	8.4	0
A alternata + *T. harzianum*	1.4	30 (33.21)	1.4	74.5 (69.67)	1.4	83.3 (65.88)
LSD at 0.05 Treatments Days Interaction	 0.337 0.413 0.748	 7.95 9.73 17.61				
Each value is a mean of 5 replicates. Values between brackets are arcsine square root of transformation percentage of inhibition.						

Effect of culture filtrate of the tested bioagent against *A. alternata*

This experiment was carried out to investigate the inhibitory effect of culture filtrate of *T. harzianum* at different concentrations on the linear growth of *A. alternata*. Increasing the concentration significantly increased the inhibitory effect of the cultures filtrate. The inhibitory effect of *T. harzianum* filtrate at three concentrations, *i.e.* 0, 10, 20 and 30% against linear growth of *A. alternata* was evaluated. Results in Figure (7) show that all tested concentrations of *Trichoderma* filtrate had inhibitory effect on fungal pathogen. The

inhibition zone was observed around mycelial disc. The diameter of inhibition zone was reduced with increase in concentration.

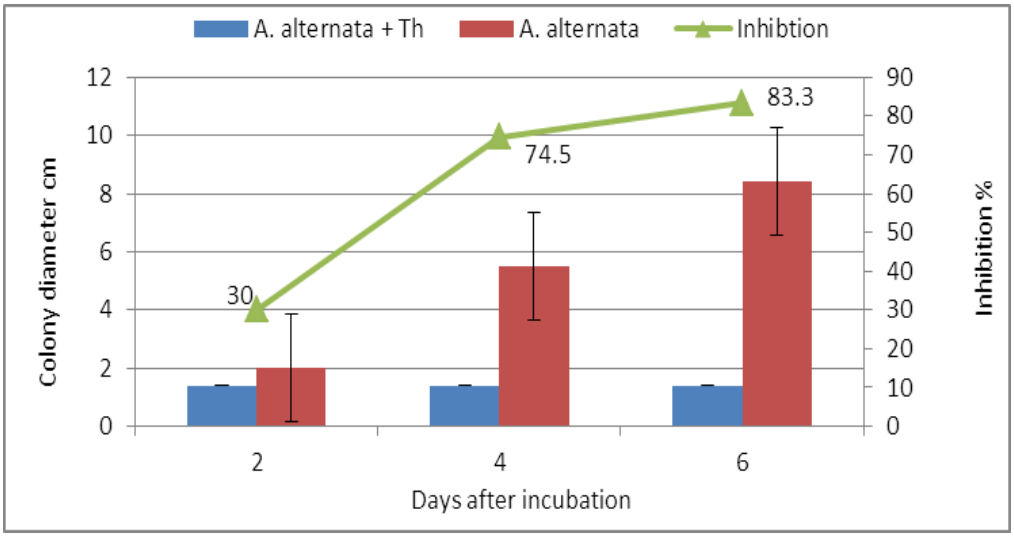

Fig. 6. Inhibition of *A. alternata* growth by *Trichoderma* conidial suspension. The Bars indicated to standard deviation.

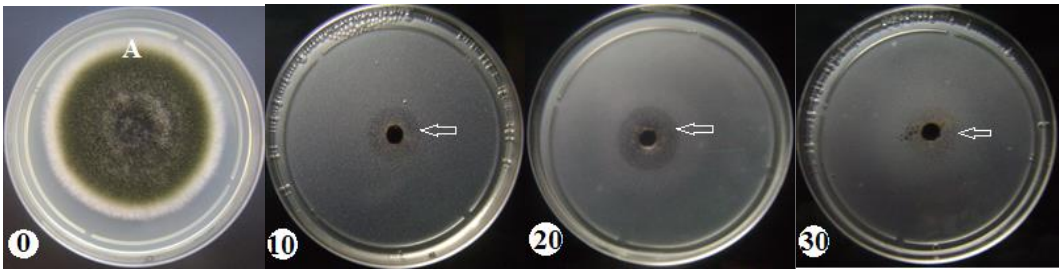

Fig. 7. Inhibition of *A. alternata* by culture filtrate (antibiotic substance) of *T. harzianum* at different concentrations. Inhibition zone at arrow.

Table. 3: Effect of different concentrations of *Trichoderma* filtrate on *Alternaria* linear growth.

Treatments	Filtrate concentration (%)	Diameter of inhibition zone (cm)	Diameter of fungal colony (cm)	Inhibition (%)
Trichoderma harzianum	0	0	7.87	0
	10	2.4±0.1	1.33±0.29	83.1 (65.73)
	20	1.8±0.1	1.26±0.06	83.9 (66.34)
	30	1.4±0.1	0.89±0.32	88.7 (70.36)
LSD at 0.05				NS

Each value is a mean of 3 replicates.
Values between brackets are arcsine square root of transformation percentage of inhibition.
NS: Non Significant

The results tabulated in Table (3) indicated to decreased in colony diameter with increasing filtrate concentration in PDA medium comparison with control treatment . It was reached to 1.33 cm at conc.10% and decreased to 0.89 cm at conc. 30% compared with control (7.87 cm). Also the diameter of inhibition zone was recorded 2.4 cm then decreased to 1.4 cm at concentrations 10% and 30% respectively. Data in Figure (8) showed that the inhibition with a different ratios, it was increase from 83.1% at 10% conc. to 88.7% at 30% conc.

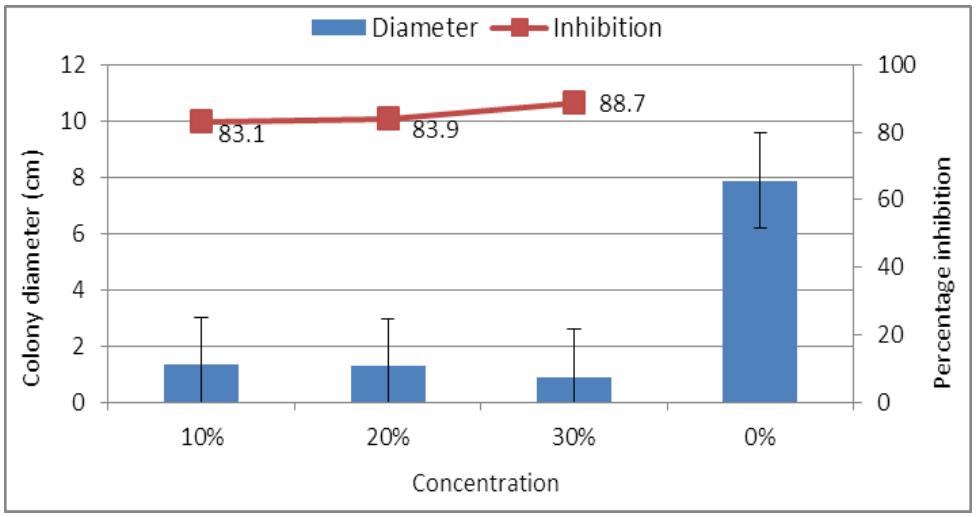

Fig. 8. Rates of growth inhibition of *A. alternata* by different concentrations of *Trichoderma* filtrate. The Bars indicated to standard deviation

DISCUSSION

Some microorganisms may play an important role in the controlling of some plant diseases. The obtained data showed that there was a promising antagonistic species of fungi prevalent on carob leaves, which could be exploited for the control of Alternaria leaf spot (El-Gali, 2014c). *T. harzianum* inhibited the growth of the target organisms through its ability to grow much faster than the pathogenic fungi thus competing efficiently for space and nutrients. Starvation was the most common cause of death for microorganisms, so that competition for limiting nutrients resulted in biological control of fungal phytopathogens. The genus *Trichoderma* comprises a great number of fungal strains that act as biocontrol agents (El-Gali, 2003; Bendahmane and Mahiout , 2012; El-Gali, 2015). Fungi of the genus *Trichoderma* have long been recognized for their ability to act as biocontrol agents against plant pathogens. During this time, research has described their mechanisms of action and how they might be used for various purposes (Harman, 2006). A second mechanism of pathogen control was mycoparasitism. Microscopic observation of the interaction region between *A. alternata* with *T. harzianum* showed that the mycelia of *T. harzianum* grew on the surface of

the pathogens always coiling round their mycelia and later penetrating their cell walls directly without formation of appresorium structures. The pathogen mycelia then disintegrated suggesting enzyme action. Lorito *et al.* (1998), Metcalf *et al*. (2001) and demonstrated possible role of chitinolytic and/or glucanases enzymes in bio-control by *Trichoderma.* These enzymes function by breaking down the polysaccharides, chitin, and glucans that are responsible for the rigidity of fungal cell walls, thereby destroying cell wall integrity limiting the growth of the pathogen. A mixture of several enzymes might be necessary for efficient cell wall lysis. *T. harziunum* has been reported to apply high chitinase and β-1, 3- glucanase activities (Sivan *et al*., 1984).

The third mechanism of pathogen control by conidial suspension of *Trichoderma* was through abnormal growth of fungal pathogen. The mycelia of *Alternaria* showed abnormal morphology such as The *A. alternata* conidia germination was strongly suppressed by chitinase derived from *Trichoderma spp*. for eight hours, resulting in the complete inhibition of germination, abnormalities, or breaking the germination tubes (Gang et al., 2004). Hyphal shrinking, cell shortening and septa thickening were found by Barbosa et al. (2001) in *Cladosporium herbarum*, in the presence of *Trichoderma.* Exactly as we observed, spores of *Trichoderma* were visible in the microscopic preparations. Morphological abnormalities in the pathogens's structure are confirmed by Ghildyal and Pandey (2008). Induction of deformities is causing by ability of *Trichoderma* to develop the direct interaction with a pathogen and produce antimicrobial substances as well as mycoparasitism involving a physical contact and production of hydrolytic enzymes, toxic components and antibiotics. breakdown the mycelium and cause a reduction of sporulation on the edge of the zone of inhibition due to secretion of antibiotic substances circulating in the culture medium.

A forth mechanism of pathogen control by *Trichoderma* was through antibiosis. Culture filtrates of the isolates had an inhibitory effect on the radial growth of the pathogens and mycelial accumulation suggesting action of non-volatile antibiotics in the filtrates. This agrees with the findings of Sivan *et al.*, (1984) who noted that culture filtrates of *T. harzianum* strongly inhibited the growth of *Pythium aphanidermatum* whereas *T. hamatum* filtrates caused only minor inhibition of growth. Antibiotic inhibitions have been documented by Claydon *et al*. (1987), Dubey and Suresh (2006), Kucuk and Kivanc (2003) and Lynch (1990). Claydon *et al*. (1987) reported inhibition due to antibiotics trichodermin, harzianum

A and harzianolide. Dubey and Suresh (2006) found that non-volatile substances produced by *T. harzianum* are inhibitory to *F. oxysporum f. sp. ciceris* causing chickpea wilt.

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