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Charcoal Disease (*Biscogniauxia mediterranea*) Control Using Biological and Chemical Compounds in Vitro

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Abstract: *Biscogniauxia mediterranea caused a lot of damages to the forests of Zagros and Hyrcanian of Iran, process and host range of this disease is increasing. To control the B. mediterranea, amples of affected tissue to charcoal disease in the Khorramabad forests were collected and transferred to the laboratory. The experimental design was completely randomized design with four replications, and Duncan test was used to compare means. Results of dual culture showed B. mediterranea in propiconazole and Trichoderma harzianum treatments had the lowest area during the growth period respectively with 1.02 and 4.6 cm^2. Treatments of propiconazole and T. harzianum respectively with 95.57 and 80.14 percent have highest growth inhibitory and treatments of copper oxychloride and T. virens have lowest growth inhibitory. The results of this study showed that the treatments of propiconazole and T. harzianum have best performance for control the B. mediterranea. Given that the function of biological agents and industrial components closer together and also the use of chemical pesticides in forests is limited Therefore, T. harzianum and T. virens can be used in the forest.*

Keywords: *Charcoal disease, Biscogniauxia mediterranea, Trichoderma harzianum, propiconazole, Zagros*

1. Introduction

From 1900 until now, decline and mortality of oak species have been reported in large area of forests in the world. Outbreak of this event with different names such decline, wilting and mortality, is caused by reaction pests and diseases to environmental stresses [16].

Endophytic fungi are sensitive to changes in temperature, humidity and host physiology. These changes makes endophytic fungi change their population size, reproduction and distribution and they can as a live indicator of climate change are known [2]. Oak charcoal disease pathogen (*Biscogniauxia mediterranea*) lives in tissues such as skin of trees, so that in trees under stress, *B. mediterranea* quickly occupied the xylem and bark and it makes reduce the growth rate, rate of photosynthesis and stomatal conductance, induce necrosis and canker, finally, the weakening of the host and mortality of oak trees in large areas and disrupt the composition, structure and function of the forest [4, 6, 15]. The genus of *Biscogniauxia* due to create cankers in the Mediterranean and semi-Mediterranean oak trees, has become one of the main problems oak forests of the world (United States, Africa, Italy, Spain, Portugal, Turkey and Iran), especially in areas that have been climate change [10, 11, 14]. Charcoal disease in Iran, first time reported in the Zagros Oak Forests [13], but with the rise of climate change and environmental stresses, outbreak also expanded to other species include *Zelkova carpinifolia* in Golestan province [14] and *Amygdalus scoparia* in Lorestan province [18] were also affected. In relation to the control against the *B. mediterranea* pathogen, no study has been done.

Campanile *et al*., (2006), in order to control oak canker disease in the forests of Italy, relationships antagonistic endophytic fungi *Trichoderma viride* ، *Epicoccum nigrum* ،*Sclerotina sclerotiorum* ، *Fusarium tricinctum* ،*Alternaria alternate* were evaluated with the causative agent (*Diplodia corticola*). To investigate the inhibitory effect of the antagonistic fungi, the dual culture on PDA was used. The results showed that *T. viride* with 28.5 percent and *F. tricinctum* with 4.2 percent had the highest and lowest inhibitory effect, respectively [3].

Martinez *et al*., (2015), with dual culture method, tested 154 isolates of endophytic fungi against *Fusarium circinatum* (Canker disease agent of pine trees). 138 isolates were antagonistic relationship with *F. circinatum*, among them 6 isolates were causes the most reduced growth of the *F. circinatum*. The 6 isolates on infected seedlings of *Pinus radiata*, *P. sylvestris*, *P. pinaster*, *P. nigra* and *P. pinea* were inoculated. The results showed that *Chaetomium aureum* and *Alternaria sp.* had the highest inhibition of the growth of the *F. circinatum* in host plants and these two fungi can be used as biological control [12].

Berger et al, (2015), evaluated the effects of chemical and biological control of *Phytophthora* spp. in the oak and beech forests of Poland. In this research evaluated *Trichoderma harzianum*, two strain of *T.atroviride* and *Bacillus amyloliquefaciens* as biological agents and phosphite (H3PO3) as chemical agent. Results showed that *T. harzianum* with 56.78 percent, *T. atroviride* with 54.56 percent and *B. amyloliquefaciens* with 34.31 percent have the highest growth inhibition relative to *P. cactorum*, *P. plurivora* and *P. quercina* respectively. Phosphite, *T. atroviride* and *T. harzianum* with 85.77 percent, 83.92 percent and 77 percent have the highest reducing the level of necrosis in the leaves of oak and beech trees relative to the control. Finally, due to the high impact species of *Trichoderma*, their use for control Phytophthora species in forests were suggested [1]. In the research of Vijayaraghavan et al., (2007), dual culture method was used for biological control of *Phytophthora* diseases. In this experiment of the 22 fungi, 20 bacteria and 5 actinomycetes were used. All the isolates of fungi and 5 bacteria prevents with various degrees of the disease development. The results showed that *T. harzianum* have highest percentage inhibition (100%) among all antagonistic [20].

Due to the spread and high damage of charcoal disease in the forests of Iran, it is necessary to adopt strategies to deal with it. Biological control can be useful method for the inhibition of charcoal disease. The aim of this study is to comparison of the effects the use of antagonistic fungi and industrial fungicides in deal with charcoal disease in the in vitro conditions.

2. Material and methods

The study area is the Kakasharaf forest of Lorestan is located on the Zagros Mountain, western of Iran. The study area has, 430 hectares area, and is positioned between 33° 20′ 54″ to 33° 22′ 10″ northern altitude and 48° 29′ 23″ to 49° 30′ 9″ eastern longitude (*See* Figure 1). Its mean annual precipitation is 496.4 mm. The altitudes of the region are ranging from 1390 to 1510 meters from mean sea level. The main forest stand types in the study area are quercus brantii in northern slopes and Amygdalus scoparia in southern slopes. The study area is an infected stand to charcoal disease.

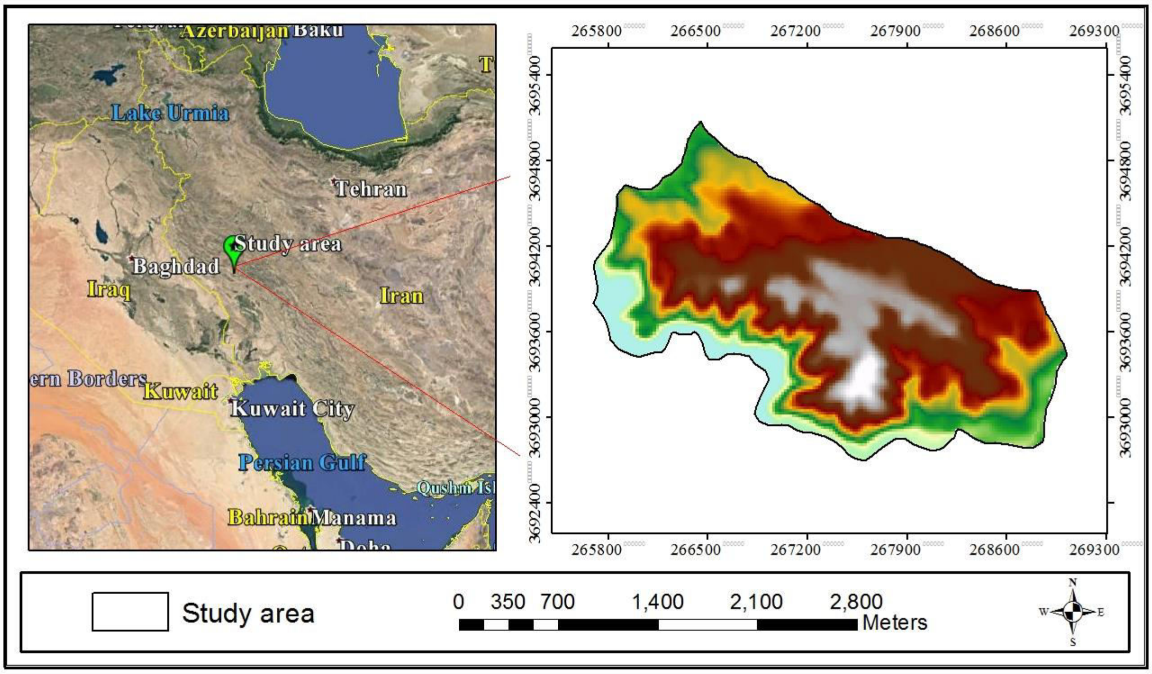


Fig. 1. The region under study

To prepare isolates of the *B. mediterranea* samples of lesions tissue from oak trees were collected and then transferred to the laboratory. After washing with water, samples in ethanol 70% for one minute, sodium hypochlorite 3% for one minute and finally for one minute in distilled water were washed (Martinez *et al*. 2015), then these samples dried with filter paper and 3 to 5 pieces of each sample were cultured on PDA and then were purified [9, 17].

In this study, two species *T. harzianum* and *T. virens* as antagonistic fungi for biological control with dual culture and volatiles culture methods and two propiconazole and copper oxy chloride as fungicides industrial were used. Due to the low growth of the pathogen in comparison with *Trichoderma*, five millimeters pieces from the edge of *B. mediterranea* colonies on PDA were placed then petri dishes incubated for 96 hours at a temperature of 24 degrees Celsius. After this time five millimeters pieces from the edge of the three-day culture of *Trichoderma*, on the other hand of petri dishes, was placed.

Percentage of inhibition of mycelial growth of *B. mediterranea* was calculated using the following equation [7].

Percentage of inhibition of the fungi growth =

$$\frac{\text{Growth area in the control} - \text{Growth area in the treatment}}{\text{Growth area in the control}} \times 100$$

In the volatiles culture method due to the low growth of the pathogen in comparison with *Trichoderma*, five millimeters pieces from the edge of the ten-day culture of *B. mediterranea* on center of petri dishes was placed then incubated for 96 hours at a temperature of 24 degrees Celsius. After this time five millimeters pieces from the edge of the three-day culture of *Trichoderma*, on the center of other petri dishes was placed. Then, the doors of Petri dishes include *B. mediterranea* and *Trichoderma* was put together and with parafilm strip were closed [5, 8]. In chemical control methods, fungicides with standard concentration (0.02%) were added to PDA, then the 5 mm parts of *B. mediterranea* were placed in it.

Using Digimizer software calculated growth area of fungi in each treatment on a daily basis. Measurements were performed until the ninth day, because after 9 days growth of fungi in the control sample reaches the maximum. The experimental design was completely randomized design with four replications, and Duncan test was used to compare means.

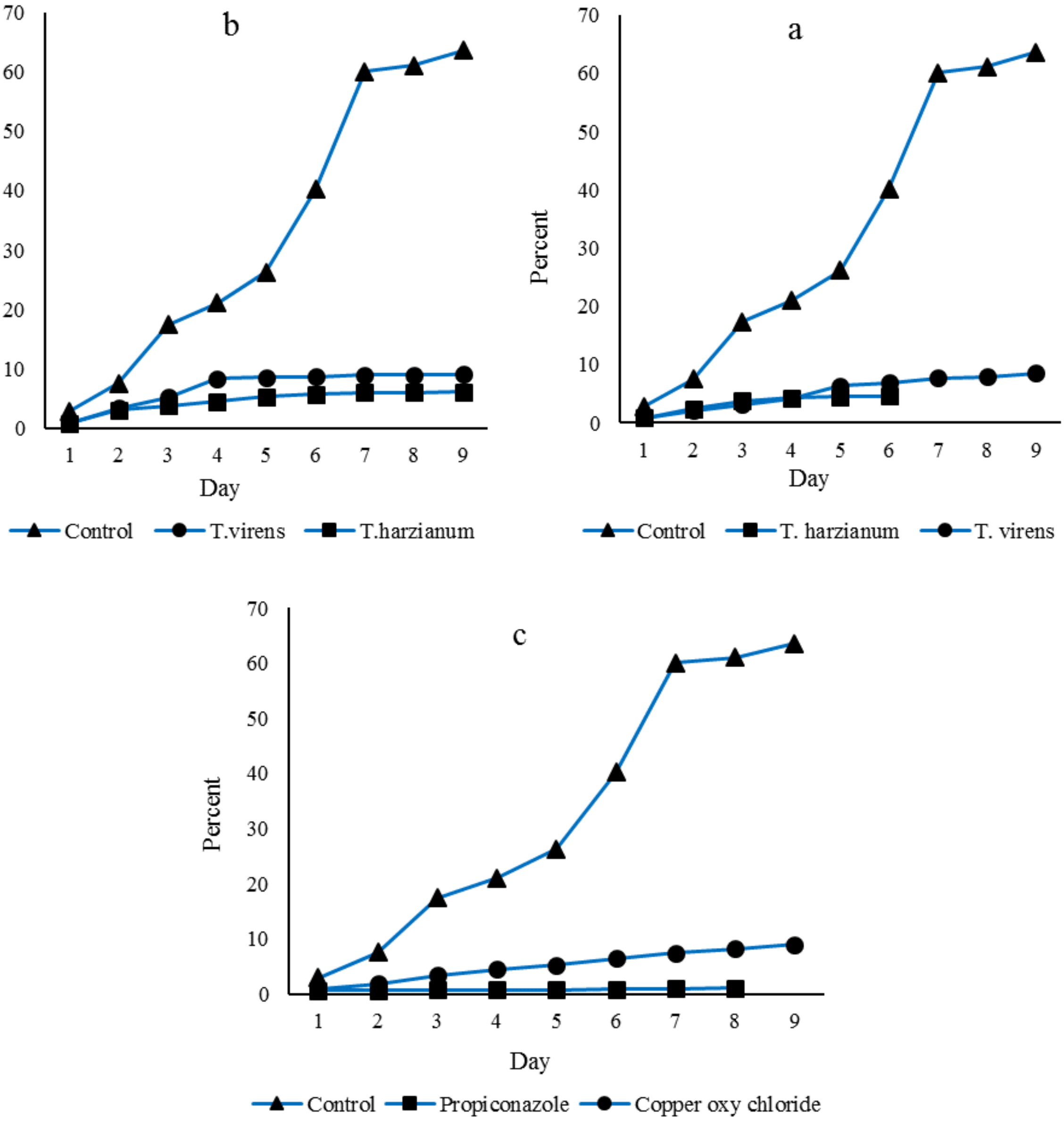
3. Result

Results showed in dual cultures and volatiles culture methods growth of control treatment (*B. mediterranea*) is lower than *Trichoderma* spp. treatments. After 9 days stop the growth of *B. mediterranea* was observed, its growth size was equal to 23.162 cm^2. In dual culture method *Trichoderma harzianum* has a better performance than *Trichoderma virens*, so that growth size of *B. mediterranea* in *T. harzianum* treatment equal to 4.6 cm^2 and in *T. virens* treatment equal to 8.52 cm^2 were measured. In volatiles culture, growth size of *B. mediterranea* in *T. harzianum* and *T. virens* treatments were equal to 6.09 cm^2 and 9.08 cm^2, respectively (Fig. A-2 and B-2).

In the fungicide treatments growth of *B. mediterranea* was lower than control treatment, so that growth size of *B. mediterranea* in propiconazole treatment and copper oxy chloride treatment were equal to 1.02 cm^2 and 8.97 cm^2, respectively (Fig. C-2).

Percent

Growth size of *B. mediterranea* in dual cultures, volatiles culture and fungicide culture methods, as well as control has been shown in figure 3.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Day
Control
T. harzianum
T. virens
c
Percent
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Propiconazole
Copper oxy chloride

Fig. 2. The growth trend for *B. mediterranea* in; a: Dual culture treatment; b: Volatiles culture treatment; c: Fungicide treatment

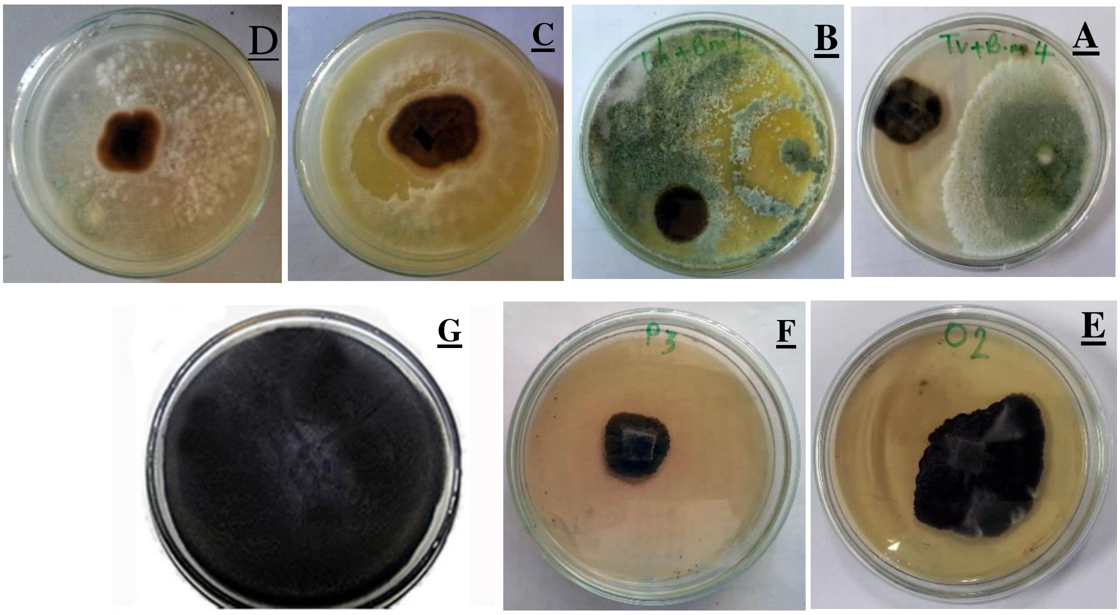

Fig. 3. The growth for *B. mediterranea* in; A: Dual culture with *T. virens*; B: Dual culture with *T. harzianum*; C: Volatiles culture with *T. virens*; D: Volatiles culture with *T. harzianum*; E: Chemical culture with copper oxy chloride; F: Chemical culture with propiconazole; G: Control treatment

3.1. Average daily growth of *B. mediterranea* in the treatments

Analysis of variance show there is a significant differences in the level of 99 percent between Average daily growth of *B. mediterranea* in the treatments (Table 1). According to the results, *B. mediterranea* in the treatments of propiconazole with 0.13 cm^2 and *T. harzianum* with 0.76 cm^2 has lower average daily growth. In the control treatment *B. mediterranea* has highest average daily growth compared to another treatments (Fig. 4).

Table 1. Analysis of variance of daily growth area for *B. mediterranea* in different treatments

Source	SS	DF	MS	F
Treatment	215.027	6	35.838	19.837 **
Errore	413.704	229	1.807	
Total	628.731	235		

** Indicates a significant difference in the level of 99 percent

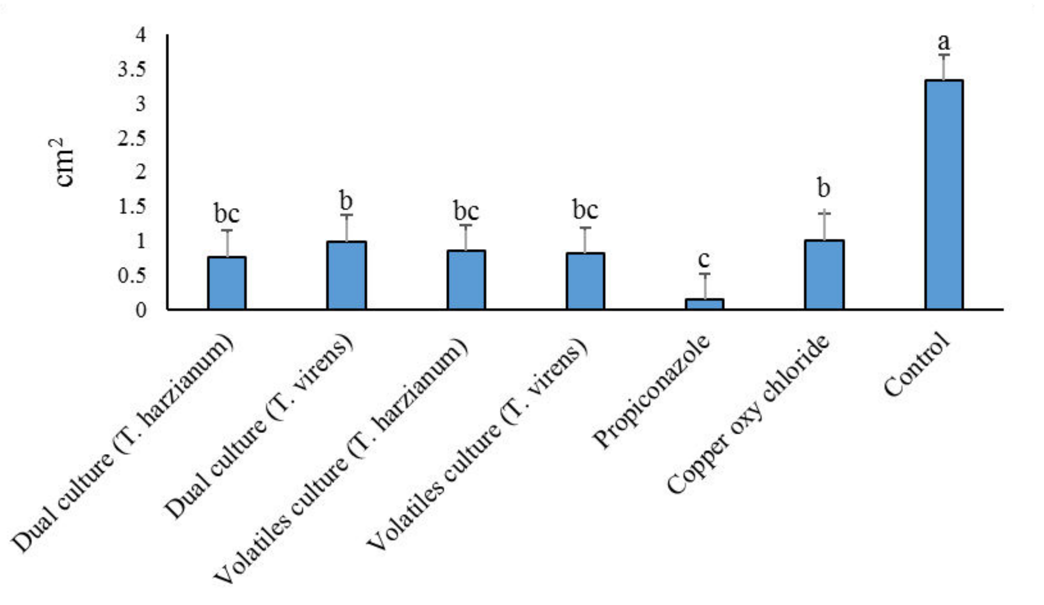

Fig. 4. The average growth rate of *B. mediterranea* in biological and chemical treatments

3.2. Growth inhibition percentage of *B. mediterranea* in biological and chemical treatments

X^2 test show significant differences between Growth inhibition percentages of *B. mediterranea* in all treatments (table 2).

Table 2. Chi-square test for growth inhibition percent of *B. mediterranea* in different treatments

X^2	Df	Sig
13.317	5	0.021

In the dual culture method with the passage of time, inhibition ability of *T. harzianum* and *T. virens* treatments will increase. In the nine day after culture *T. harzianum* with 80.14 percent than *T. virens* with 63.2 percent has a better performance. While growth inhibition fluctuations in *T. harzianum* is less than *T. virens* treatment (Fig. 5).

In Volatiles culture method, 9 day after culture growth inhibition of *B. mediterranea* in *T. harzianum* and *T. virens* were 73.69 percent and 60.77 percent respectively. In terms of inhibition ability *T. harzianum* has a better performance and as well as it has less growth inhibition fluctuations than *T. virens* (Fig. 6).

Growth inhibition trend of *B. mediterranea* in chemical treatments showed that propiconazole with 95.57 percent than copper oxy chloride with 61.24 percent has better performance and as well as propiconazole has less growth inhibition fluctuations than copper oxy chloride (Fig. 7).

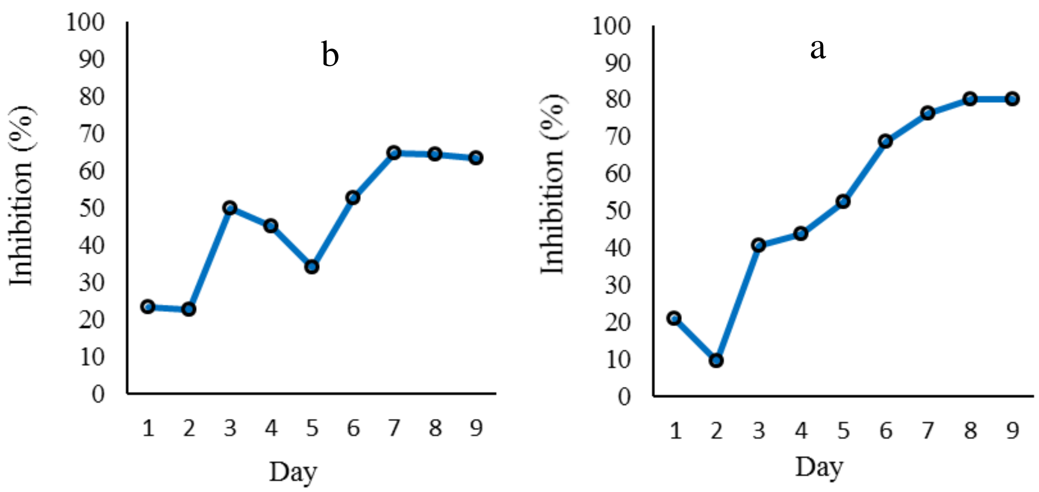

Fig. 5. Inhibition trend of B. mediterranea in dual culture method, a: *T. harzianum*, b: *T. virens*

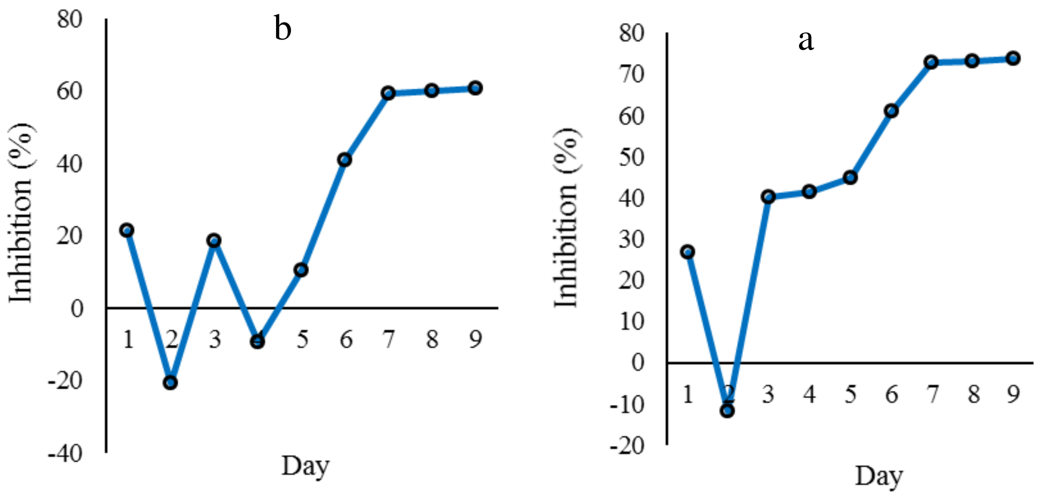

Fig. 6. Inhibition trend of B. mediterranea in Volatiles culture method, a: *T. harzianum*, b: *T. virens*

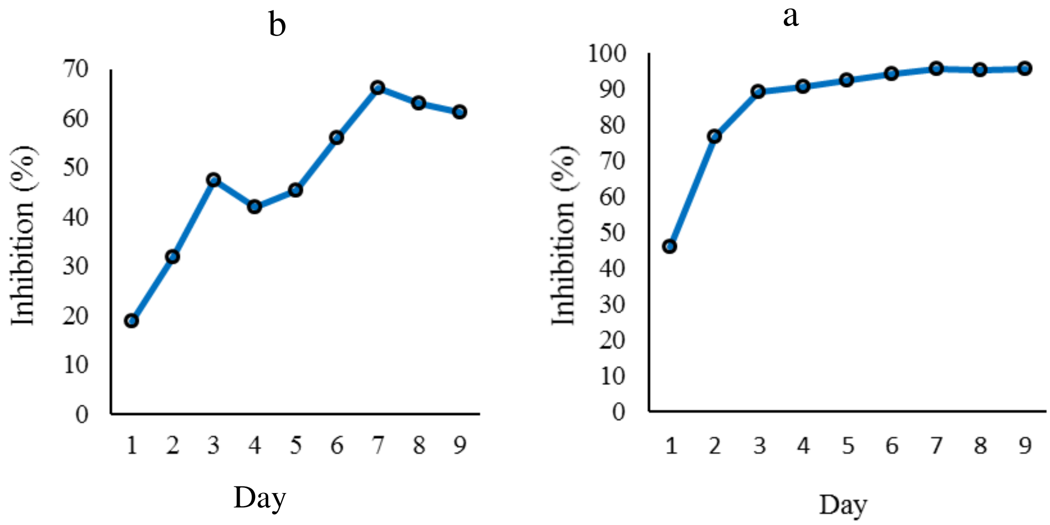

Fig. 7. Inhibition trend of *B. mediterranea* in chemical treatments, a: Propiconazole, b: copper oxy chloride

4. Discussion

For different reasons Zagros forests are influenced by environmental stresses. Some of these reasons include weather and climatic factors such as drought and high temperatures, soil erosion, understory planting, overgrazing livestocks, deforestation by man, fire and etc. can be mentioned which makes are physiological weakness trees. Charcoal disease outbreak following environmental stresses and physiological weakness trees and it is causing pollution and dieback trees at widespread. In forest regions, biological control is one of the effective and importance methods for plant diseases inhibition.

According to results *B. mediterranea* has lower growth area in propiconazole treatment. In dual culture method and *T. harzianum* treatment growth of *B. mediterranea* was stoped after 6 days.

Treatments used were cause low growth *B. mediterranea* compared to control treatment. Results show in total, propiconazole and *T. harzianum* treatments has a better performance compar to *T. virens* and copper oxy chloride treatments. propiconazole and *T. harzianum* treatments has highest inhibition of *B. mediterranea* compar to control treatment and showed significant differences with *T. virens* and copper oxy chloride treatments. Results of another researchers as well as shows the *Trichoderma* especially *T. harzianum* has optimal performance in ordre to diseases control (Campanile *et al*., 2006; Berger *et al*., 2015; Vijayaraghavan *et al*., 2007; Savage *et al*., 2003).

Due to the performance of biological and chemical factors are almost close to each other and on the other hand the use of chemical materials is prohibited, it can be suggested that the two species of *Trichoderma* used in this research can be used to charcoal disease control in the forests.

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