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Antifungal activity of some plant extracts and *Trichoderma* spp. against cucumber damping off caused by *Pythium aphanidermatum*

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Abstract

The present study aimed to use some plant extracts and two different species of *Trichoderma* spp. to control damping off disease in cucumber caused by *Pythium aphanidermatum* instead of using chemicals where serious environmental problems caused by several chemical fungicides. Some plant products such as plant metabolites and plant based pesticides considered to be better alternatives as they are known to have environmental impact as opposed to chemical pesticide. In the antagonistic assay that carried out with *Trichoderma* spp., the results revealed high inhibition on the tested pathogen, but in an assay of plant extracts somerplant extracts with different concentrations were tested *in vitro* for their activity against *P. aphanidermatum*. Some of the tested plant extracts significantly reduced the radial growth of the pathogen. Cinnamon, carnation and camphor were the most affective from all the eight tested plant extracts than the other extracts, where with the concentration of 30.000ppm Cinnamon, Carnation and Camphor gave the highest inhibition values against *P. aphanidermatum*, while mango and mint extracts did not give any inhibition for the radial growth of *P. aphanidermatum*.

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Introduction

Cucumber (*Cucumis sativus* L.) is one of the most important vegetable crops which grown all over the world in open fields, tunnels and greenhouses (Wang *et al*., 2007). There are serious seed-borne and soil-borne pathogens exist at the rhizospher area, i.e. *Fusarium* spp., *Rhizoctonia* spp., *Macrophomina phaseolina*, *Seclrotinia sclerotiorum* and many species of *Pythium*, can cause seed rot and damping off disease, so cucumber seedlings are susceptible to most of these pathogens which lead to significant economic losses in the quality and yield of cucumber crop (Messiaen *et al*., 1995). The application of plant extracts and biological agents as an alternative ways for the management of plant diseases has recently become an important variable component for Integrated Pest Management (IPM), so plant metabolites regarded as eco-friendly where plants play an important role in the control of diseases (Sahayaraj *et al*., 2009). So the widespread of using chemical fungicides to control plant diseases is leading to an increase of human being hazards due to phytotoxic residual and environmental pollution effects, therefore, applying other method like using biochemical agents and plant extracts are strongly recommended, beside the biological control of plant diseases has received considerable attention as an alternative strategy (Salih M. R. and Ismael J. H. S., 2016).

Trichoderma spp. isgone of the most important factors used in biological control against pathogens, it has been widely used and proved to be a great success on some diseases because of the ease of getting it from the soil and its quick isolation as well as the availability requirements of its growth and reproduction, also their growth in temperatures and humidity can be easily obtained (Harman, 2000).

Application of plant extracts triggers plants' latent defense mechanism in responses to infection by pathogen, and also an aqueous plant extract is rich with nutrient and microorganisms so this application can stimulate growth, protect plants from disease and help suppress of soil-borne pathogen (Quarles, 2011). use of carnation and cinnamon extracts are significantly reduced the pre and post-emergence damping off of faba bean caused by *Rhizoctonia solani* and *Fusarium solani* compared with the control treatment, so they showed superior reducing effect on the pre-emergence damping off incidence when applied as powder or extracted materials (El-Mougy *et al*., 2007). An aqueous extract of *Cinnamomum camphora* has an important role of using its oil as natural pesticides in the view of the environmental and toxicological inference of the haphazard use of artificial pesticides and reducing the trouble of increasing pest resistance (Batish *et al.* 2000). Using of the oil of carnation in inhibiting the radial growth of *P. aphanidermatum* where the rate of inhibition reached to 100% at 500ppm concentration (Kareem, *et al*., 2009). When testing the Eugenol and Isoeugenol compounds which regard the main components of the essential oils of carnation, that these substances have inhibited the growth of *Fusarium oxysporium* and *F. spranilla* which cause stem rot of flannel trees, and also *Aspergillus* spp. and *Penicillium* spp. where the inhibition of the growth of these fungi was 100% (Mansour *et. al*., 1996).

The aim of the present study is to evaluate the effect of some plant extracts and biological agents particularly *Trichoderma* spp. against the soil-borne pathogen *Pythium aphanidermatum* on cucumber seedlings under laboratory conditions.

Materials and methods

Isolation and identification of P. aphanidermatum and the accompanied fungi

P. aphanidermatum was isolated by using the baiting technique, with some modifications. Cucumber fruits were used as a natural medium to obtain pure isolates of the pathogen (Dewan, 1989) also by using dilution method till reach to 10^{-4} and 10^{-5} dilutions from sample of soil with and without plant residues (Belete *et al*., 2015 ; Killani *et al*., 2011). Two isolates of the pathogen were obtained (X1 and X2) in addition to high frequencies of the other accompanied fungi which were purified individually in the sterile petri dishes containing PDA. The frequency ratio was determined according to the following equation:

$$\text{Percentage of frequency (\%)} = \frac{Number\ of\ fungal\ colonies}{Total\ number\ of\ colonies} \times 100$$

The isolates of pathogen *P. aphanidermatum* identified using morphological features (Van der Plaats-Niterink, 1981) and the other fungi identified as mentioned by (Domsch *et al.*, 1980).

Two biological agents were used in this study, *Trichoderma harzianum* was obtained from Centre of Scientific Research, Ministry of Science and Technology, Iraq, and *Trichoderma hamatum* strain (T- 113) was obtained from the Laboratory of Field Crops, Faculty of Agriculture, University of Wasit, Iraq. Then have been grown in slant, with PDA and saved in Refrigerator at 5 °C to use it when needed.

Preparation of plant crude extracts

The study involves using of eight plant extracts as treatments include: Camphor, Oleander, Mint, Mango, Guava, Henna, Cinnamon and Carnation (Table 1), were collected, washed thoroughly with tap water, and allowed to dry under shade until completely dry, then extracted according to (Bajwa *et al.*, 2003). The filtrates were then preserved in dark glass bottles in the freezing temperature. While the extracts that obtained were stored as a stock of 100.000ppm in the refrigerator at - 5°C to use when needed.

Table 1. The Plant extracts were used in the study.

Common name	Scientific name	Family	Extracted plant part
Camphor	*Cinnamomum camphora*	Lauraceae	leaves
Carnation	*Dianthus caryophyllus*	Umbelliferae	flowers
Cinnamon	*Cinnamomum zeylanicum*	Lauraceae	bark
Guava	*Psidium guajava*	Myrtaceae	leaves
Henna	*Lawsonia inermis*	Lythraceae	leaves
Mango	*Mangifera indica*	Anacardiaceae	leaves
Mint	*Mentha piperita*	Labiatae	leaves
Oleander	*Nerium oleander*	Apocynaceae	leaves

In-vitro experiments

To examine the sensitivity of *P. aphanidermatum* to the biological agents and plant extracts, a laboratory study of the plant extracts was performed with three concentrations 10.000, 20.000, 30.000ppm for each extract and an antagonistic study of *P. aphanidermatum* with bio-agents *T. harzianum* and *T. hamatum* by using mycelial discs 0.5 cm Diameter using dual culture technique were inoculated simultaneously in the right half of the plates contained solidified PDA (Potato Dextrose Agar) and 0.5 cm Diameter mycelial growth discs for each of the two isolates of *P. aphanidermatum* (X_1 and X_2), with an equal distance of 4cm between both discs of the pathogen and the bio-agents (three replicates for each treatment). As well as the control treatments have the two isolates of the pathogen *P. aphanidermatum* only. All plates were incubated at 25±2°C, when the mycelial growth of *P. aphanidermatum* in the control treatment filled the Petri plate after 3 days, percentage of the fungal growth inhibition was calculated by using the following formula: (Abd -El- Moity T.H. 1985).

$$X = \frac{G1-G2}{G1} \times 100$$

Where

X: fungal growth inhibition.

G1: Radial growth of the pathogen inoculated alone.

G2: Radial growth of the pathogen inoculated against the antagonistic fungus.

Pathogenicity test

HAYEL 2500 seeds of cucumber were sterilized by soaking it in 1% sodium hypochloride for 3 minutes, then it was washed several times with sterile distilled water to eliminate all traces of the sodium hypochloride, then dried it on blotting paper till the completely dried. Seeds were sown in plastic pots 10 cm in diameter and each pot contains 6 seeds with 3 replicates after contaminated by using the bio-mass of *P. aphanidermatum* (X_1) half petri plate 9cm for each pot. Plants were left to grow and irrigated with normal tap water when necessary. Control plants 3 replicates were left without added the pathogen. Seven days after sowing (DAS). The pathogenicity of the isolate (X_1) was evaluated by calculated the percentage of seed rot, pre and post – emergence damping off and the percentage of the survival seedlings. Disease assessment was recorded at 7 days after sowing according to a scale of (0–4) that described below (Decal, 1997) by recording the percentage of the infection.

Then the percentage of disease severity (D.S.) was recorded depending on the percentage of different symptoms which were seed rot, pre and post – emergence damping off using a scale of 5 scores (0 – 4) where the percentage of the infection was the same as the percentage of disease severity which was 100%.

Score	Infection
0	No Infection (0%)
1	Infection (1 – 25%)
2	Infection (26 – 50%)
3	Infection (51 – 75%)
4	Infection (76 – 100%)

Disease severity (D.S.) was measured depending on the equation of Mickenny that mentioned in (Al-Waely, D.S.A. 2004):

$$D.S\% = \frac{(n \times v)}{4N} \times 100$$

Where:

(n)= Number of plants in each category.

(v)=Numerical values of percentage infection in each category.

(N)=Total number of plant.

(4)= maximum numerical value of percentage infection category.

Effect of plant extracts on the radial growth of P. aphanidermatum

In-vitro, an aqueous plant extracts (Table 1) were screened based on the inhibition of radial growth of the pathogenic fungus *P. aphanidermatum*. Highly pathogenic isolate of *P. aphanidermatum* was chosen to evaluate the effectiveness of the extracts in inhibiting the radial growth of the pathogen. Three concentrations 10.000, 20.000, 30.000ppm have been made for each extract to determine the most effective concentrations in controlling of damping off disease in cucumber. Extracts were also sterilized by evaporation process using ethylene chloride by placing the extracts in small open-flask bottles and then placed in a large beaker contained in the bottom ethylene chloride and then covered with a sterile plastic food packaging then left the stocks of extracts in the laminar flow cabinet for 24 hour to sterilize the extracts before using them.

Extracts were added with the above concentrations to the PDA medium before the solidify of the medium at 45°C using a Food Poisoning Technique (FPT), mixed well until the medium takes the colour of the added extracts. Chloramphenicol 100mg/liter were added to the medium to ensure non-growth of bacteria.

Then 0.5 cm mycelial plugs of *P. aphanidermatum* (X_1) were put in the center of the tested plates. Three replicates for each concentration of the using extracts were incubated at 25± 2°C for 3 days, with 3 plates which were contain sterile distilled water with the same concentrations were put instead of the mentioned extracts as control treatments. After 3 days when the mycelial growth of the control treatments reach to the edge of the plates, the radial growth of *P. aphanidermatum* (X_1) were recorded.

Statistical analysis

The Statistical Analysis System- SAS (2012) program was used to effect of difference treatments in study parameters (Randomized completely Block Design-RCBD in farm experiments and Completely Randomized Design-CRD in laboratory experiments). Least significant difference LSD test (ANOVA) was used to significant compare between means in this study.

Results and discussion

Isolation of the accompanied fungi

The results showed that number of different microorganisms were isolated from two samples of soil with and without plant residues by using dilution method (Table 2) (Belete *et al.*, 2015; Killani *et al.*, 2011). The results of serial dilutions showed variation of different fungal species in addition to *P. aphanidermatum*, which were the highest proportion of the fungal appearance in the soil that is with and without plant residues was *Rhizoctonia solani* where it recorded the highest percentage of frequency which was 81.4% and 53.1% respectively and the lowest appearance in the same samples of soil was 8.7% and 11.5% respectively which were recorded for *T. harzianum*. While the ratio of the appearance of *P. aphanidermatum* was 33.8% and 15.2% respectively.

These results give an indication of the numbers and species of different fungi that lived at the same habitat where the pathogens present.

Table 2. Percentage of the accompanied fungi for *P. Aphanidermatum.*

The fungi	Appearance of fungi%	
	Soil with plant residues	Soil without plant residues
Alternaria alternate	20.3	27
Aspergillus niger	25.5	41.6
Fusarium solani	50.7	47.8
Rhizoctonia solani	81.4	53,1
Trichoderma harzianum	8.7	11.5
Pythium aphanidermatum	33.8	15.2

The isolation results revealed that the fungus *Rhizoctonia solani* was the highest frequency in the two samples of soil and was found in all replicates and ranged by 81. 4% in the soil of plant residues, followed by *Fusarium solani* with 50.7% in the soil sample containing plant residues, so this is consistent with what was recorded by (Jebir K. S., 2001) that these two species of fungi were settler in the soil and attack the roots of different plant families, furthermore the repeated cultivation of the same plants annually and continuously in the same areas and the appropriate environmental conditions lead to the accumulation of the same pathogens in the soil. While the lowest frequency was *T. harzianum* with 8.7% in the soil sample containing plant residues, while the frequency of the target fungus *P. aphanidermatum* was 33.8% and 15.2% in the sample of soil with and without plant residues respectively, this result explains and confirms that the plant residues contain mycelium or spores of the pathogenic fungus *P. aphanidermatum* from the previous season. Both isolates of *P. aphanidermatum* have been grown and purified and labeled as (X_1 and X_2) for subsequent studies.

Effect of antagonistic fungi of biological agents on the radial growth of two isolates of P. aphanidermatum

The two isolates of *P. aphanidermatum* (X_1 and X_2) were isolated and tested in-Vitro with two biological agents *T. harzianum and T. hamatum* to determine the most antagonistic isolate against the biological agents by measuring the colony diameter of *P. aphanidermatum*. The results (Table 3) showed that the isolate (X_1) gave high antagonistic effect against the two biological agents which was 27.77% and 29.63% for the biological agents *T. harzianum and T. hamatum* respectively. While the isolate (X_2) showed less antagonistic effect against the same tested biological agents which were 45.55 and 49.26% respectively, so the isolate (X_1) will use for subsequent studies.

These results revealed that one of the two tested isolates of *P. aphanidermatum* has high ability of the antagonistic effect with the tested biological agents due to the secretion ability of different kinds of enzymes and toxins which act as an antibiotic and inhibit the radial growth of the pathogen.

Table 3. Antagonistic effect of Biological agents on the radial growth of two isolates of *P. aphanidermatum.*

Treatment		Inhibition%	Colony diameter (cm)
X_1	T. hz.	27.773	6.5
	T. hm.	29.627	6.333
	Control	0.000	9.00
X_2	T. hz.	45.553	4.9
	T. hm.	49.257	4.567
	Control	0.000	9.00
L.S.D at 0.05		0.6523	7.2478

The results of dual culture of antagonists and pathogen (Table 3) showed that both *T. harzianum* and *T. hamatum* had antagonistic effect on the two isolates of *P. aphanidermatum* (X_1 and X_2). The results produced by the antagonists have significantly differences with control as well as within them. *T. hamatum* strain (T-113) showed high inhibited the growth of the tested pathogen than *T. harzianum* with both of the two isolates of *P. aphanidermatum* (X_1 and X_2) compared with the control treatment, and the percentage of inhibition was more with the isolate (X_2) which revealed that the isolate (X_1) has antagonistic activity stronger than the isolate (X_2), this result was consistent with the similar results that recorded by (E. Christy Jeyaseelan *et al.*, 2012). So the expansion of the colony diameter of *P. aphanidermatum* was more with the isolate (X_1) than the isolate (X_2).

Disease severity was measured depending on the percentage of different symptoms which were seed rot, pre and post–emergence damping off using a scale of 5 scores (0 – 4) where the percentage of the infection was the same as the percentage of disease severity which was 100%, that represented under the score 4, which means that the death of the tested seedlings was 100%.

Pathogenicity test

One of the two isolates of *P. aphanidermatum* (X_1) which showed high antagonism against the tested biological agents was used for testing the pathogenicity of *P. aphanidermatum*, pots experiment was carried out in laboratory Conditions, where the sterilized cucumber seeds were sown in plastic pots 10 cm diameter. Some pots 3 replicates were contaminated with the bio-mass of *P. aphanidermatum* isolate (X_1), and others were not (Table 4). The experiment showed different symptoms of damping off disease represented by seed rot of some tested seeds where the percentage of seeds rotting was 66.66%, and the other symptoms was pre-emergence damping off of the growing seedlings which was 33.33%. While the control treatment did not show any symptoms of damping off disease in the tested pots. So the percentage of survival seedlings was 0.00%. The results showed high pathogenicity of *P. aphanidermatum* isolate (X_1) which was showed severe infection in cucumber crop. Thus the isolate of the pathogenic fungi *P. aphanidermatum* (X_1) showed high efficacy of pathogenicity, then it was used in all the subsequent studies.

Table 4. Evaluation of damping off disease on cucumber seedlings in pots.

Treatment	Seed rot %	Damping off of Cucumber seedlings%		Survival seedlings %
		Pre – Emergence	Post – Emergence	
Pots infested with *P.aphanidermatum* (X1)	66.66	33.33	0.00	0.00
Control without *P. aphanidermatum* (X1)	0.00	0.00	0.00	100

These results were identical to the previous studies carried out in Egypt on damping off disease of cotton which indicated that the main preceding pathogens implicated with this disease is *Pythium* spp. (Omar, MR. *et al*., 2007). And there is a study showed that all the isolated fungi of *Pythium* spp., *Fusarium* spp., reduced seeds variably of cucumber after ten days of infection. The pathogenic fungi are transmitted from the germinated seeds to the growing seedling causing pre- and post- emergence death. The transmission rate of the tested fungi causing seed rot or pre-emergence death was higher than that causing seedling mortality (Eman, *et al*., 2013). The effecting isolate (X_1) of *P. aphanidermatum* was selected for the subsequent studies.

Plant extracts

The results of using eight plant extracts with different concentrations, using fungal inhibition diameters technique (FID) were evaluated to control damping off disease caused by *P. aphanidermatum* (X_1) in-vitro which obtained, were statistically analyzed and observed in (Table 5). Three extracts from all of the tested plant extracts Cinnamon, Carnation and Camphor gave the highest inhibition (100% inhibition) of the radial growth of *P. aphanidermatum*, at 30.000ppm, while the extracts of Mango and Mint did not give any inhibition of the radial growth of *P. aphanidermatum* 0.00% at all the tested concentrations. While Oleander extract gave the best result at 20.000ppm which were 44.44%, and Guava and Henna extracts at 30.000ppm gave the highest inhibition of the growth of *P. aphanidermatum*, which were 86.66%, 57.77% respectively. The best tested plant extracts were Cinnamon, Carnation and Camphor which showed high antifungal activity, and the results which obtained in the present study showed that plant crude extracts possess high potential antifungal activity against *P. aphanidermatum*; however, the concentration of 30.000ppm was used to get high effective antifungal agents than the other concentrations.

The results showed significant differences between the tested extracts and the different concentrations and the interaction between them.

The high antifungal activity of the plant extracts of Cinnamon, Carnation and Camphor may be belongs to the influence on the defense mechanisms in responses to infection by *P. aphanidermatum*, and also an aqueous plant extract is rich with nutrient and microorganisms so this application can stimulate growth, protect plants from disease and the ability of the suppress of soil-borne pathogen (Quarles, 2011). So, in the present study the aqueous plant extracts of Cinnamon, Carnation and Camphor showed high antifungal activity when the results obtained showed that there were high antifungal activity of the mentioned plant extracts against *P. aphanidermatum*, where the concentration 30.000ppm was found to be more effective antifungal agents than using the other concentrations 10.000, 20.000ppm.

Table 5. The inhibition of plant extracts on the radial growth of *P. aphanidermatum*.

Extract	Inhibition% Concentrationppm			
	10.000	20.000	30.000	mean
Mango	0.00	0.00	0.00	0.0000
Mint	0.00	0.00	0.00	0.0000
Camphor	32.40	86.11	100	0.7284
Carnation	81.51	96.29	100	0.7160
Oleander	37.033	44.44	0.00	0.2716
Cinnamon	10.18	96.29	100	0.6883
Guava	9.26	81.48	85.18	0.5864
Henna	0.00	24.44	57.78	0.2741
Water	0.00	0.00	0.00	0.0000
L.S.D. 0.05 For extract	7.762			
mean	0.0618	0.1070	0.1854	
L.S.D. 0.05 For con.	4.482			
L.S.D. 0.05 For interaction between extract and con.	2.587			
Angular transformation Data L.S.D.	0.0618	0.1070	0.1854	

So the importance of these three extracts in various antibiotics used in treating common soil-borne pathogens such as *P. aphanidermatum* has recently been reported by (El-Mougy, *et al*., 2007) where they reported when using carnation and cinnamon extracts that the tested products significantly reduced the pre and post-emergence damping off of faba bean caused by *Rhizoctonia solani* and *Fusarium solani* compared with the control treatment.

On the other hand, using of plant extract and essential oil of camphor has high efficiency as an antimicrobial activity and can use them as pesticides, as mentioned by (Grieve 1992). So, in another study *T. harzianum* strain T969 and the pathogen species in simultaneous dual cultures, *Trichoderma* had a marked inhibitory effect on the pathogen growth compared with the control, however, with different efficiency in the in-vitro test (Zamanizadeh, *et al*., 2011), and the antagonistic activity of the genus *Trichoderma* versus *Pythiym* species has been widely demonstrated (Yedidia, *et al*.; 2001).

Conclusion

In conclusion, according to the results obtained from this in-vitro antagonistic study and *in-vivo* seedling incidence study, the tested both of the *Trichoderma* spp. and the eight plant extracts are potent sources for further greenhouse and field studies against *P. aphanidermatum* causing damping off disease.

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