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A Study of Antifungal Activity of Chrysophanol from *Trichoderma viride* Against some Phytopathogenic Fungi

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Abstract

This work aimed to evaluate the antifungal activity of chrysophanol derived from *Trichoderma viride* against some plant pathogenic fungi. A total of 160 infected plant samples were collected during the agricultural season of 2020-2021 from six plastic houses: two plastic houses (tomato plants) in Al-Rashidiya district, two plastic houses (eggplant plants) in Al-Nahrawan district and two plastic houses (cucumber plants) in Abu Ghraib district, Baghdad governorate. Five species of phytopathogenic fungi were isolated from infected plants and identified depending on morphological and microscopic characteristics. The results of microscopic examination showed that there were two species (*Fusarium oxysporum* and *Rhizoctonia solani)* from the Al-Rashidiya tomato field, three species (*Fusarium solani, R. solani,* and *Sclerotinia sclerotiorum)* from Al-Nahrawan eggplant field and three species from Abu Ghraib cucumber field (*F. oxysporum, R. solani,* and *Pythium aphanidermatum)*. Among 160 infected plant samples, there were 42 isolates of *F. oxysporum*, 24 isolates of *F. solani*, 54 isolates of *R. solani*, 14 isolates of *S. sclerotiorum,* and 26 isolates of *P. aphanidermatum*. All isolates were subjected to a pathogenicity test by exposing sterilized local cress seeds to phytopathogenic fungal isolates. The results of this test showed moderate to high pathogenicity, but only 32 isolates recorded 100% pathogenicity, and 15 isolates from these isolates were selected for the antifungal test. Thirty *Trichoderma spp.* isolates were collected from the soil of three agriculture regions: Al-Rashidiya, Al-Nahrawan , and Abu Ghraib districts, and they were identified morphologically. These isolates were processed with ethyl acetate using the standard extraction method and then screened for producing chrysophanol by High-performance liquid chromatography (HPLC). The results showed that only 11 isolates produced chrysophanol, and isolate number 10 (*Trichoderma viride*) produced higher chrysophanol (7.74µg/ml) than the rest. Chrysophanol from *T. viride* was purified by preparative Thin-layer chromatography (TLC), and the final quantity was 6.5mg. Different concentrations of chrysophanol (20, 40, 80, and 120 ppm/ml) were prepared and then used against the isolated phytopathogenic fungi compared with the fungicide Beltanol at 40 ppm/ml. The chrysophanol concentrations 20, 40, and 80 ppm/ml significantly achieved a higher percentage of growth inhibition against *S. sclerotiorum* 1 followed by *F. oxysporum* 2, which recorded (48 and 44.75%) respectively, while *P. aphanidermatum* 2 was the least affected, which recorded 25%. The chrysophanol concentration of 120ppm/ml achieved 100% growth inhibition for all phytopathogenic fungi except for *R. solani* 2 and 5, which achieved 99%. Beltanol fungicide 40ppm/ml significantly achieved a higher percentage of growth inhibition against *F. solani* 4 followed by *F. solani* 1 recorded 94.25 and 93%, respectively, while *S. sclerotiorum* 1 was the least affected with 75.25% growth inhibition. When comparing the results of chrysophanol at the concentration of 80 ppm/ml with the concentration of 40ppm/ml of Beltanol fungicide, we noticed that chrysophanol is significantly superior to Beltanol in the percentage of growth inhibition of most of the phytopathogenic fungi under test.

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While at the concentration of 120 ppm/ml of chrysophanol, we noticed a significant superiority in the growth inhibition of all phytopathogenic fungi under this study, showing 100% growth inhibition. The results of the current study showed that chrysophanol from *T. viride* can be used as an effective fungicide to eliminate phytopathogenic fungi rather than the use of a chemical fungicide.

Keywords: Chrysophanol, *Trichoderma viride,* Antifungal activity

دراسة الفعالية الضدية للفطريات لمادة الكرايسوفينول المستخلص من فطر *Trichoderma* ضد بعض الفطريات المسببة لامراض النبات

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الخلاصة:

يهدف هذا العمل إلى تقييم الفعالية المضادة للفطريات للكرايزوفينول المشتق من *Trichoderma viride* ضد بعض الفطريات المسببة للأمراض النباتية. تم جمع 160 عينة من النباتات المصابة خلال الموسم الزراعي 2020−2021 من ستة بيوت بلاستيكية. بيتان (نباتات الطماطم) في ناحية الراشدية وبيتان (نباتات الباذنجان) في ناحية النهروان وبيتان (نباتات الخيار) في قضاء أبو غريب بمحافظة بغداد. تم عزل خمسة أنواع من الفطريات المسببة للأمراض النباتية من النباتات المصابة وتم تحديدها اعتمادا على الخصائص المظهرية والمجهرية. أظهرت نتائج الفحص المجهري أن هناك؛ نوعان (*Fusarium oxysporum* و *Rhizoctonia solani*) من حقل طماطم الراشدية، وثلاثة أنواع (*Fusarium solani* و *R. solani* و *Sclerotinia sclerotiorum*) من حقل باذنجان النهروان وثلاثة أنواع من حقل خيار أبو غريب (*F.* oxysporum و *R. solani* و *Pythium aphanidermatum*). من بين 160 عينة نباتية مصابة، كان هناك: 42 عزلة من *F. oxysporum* و 24 عزلة من *F. solani* و 54 عزلة من *R. solani* و 14 عزلة من *S. sclerotiorum* و 26 عزلة من *P. aphanidermatum*. خضعت جميع العزلات لاختبار الإمراضية عن طريق تعريض بذور الرشاد المحلية المعقمة لعزلات فطرية ممرضة للنباتات. أظهرت نتائج هذا الاختبار إمراضية متوسطة إلى عالية، ولكن 32 عزلة فقط سجلت إمراضية بنسبة 100٪ وتم اختيار 15 عزلة من هذه العزلات للاختبار المضاد للفطريات. تم جمع ثلاثين عزلة من *Trichoderma spp.* من التربة لثلاث مناطق زراعية وهي منطقة الراشدية والنهروان وأبو غريب وتم تشخيصها مظهريا. تمت معالجة هذه العزلات بأسيتات الإيثيل باستخدام طريقة الاستخلاص القياسية ثم تم فحصها لإنتاج الكرايزوفينول بواسطة HPLC. أظهرت النتائج أن 11 عزلة فقط انتجت الكرايزوفينول وأن العزلة رقم 10 (*Trichoderma viride*) أنتجت كرايزوفينول اعلى من البقية (7.74 ميكروغرام / مل). تم تنقية الكرايزوفينول من *T. viride* بواسطة TLC التحضيري وكانت الكمية النهائية 6,5 ملغم. تم تحضير تراكيز مختلفة من الكرايزوفينول (20 و 40 و 80 و 120 جزء في المليون/مل) ثم استخدمت ضد الفطريات المسببة للأمراض النباتية المعزولة وتم مقارنتها مع مبيد الفطريات البيلتانول عند التركيز 40 جزء في المليون/مل. حققت تراكيز الكرايزوفينول (20 و 40 و 80 جزء في المليون/مل) نسبة أعلى من تثبيط النمو ضد الفطر *S. sclerotiorum* 1 يليه *F. oxysporum* 2 حيث سجلوا (48 و 44.75٪) على التوالي ، في حين كان *P. aphanidermatum* 2 هو الأقل تأثرا حيث سجل (25٪). حقق تركيز الكريسوفانول 120 جزء في المليون/مل تثبيط نمو بنسبة 100٪ لجميع الفطريات المسببة للأمراض النباتية باستثناء *R. solani* 1 و 5 حيث حققوا نسبة 99٪. حقق المبيد الفطري البلتانول 40 جزء في المليون/مل تثبيطا معنويا للنمو بنسبة أعلى ضد الفطر *F. solani* 4 يليه *F. solani* 1 حيث سجلوا (94.25 و 93٪) على التوالي ، في حين كان *Sclerotinia sclerotiorum* 1 هو الأقل تأثرا بتثبيط النمو حيث كانت نسبته (75.25٪). عند مقارنة نتائج الكرايزوفينول عند تركيز 80 جزء في المليون/مل بتركيز 40 جزء في

المليون/مل من المبيد الفطري البيلتانول ، لاحظنا أن الكرايزوفينول يتفوق بشكل ملحوظ على البيلتانول في النسبة المئوية لتثبيط نمو معظم الفطريات المسببة للأمراض النباتية قيد الاختبار. بينما عند تركيز 120 جزء في المليون / مل من الكريسوفانول ، لاحظنا تفوقا كبيرا في تثبيط النمو لجميع الفطريات المسببة للأمراض النباتية في إطار هذه الدراسة حيث ثبط النمو بنسبة 100٪. أظهرت نتائج الدراسة الحالية أنه يمكن استخدام الكرايزوفينول المشتق من *T. viride* كمبيد فطري فعال للقضاء على الفطريات المسببة للأمراض النباتية بدلا من استخدام المبيدات الفطريات الكيميائية.

INTRODUCTION

The main cause of plant diseases in the world is fungi, as they cause great losses in agricultural crops and threaten global food security by reducing the supply of the global food chain [1]. As a result of the large increase in global population growth, it has become necessary to solve this problem by controlling fungal plant diseases to secure access to food products in large quantities and reduce economic losses [2]. Where farmers used chemical fungicides widely over a long period, which led to an increase in the resistance of fungi to chemical pesticides, in addition to killing non-pathogenic fungi to plants and the damages they caused to the environment and human and animal health [1,3]. Therefore, in the last twenty years, research has begun significantly to find alternative ways to control fungal plant diseases using bio-fungicides, as these pesticides are considered mostly facultative pesticides (they only kill fungi that cause plant diseases without affecting other fungi present in the agricultural environment) and are environmentally friendly and relatively low cost when compared to other non-sustainable traditional control methods [3,4]. Currently, biocontrol agents and their secondary metabolites are considered one of the most important applications used today in controlling agricultural pests. *Trichoderma spp.* is a well-known biocontrol specialist that utilizes all inclusive. Numerous *Trichoderma* species are the foremost unmistakable makers of secondary metabolites (SMs) with antimicrobial movement against phytopathogenic parasites such as pachybasin, chrysophanol, emodin, terpenes, pyrones, gliotoxin, gliovirin, and peptaibols may be included [5,6].This work aimed to evaluate the antifungal activity of chrysophanol derived from *Trichoderma viride* against some plant pathogenic fungi.

Material and Methods

Isolation and identification of phytopathogenic fungi

The infected plant (damping off, wilt, rot root, and white mold diseases) samples were collected during the agricultural season of 2020 - 2021 from tomato, eggplant, and cucumber plants in Baghdad governorate in six plastic houses as follows: two plastic houses (tomato plants) in Al-Rashidiya district, two plastic houses (eggplant plants) in Al-Nahrawan district and two plastic-houses (cucumber plants) in Abu Ghraib district. Plant samples were collected in two periods, the first at the start of seedling growth and the second at the beginning of the production stage. The infected plant was identified by the disease signs that appeared on the vegetative parts and roots, where the signs appeared on the leaves in the form of yellowing, wilting, and falling, as well as some dead branches, the wooden vascular bundles in the stem and root of brown color when cut with a knife, sometimes the appearance of fungal threads around the crown area, and the presence of knots on the total root sometimes. Samples were taken for the study from plants located within the intersection of the diameters (cross diagonal method) in each plastic house, and sample numbers ranged from 30 to 50 plants for each plastic house for two periods. The total number of samples reached 160 infected plants [7]. Infected plants were placed in polyethylene bags, marked with the collection area, the plastic-house number, and the collection date, and then transported to the laboratory for isolation and diagnosis of the pathogens. The infected plants' roots, stems, and leaves were rinsed with tap water to remove suspended mud. Then, they were chopped into small pieces (5mm) and

sterilized by soaking them in a sodium hypochlorite solution (1% free chlorine) for 2 minutes. The pieces were subsequently washed three times in sterilized distilled water and then placed on sterile filter paper to dry. Four pieces of each infected plant were cultured in Petri dishes (90mm) containing autoclaved potato dextrose agar (PDA) (OXOID, England) and incubated for 5 days at 28±2˚C. Fungal isolates were purified by taking mycelial plugs with a 10mm diameter from the growing margin, put overhead onto fresh PDA in the center of the Petri dish, and incubated for 5 days at 28±2˚C. The purified isolates were diagnosed based on morphological characteristics according to [8,9].

Pathogenicity test

The cress seeds that had not been treated with fungicide were subjected to surface sterilization by being submerged in a 2% free chlorine solution of sodium hypochlorite for 2 minutes. Following this, the seeds were thoroughly rinsed three times with sterile distilled water and subsequently dried using sterile filter paper. For each of the four replicates, ten seeds were then placed onto the sterile filter paper inside a Petri dish containing moisture, achieved by adding drops of sterile distilled water. These seeds were then left for 7 days, then account for the percentage of seed germination according to the following formula:

P%= Number of germinating seeds / Total number of seeds planted×100

P = The percentage of seed germination

All fungal isolates were grown on PDA plates for 7 days at 28±1˚C. Five ml of sterile water was poured into each plate containing a fungal colony to obtain a fungal suspension. Ten seeds of local cress seed untreated with fungicide were surface sterilized by immersion in sodium hypochlorite (2 % free chlorine) for 2 minutes and then rinsed 3 times with sterile distilled water and dried with sterile filter paper. The sterile seeds were soaked in the fungal suspensions for 30 minutes, then inoculated seeds were placed on the sterile filter paper inside a Petri dish containing moisture (by putting drops of fungal suspension) and left for 7 days at room temperature, then the percentage of infection was account according to the following formula [10]:

P %=Number of non-germinating seeds/ Total seeds number ×100

P = the percentage of infection percentage

Isolation and identification of *Trichoderma* species

Forty-five soil agricultural samples were collected from Al-Rashidiya, Al-Nahrawan, and Abu Ghraib districts (15 samples from each district) at a depth of 5-10 cm using a metal spatula sterilized every time with 70% alcohol. The samples were secured in fresh plastic bags, sealed, and promptly taken to the laboratory for mycological testing. Ten grams of soil samples were placed in a 250ml conical flask with 100ml of sterile distilled water. The flask was shaken using an electric shaker to create a uniform suspension, and serial dilutions of the soil sample were prepared. One milliliter of the 10^{-3} dilution for each sample was added to a Petri dish with sterilized modified potato dextrose agar (PDA) medium (OXOID, England) and then left to incubate at 28±1°C for five days [11]. After various colonies had developed on the PDA plates, fungal cultures were transferred and sub-cultured to obtain a pure culture. The identification of *Trichoderma* spp. was based on the characteristics of the colony morphology and microscopic examination according to [12].

Screening of *Trichoderma spp.* isolates producing chrysophanol by HPLC

Trichoderma isolates were grown on PDA for 4 days, three blocks of 10mm in diameter from each *Trichoderma* agar culture were added to a flask containing 500ml sterilized modified Czapek liquid medium (Na_2HPO_4 2g, $MgSO_4.7H_2O$ 1.5g, KH_2PO_4 7g, $FeCl_3$ 0.2g, $ZnSO_4.7H_2O$ 0.1g, $CaCl_2$ 0.1g, $(NH_4)_2SO_4$ 0.5g, Glucose 15g and Sugarcane bagasse 100g),

then incubated in the dark for 14 days at 28±1°C with shaking. A volume of 500ml of ethyl acetate (EtOAc) was added to all *Trichoderma* isolates that were grown on modified Czapek's broth media and then placed on a shaker at 121 rpm (overnight). The extraction was completed within the 24-hour static period. Extraction of antifungal crude extract was employed by using a rotary evaporator (Gallen-Hamp, England) at 37 ^{0}C, taking into consideration the boiling point of the solvents (EtOAc, 77.1°C) [13]. Detection *Trichoderma* isolates that produce chrysophanol achieved by high-performance liquid chromatography (HPLC). In the HPLC analysis, the stationary phase column specifications were 250*4.6 mm of pursuit xfs 3µ-C18 at 35◦C. The mobile phase consisted of acetonitrile: water at a ratio of 50:50 and pumped at a flow rate of 0.8 mL/min. The photodiode array detector was set at a wavelength of 280 nm, and an injection volume of 20 µL was used. The data from the HPLC analysis of the sample was separated from *Trichoderma* spp. culture and that of the authentic sample showed that chrysophanol was separated at the same retention time (Rt = 19.8 min) [14].

Extraction and purification of chrysophanol by preparative TLC

According to the production of high chrysophanol detected by HPLC, one *Trichoderma* isolate number 10 (*Trichoderma viride*) was selected to be extracted and submitted to the purification process for the chrysophanol compound. The antifungal compound's partial purification was achieved by using thin layer plate preparation. The stationary phase (Silica gel) was prepared as a slurry with water (or) buffer at a 1:2 ratio, and it was applied to a glass plate or aluminum sheet by using a glass rod using a TLC applicator (0.25mm) thickness for analytical separation and 2.5m thickness for preparative separations are prepared. Calcium sulfate ($CaSO_4$) ½ H_2O (Gypsum) (10.15%) was integrated into the adsorbent; it was a binder because it facilitated the adhesion of the adsorbent to the plate. After applying the adsorbent, the plate was air dried for 10-15 min. The process is also known as the activation of the absorbent. The plate could be used immediately or stored in desiccators [15]. For preparative TLC, the sample was applied as a band across the layer rather than as a spot. From the preparative TLC plates, major bands at the desired retardation factor (RF) were scrapped out and extracted individually with ethyl acetate [16].

Antifungal Assay

The antifungal activity of chrysophanol was tested against *Fusarium solani, Fusarium oxysporum*, *Rhizoctonia solani, Sclerotinia sclerotiorum,* and *Pythium aphanidermatum* isolates (selected based on recording high pathogenicity) described by [4,17]. Briefly, pathogen pieces (1cm in diameter) were put in the middle of PDA Petri dishes containing chrysophanol at concentrations of 20, 40, 80, and 120 ppm/ml (Stocks of 200 ppm were made by dissolving 2 mg of partially purified chrysophanol in 20 ml of DMSO and then the concentrations used were obtained by dilution). The pathogen growth was determined by measuring the diameter (mm) of the mycelial plug after 4 days of incubation for *F. solani, F. oxysporum,* and *R. solani* while after 96 hours for *S. sclerotiorum* and *P. aphanidermatum* at 28±1°C and compared with a fungicide called Beltanol at concentrations of 40ppm/l. Experiments were done in four replicates. The percentage of mycelia growth inhibition was measured and calculated using the following formula [18]:

% mycelia growth inhibition = $(G_1 – G_2) / G_1 \times 100$

G_1 = a means of mycelia radius of phytopathogenic fungi in a Petri dish (control).

G_2 = a mean of mycelia radius of phytopathogenic fungi in a Petri dish with chrysophanol and Beltanol concentrations.

Analysis of results

Results were analyzed using the program statistical analysis system (2012) to calculate the antifungal activity of different concentrations of chrysophanol in this study. To significantly compare between means in this study, the least significant difference (LSD) test was used.

Results and Discussion

Pathogens isolation

Five species of phytopathogenic fungi were isolated from infected plant fields as follows: two species of *Fusarium oxysporum* and *Rhizoctonia solani* from Al-Rashidiya tomato plastic house, three species of *Fusarium solani, R. solani,* and *Sclerotinia sclerotiorum* from Al-Nahrawan eggplant plastic house and three species from Abu Ghraib cucumber plastic house *F. oxysporum, R. solani* and *P. aphanidermatum.* Among 160 infected plant samples, there were *F. oxysporum* (42 isolates), *F. solani* (24 isolates), *R. solani* (54 isolates), *S. sclerotiorum* (14 isolates), and *P. aphanidermatum* (26 isolates). Results showed that the main pathogen of tomato and eggplant plants in the seedling stage was *R. solani,* while *P. aphanidermatum* was the main pathogen of cucumber plants. At the fruiting stage, the main pathogen of tomato and cucumber plants was *F. oxysporum,* while *F. solani* was the main pathogen of eggplant plants, as shown in Table 1. These fungi are one of the main causes of root rot, wilting, and plant death of crops in the world [19]. These results agree with previous studies that mentioned *Fusarium oxysporum* and *Rhizoctonia solani* cause wilt and damping off disease in tomato plants, also, *F. solani, S. sclerotium*, and *R. solani* cause rot root, white mold, and damping off disease in eggplants, in addition, *P. aphanidermatum, R. solani* and *F. oxysporum* cause damping off and wilt disease for cucumber plants [20-24].

Table 1: Distribution of fungal species among plastic-house fields**.**

Region name	Plastic House NO.	Number of samples		Seedling stage	Total	Fruits stage	Total
		Seedling stage	Fruit stage	Fungal isolates	NO.	Fungi isolates	NO.
Al-Rashidiya Tomato	1	12	11	*Fusarium oxysporum* *Rhizoctonia solani*	2 10	*F. oxysporum*	11
	2	24	16	*F. oxysporum* *Rhizoctonia solani*	7 17	*F. oxysporum*	16
Al-Nahrawan Eggplant	1	17	13	*Fusarium solani* *Sclerotinia sclerotiorum* *R. solani*	3 5 9	*F. solani* *S. sclerotiorum*	9 4
	2	14	11	*F. solani* *S. sclerotiorum* *R. solani*	4 2 8	*F. solani* *S. sclerotiorum*	8 3
Abu Ghraib Cucumber	1	18	4	*Pythium aphanidermatum* *R. solani*	12 6	*P. aphanidermatum* *F. oxysporum*	3 1
	2	15	5	*P. aphanidermatum* *R. solani*	11 4	*F. oxysporum*	5
Total		100	60		100		60
		160					

Pathogenicity test

The results of this test detected that all isolates (160 isolates) showed moderate to high pathogenicity, but only 32 isolates recorded 100% pathogenicity, as demonstrated in Table 2. Therefore, 15 isolates from these isolates were selected for the antifungal test.

Table 2: Pathogenicity test of phytopathogenic fungi on local cress seeds.

Fungal isolates	Pathogenicity %	Fungal isolates	Pathogenicity %
*s Fusarium oxysporum 1	100	*s Rhizoctonia solani 3	100
*s Fusarium oxysporum 2	100	*s Rhizoctonia solani 4	100
*f Fusarium oxysporum 3	100	**s Rhizoctonia solani 5	100
*f Fusarium oxysporum 4	100	**s Rhizoctonia solani 6	100
*f Fusarium oxysporum 5	100	**s Rhizoctonia solani 7	100
*f Fusarium oxysporum 6	100	**s Rhizoctonia solani 8	100
***f Fusarium oxysporum 7	100	***s Rhizoctonia solani 9	100
***f Fusarium oxysporum 8	100	***s Rhizoctonia solani 10	100
**s Fusarium solani 1	100	**s Sclerotinia sclerotiorum 1	100
**s Fusarium solani 2	100	**s Sclerotinia sclerotiorum 2	100
**f Fusarium solani 3	100	***s Pythium aphanidermatum 1	100
**f Fusarium solani 4	100	***s Pythium aphanidermatum 2	100
**f Fusarium solani 5	100	***s Pythium aphanidermatum 3	100
**f Fusarium solani 6	100	***s Pythium aphanidermatum 4	100
*s Rhizoctonia solani 1	100	***s Pythium aphanidermatum 5	100
*s Rhizoctonia solani 2	100	***s Pythium aphanidermatum 6	100

* Al-Rashidiya district (Tomato) s Seedling stage
** Al-Nahrawan district (Eggplant) f Fruits stage
*** Abu Ghraib district (Cucumber)

These findings are consistent with previous research [20-25], that has shown *F. oxysporum, F. solani, R. solani, S. sclerotiorum,* and *P. aphanidermatum* have a strong capacity to infect plants in pathogenicity tests.

Isolation and identification of *Trichoderma* spp.

Thirty *Trichoderma* spp. isolates were obtained from 45 soil samples of the three agricultural regions (Table 3).

Table 3: *Trichoderma* spp isolates from three agriculture regions.

Region name	Number of samples	Fungal isolates	Number of isolates
Al-Rashidiya	15	*Trichoderma* spp.	10
Al-Nahrawan	15	*Trichoderma* spp.	8
Abu Ghraib	15	*Trichoderma* spp.	12
Total	45		30

Results of high-performance liquid chromatography

Thirty *Trichoderma* spp. isolates were screened by HPLC for producing chrysophanol. The results showed that only 11 isolates produced chrysophanol, and isolate number 10 produced higher chrysophanol 7.74 µg/ml, than the rest.

Table 4: HPLC results for chrysophanol from *Trichoderma* species.

Number of *Trichoderma* spp. isolates	HPLC result (Qualitative)	HPLC result (Quantitative µg/ml)
1	Negative	0
2	Negative	0
3	Negative	0
4	**Positive**	0.72
5	Negative	0
6	Negative	0
7	**Positive**	2.36
8	**Positive**	4.68
9	**Positive**	1.33
10	**Positive**	7.74
11	**Positive**	0.81
12	Negative	0
13	Negative	0
14	**Positive**	3.81
15	Negative	0
16	**Positive**	1.61
17	Negative	0
18	Negative	0
19	Negative	0
20	Negative	0
21	Negative	0
22	Negative	0
23	Negative	0
24	**Positive**	0.12
25	**Positive**	0.02
26	Negative	0
27	Negative	0
28	Negative	0
29	Negative	0
30	**Positive**	0.32

Quantification of chrysophanol compound

The final production of chrysophanol was 6.5mg per 15g/L (modified Czapek liquid medium), equal to about 433mg per Kg sugarcane bagasse medium. This result agreed with [13,26] in which the compound obtained from *Trichoderma harzianum* produced 3.2mg of chrysophanol per 15g of sugarcane bagasse medium, equal to about 213 mg per Kg sugarcane bagasse medium. Also, chrysophanol was extracted from *Rheum emodi* with a final quantity of 147.4 mg per kg roots and this extraction occurred through long procedures that required many years of growth [27]. Obtaining this compound from *Trichoderma viride* provides a high yield with a much shorter growth period than that of plants. Also, the modified Czapek medium

saves more energy and solvent than the routine *Trichoderma* cultivation media. So, it is a cost-efficient, environmentally safe, and energy-saving method.

Antifungal assay

This assay was carried out to evaluate the antifungal activity of chrysophanol in four concentrations: 20, 40, 80, and 120 ppm/ml compared with Beltanol fungicide at 40 ppm/ml against *F. solani, F. oxysporum*, *R. solani, S. sclerotiorum,* and *P. aphanidermatum* isolates. The results revealed that chrysophanol at all concentrations and Beltanol have antifungal activity against all phytopathogenic fungi under this study and showed significant differences in the antifungal activity between chrysophanol and Beltanol concentrations against phytopathogenic fungi. Also, a significant difference was noticed in the antifungal activity among chrysophanol concentrations. In addition, results showed a considerable difference among phytopathogenic fungi at each concentration. In general, results revealed that the phytopathogenic fungi inhibition growth increased with increasing chrysophanol concentrations (Table 5) and (Figure 1). The chrysophanol concentrations 20, 40, and 80 ppm/ml significantly achieved a higher percentage of growth inhibition against *S. sclerotiorum* 1 followed by *F. oxysporum* 2, which recorded (48 and 44.75%) respectively, while *P. aphanidermatum* 2 was the least affected which recorded 25%. The 120 ppm/ml concentration of chrysophanol achieved 100% growth inhibition for all phytopathogenic fungi except for *R. solani* 2 and 5, which achieved 99%. Beltanol fungicide at 40ppm/ml significantly achieved a higher percentage of growth inhibition against *F. solani* 4 followed by *F. solani* 1 recorded 94.25 and 93%, respectively, while *S. sclerotiorum* 1 was the least affected with 75.25% growth inhibition. When comparing the results of chrysophanol at the concentration of 80 ppm/ml with the concentration of 40ppm/ml of Beltanol fungicide, the chrysophanol was significantly superior to Beltanol in the percentage of growth inhibition of most of the phytopathogenic fungi under test. While at the concentration of 120 ppm/ml of chrysophanol, A significant superiority in growth inhibition was noticed in all phytopathogenic fungi in this study, showing 100% growth inhibition (Table 5) and (Figure 1).

The inhibitory effect resulted from the disintegration of the vascular wall of microbial cells, swelling and collapse of hyphal tissue, deformation, delay, and reduction of fungal sporulation, spore germination, and penetration. Structural changes such as haustorium opening, vacuolization, abortion, and necrosis were observed in fungi treated with chrysophanol [28,29].

These results were compatible with [4,30] who found that chrysophanol significantly inhibited the development of *Rhizoctonia solani*, *Botrytis cinerea*, *Blumeria graminis* f. sp. *hordei*, *Magnaporthe oryzae* growth from 75 to 100% at a concentration range from 75 to 500µg/ml., Also, chrysophanol obtained from the upper part of Rumex chinensis demonstrated a dose-dependent effect in laboratory conditions and effectively suppressed the growth of phytopathogenic fungi (red pepper anthracnose, wheat leaf rust, and barley powdery mildew) at concentrations between 32 and 512 µg/mL [31]. Recently, the research mentioned that chrysophanol has good antifungal activity for dermatophyte fungi (*Microsporum canis* and *Trichophyton rubrum*), which inhibited growth at 250 and 2000 (µg/ml), respectively [32].

Table 5: Antifungal activity of different chrysophanol concentrations (20, 40, 80, and 120 ppm/ml) with one fungicide (Beltanol 40 ppm/ml) against phytopathogenic fungi on PDA after 4 days at 28 ± 2 ˚C and pH 5.5.

Fungal isolates	% Inhibition growth				
	Chrysophanol concentrations				Beltanol
	20 ppm/ml	40 ppm/ml	80 ppm/ml	120 ppm/ml	40 ppm/ml
*s *Fusarium oxysporum* 1#	42.00	67.50	94.00	100	91.25
*s *Fusarium oxysporum* 2#	44.75	70.75	96.25	100	90.00
*f *Fusarium oxysporum* 3#	44.00	66.75	93.50	100	90.75
*f *Fusarium oxysporum* 4#	41.75	69.25	93.75	100	89.25
***f *Fusarium oxysporum* 7#	43.50	70.00	94.50	100	91.00
**s *Fusarium solani* 1#	38.25	68.50	92.25	100	93.00
**f *Fusarium solani* 4#	36.75	68.00	92.00	100	94.25
*s *Rhizoctonia solani* 1#	30.00	60.25	90.00	100	88.75
*s *Rhizoctonia solani* 2#	31.25	62.00	91.00	99.00	89.25
**s *Rhizoctonia solani* 5#	33.00	68.25	89.00	99.00	92.00
**s *Rhizoctonia solani* 6#	35.00	67.50	90.25	100	91.50
***s *Rhizoctonia solani* 10#	33.50	68.00	92.00	100	86.75
**s *Sclerotinia sclerotiorum* 1##	48.00	80.50	100	100	75.25
***s *Pythium aphanidermatum* 1##	27.25	62.00	90.00	100	88.50
***s *Pythium aphanidermatum* 2##	25.00	60.25	88.00	100	90.00
L.S.D	P=0.05				
Between concentrations	0.4				
Between fungal isolates	0.7				
Between interaction	1.5				

*s: isolated from Al-Rashidiya district (Tomato) seedling stage
*f: isolated from Al-Rashidiya district (Tomato) fruits stage
**s: isolated from Al-Nahrawan district (Eggplant) seedling stage
**f: isolated from Al-Nahrawan district (Eggplant) fruits stage
***s: isolated from Abu Ghraib district (Cucumber) seedling stage
***f: isolated from Abu Ghraib district (Cucumber) fruits stage
#: Incubation period for 48hours at 28±1°C
##: Incubation period for 96 hours at 28±1°C
^**:** Each number is an average of four replicates.

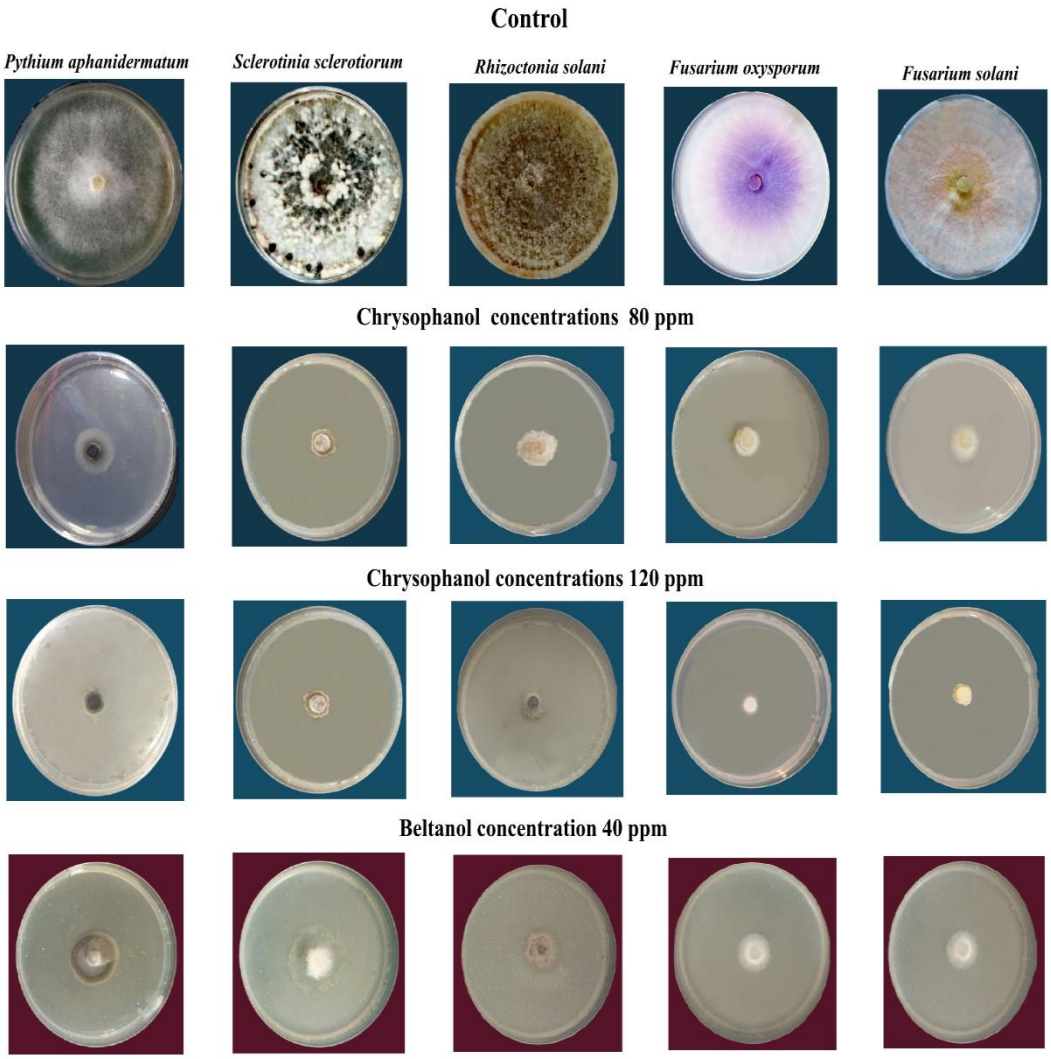

Figure.1: Growth inhibition of *Pythium aphanidermatum, Sclerotinia sclerotiorum, Rhizoctonia solani, Fusarium oxysporum,* and *Fusarium solani* using chrysophanol at concentrations of 80, and 120 ppm/ml and Beltanol (fungicide) at 40 ppm/ml on **PDA media after 72-96 hours at** 28 ± 1 ˚C and pH 5.5.

Conclusions

The seedling stage is the most vulnerable to diseases caused by fungi. *Rhizoctonia solani* (cause damping off disease) was the most commonly isolated from all plastic houses at the seedling stage, while *F. oxysporum* was the most common at the fruit stage. All pathogenic plant fungi were inhibited by chrysophanol, which showed high antifungal activity, especially at 80 and 90 ppm concentrations.

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