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BIOLOGICAL MANAGEMENT OF THE FUNGI CAUSING ROOT ROT DISEASE IN COWPEA (*VIGNA UNGUICULATA* L.)

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SUMMARY

The following study aimed to isolate some pathogenic fungi from cowpea (*Vigna unguiculata* L.) plants infected with root rot disease and determine their tenacity to pathogens using some plant resistance induction factors and the biological fungus *Trichoderma* spp. The results showed the presence of cowpea root rot disease in all areas included in the survey in the Babylon Governorate. The 13 types of fungi accompanied the roots of the cowpea plant. *Fusarium solani* was the most abundant pathogenic fungus, with a frequency rate of ***55.9***%, followed by the fungus *Macrophomina phaseolina,* with an appearance rate of ***45.55***%***.*** Isolates of the fungus *Trichoderma* spp. (*T. viride, T. harzianum, T. koningiopsis,* and *T. reesei*) achieved a high antagonistic ability against pathogenic fungi under laboratory conditions. The highest antagonistic ability was one for the *T. viride* isolate against the pathogenic fungi *F. solani* and *M. phaseolina****.*** The results revealed that adding chitosan to the culture medium at all concentrations led to growth inhibition of the fungi *F. solani* and *M. phaseolina* compared to the inhibition percentage of 0.00% in the control treatment***.***

Keywords: Cowpea (*V. unguiculata* L.), *Trichoderma* spp., chitosan, fungi, root rot diseases

Key findings: The emergence of cowpea (*V. unguiculata* L.) root rot disease caused by pathogenic fungi prevailed in contaminated soils of the Babylon Governorate, Iraq. The results revealed the effectiveness of the biological agent *Trichoderma* spp. against pathogens. The chemical inducer chitosan proved to inhibit and eliminate the two pathogenic fungi, *F. solani* and *M. phaseolina****.***

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INTRODUCTION

The cowpea crop (*Vigna unguiculata* L. Walp.), which belongs to the family Leguminaceae, is one of the most important plants of this family, grown worldwide for its high nutritional value, as its fruits contain many carbohydrates, proteins, and lipids. Cowpea cultivation seeks to obtain their dry seeds and green pods, mainly cooked as food for humans. Likewise, it can serve as green fertilizer to improve soil characteristics and increase its fertility (Langyintuo *et al*., 2003). It grows wild in Central Africa and seems to originate in the Central African regions.

The cowpea crop is one of the valuable and widespread economic vegetable crops in the world, being grown in almost all countries globally, whether for consumption or food processing (Langyintuo *et al*., 2003). The area cultivated with the cowpea crop in Iraq for the year 2021 had an estimate of 1,759.6 ha. The total production of this area in Iraq reached 26,606 tons (Iraqi Central Statistics, 2021)**.** The cowpea crop sustains infection from a group of pathogens, the most critical of which are types of fungi, bacteria, viruses, and some mycoplasma. Root rot and seedling damping off are among the diseases that most affect plants, as caused by infection with soil-inhabiting fungi (Campos *et al.*, 2019).

Several methods are applicable to control these pathogens, including the use of chemical pesticides, but the extensive treatment of these materials has led to the emergence of many risks to human and animal health and the environment. Additionally, they have high economic costs, as well as causing the emergence of resistance by pathogens toward them. This has prompted the efforts of researchers in the field of plant protection to search for less risky and safer methods; the most prominent is the use of microorganisms as vital agents to resist plant pathogens, reduce their pollen, and increase the crop quantity and quality (Saad *et al*., 2021). One of the most crucial biological agents is the biological fungus *Trichoderma* spp. The species belonging to the genus *Trichoderma* are among those antibiotics that have given encouraging results in the field.

This fungus possesses various antagonistic properties against pathogens and is vital in improving plant growth and production, as well as the ability to induce resistance (Xie *et al*., 2021; Abdullahi *et al*., 2021; Kareem and Matloob, 2021). Many recent studies have reported the use of chitosan, which is one of the components of the cell walls of some organisms in various crustaceans, such as crabs, shrimp, and insects. Chitosan induces resistance in plants against pathogens, including cowpea, cucumber, eggplant, grapes, and sugarcane (Nedved *et al*., 2022; Makhlouf *et al*., 2022)**.**

With insufficient studies on the causes of cowpea root rot diseases in Iraq and trying to find new and safe ways to combat them, this study aimed to isolate some pathogenic fungi from cowpea plants infected with root rot. It also sought to diagnose them and select their pathogenicity**.** Moreover, the study hoped to determine the resistance to the causes using some plant resistance-inducing agents and the biological fungus *Trichoderma* spp.

MATERIALS AND METHODS

Field survey

The field survey progressed in the cowpea crop season of 2022–2023 in the province of Babylon, Iraq. From March 25 to July 1, 2023, random sample collection from a plant succeeded, placing the samples in polyethylene bags. The percentage of the disease's incidence, as calculated, used the following equation**:**

% Disease incidence = (The number of infected seedlings\the total number of total seedlings) × 100.

The percentage of severity according to McKinney (1923) used the following calculation. The computation of the percentage of infection severity for the root system employed the pathological index as follows: 0 = healthy plant, 1 = more than 0%–25% of the roots are infected, 2 = more than 25%–50% of the roots are infected, 3 = more than

50%–75% of the roots are infected, 4 = more than 75%–100% of the roots are infected, and 5 = plant death.

Disease Severity (%) = ([Plants in 1 degree × 1 +… Plants in 5 degree × 5]/ all plants × 5) × 100%.

Isolation and identification of fungi associated with the cowpea plant roots

The conduct of the isolation process occurred on cowpea plant samples with symptoms of root rot disease. These symptoms are wilting, yellowing of the leaves, and general weakness in growth, revealing brown rot presence on the main and branch roots on the next day of the field survey. The roots' washing used running tap water before cutting the roots into small pieces and sterilizing these pieces with sodium hypochlorite. Using sterile forceps, we transferred the sterilized cowpea root pieces to Petri dishes, with four pieces in each dish (9-cm diameter) containing the potato dextrose agar (PDA) medium added with the antibiotic tetracycline at 250 mg/L. The dishes sustained incubation at 25 °C + 1 °C for three days. The identification of fungi relied on the taxonomic keys (Parmeter and Whitney, 1970; Booth, 1971; Ellis, 1971; Summerell and Leslie, 2006). The occurrence rate of the fungi that appeared, as calculated, followed the equation below:

The percentage of the presence of fungi = N/n × 100,

Where,
N = ***The number of root pieces in the dishes*** where ***the fungus appeared***
n = ***The total number of cut roots used for each sample***

Detection of pathogenic isolates using cabbage seeds

The pathogenicity of the fungal isolates (seven *F. solani* isolates and four *M. phaseolina* isolates) obtained through the isolation process proceeded to be tested. Employing the method of Bolkan and Butler (1974), the dishes underwent inoculation from their center after hardening the water agar medium with a 0.5-cm disk taken from the edges' colony of five-day-old fungi *Fusarium solani* and *Macrophomina phaseolina*. The incubation of dishes continued in the incubator at a temperature of 25 °C ± 1 °C for three days. Then, the seeds of cabbage were samples used after sustaining surface sterilization with sodium hypochlorite (1% chlorine) at 10 seeds per dish, with three replicates for each isolate, aside from the control treatment (without pathogenic fungi). The dishes received incubation for seven days, with the results taken by calculating the percentage of germination according to the following equation:

Germination (%) = seeds germinated/total seeds × 100

Isolates' effect on cowpea seeds *germination*

This experiment transpired at the Technical College, Al-Musayyab. The fungal inoculum of *Fusarium solani* and *Macrophomina phaseolina*, carried on local millet seeds (Dewan, 1989), succeeded in mixing with soil sterilized with an autoclave and distributed in 1 kg plastic pots at 1% (weight/weight). The planting of 10 seeds (surface-sterilized cowpeas with sodium hypochlorite solution) ensued in each pot, repeating each treatment three times, leaving three repetitions without adding the pathogenic fungus as a comparison. The pots received watering with caution, and with the complete emergence of seedlings in the comparison treatment, calculating the number of germinated seeds took place after 15 days of planting.

Antagonistic ability of the *Trichoderma* spp.

The antagonistic ability testing of some types of biological fungi, *Trichoderma* spp., advanced. The most vital species that have been useful in the antagonistic ability are *Trichoderma viride*, *Trichoderma reesei*, *Trichoderma harzianum,* and *Trichoderma*

koningiopsis, which came from the University of Karbala. Against the pathogenic fungi *Fusarium solani* and *Macrophomina phaseolina*, as obtained from the Plant Pathology Laboratory, Al-Mussaib Technical College, the experiment relied on the double-culture method. PDA petri dishes with a diameter of 9 cm, as divided into two equal parts, had the first part of the dish inoculated with the fungus inoculum. The pathogen, taken separately, researchers took a 0.5-cm diameter disc from the fungi culture at seven days old, while inoculating the other part of the plate with a 0.5-cm diameter disc from the *Trichoderma* spp. fungi culture at seven days old. The experiment continued with three replications. The dishes' placement in the incubator gained a temperature of 25 °C ± 1 °C for one week. The degree of antagonistic ability estimation depended on keys mentioned in Bell *et al*. (1982) having five-to-five degrees.

Chitosan effect in inhibiting the growth of *F. solani* and *M. phaseolina*

Chitosan use ensued in this experiment with the following steps. Dissolving 20 grams of regular chitosan in 50 ml of vinegar before adding the volume to 1000 ml of distilled water for the purpose of preparing concentrations of 5%, 10%, 15%, and 20% of regular chitosan. Taking each concentration separately and placing it in a 100 ml glass beaker succeeded in completing the volume to 100 ml of the potato dextrose agar (PDA) culture medium, using a rotary movement to homogenize the mixture. Then, their pouring into 9-cm diameter Petri dishes continued to inoculation with a disk with a diameter of 0.5 cm at the age of five days in three replicates for each concentration, along with three replicates without adding any concentration (culture medium only). As a control treatment, the dishes sustained incubation in the incubator at a temperature of 25 °C + 1 °C for seven days. The average of two perpendicular drops from each colony succeeded in measuring to calculate the percentage of fungal growth inhibition.

RESULTS AND DISCUSSION

Field survey of cowpea root rot diseases

The results (Table 1) showed the presence of cowpea root rot disease in all areas included in the survey in the Babylon Governorate, with varying rates of disease incidence ranging between 40% and 100% and infection severity ranging from 18% to 75%. The highest infection severity was in samples from the areas of Al-Hashimiya, Al-Qasim, Al-Badaa, and Taliah, with infection rates of 100% in most areas. The outcomes further showed the lowest incidence and severity of infection appeared in samples from the Al-Rashidiyah area, and this may be a result of the field being planted with the crop for the first time***.*** The reason for the high incidence of infection in these areas may be due to the areas specializing in the cultivation of cowpeas, as this crop is being grown there continuously annually. It led to the accumulation of inoculum from pathogenic fungi, especially sclerotia, which remained in the soil for a long period of time, perhaps up to five years (Vadakattu and Paterson, 2005).

Isolation and identification of fungi

Through microscopic examination of fungal growths formed from planting infected plant cuttings on PDA culture medium, it revealed the presence of 13 species of fungi associated with the roots of the cowpea plant (Table 2 and Figure 1). The fungus *Fusarium solani* was the most abundant fungus, as it appeared in most samples with varying frequency rates (55.9%), followed by the fungus *Macrophomina phaseolina,* with an appearance rate of 45.55%. Meanwhile, the fungus *F. sulphureum* emerged in samples from the Al-Qasim, Taliah, and Al-Qasim areas, and in Al-Rashidiyah, at 15.4%. This is the first recording of the fungus *F. sulphureum* on the cowpea plant in Iraq. It was evident that the dominance of some species was visible in samples of some areas despite their repeated appearance in samples of other areas. The fungus *F. solani* recorded a

Table 1. Areas included in the field survey and date of sampling in Babylon Governorate, Iraq.

No.	Region	Date of survey	Field area / 0.1 ha	Disease incidence (%)	Severity (%)
1	Al-Qasim	3/25/2023	2	100	73
2	Tabo	5/6/2023	1	80	58
3	Taliah	3/26/2023	3	100	60
4	Al Mashroa	4/27/2023	3	90	56
5	Al Azawia	5/18/2023	5	100	56
6	Al-Qasim- Al fayadia	6/10/2023	3	100	52
7	Al-Rashidiyah	6/15/2023	1	40	18.0
8	Al-Badaa	6/29/2023	2	100	62.8
9	Al-Hashimiya	7/1/2023	4	100	75

Table 2. Fungi accompanying the roots of infected cowpea plants, their locations, and their frequency in samples.

Fungus	No. sample*	Appearance rate (%) **	Highest ratio of appearance
Fusarium solani	1،4،5	55.9	92
Alternaria alternata (Fres.) Keissler	3،8، 4،5	15.5	32.9
Penicillium spp.	5،1،8	52.6	81
Aspergillus niger	3،5،6،1	29.6	18
Mucor spp.	1،4	12.33	14
Fusarium oxysporum.	6،7	1.27	1.3
Fusarium solani (Mart.) Sacc.	1-3،5-9	37.4	65.0
F. sulphureum Schlecht.	1،3،7	7.6	15.4
Macrophomina phaseolina (Tassi) Goid.	1-3،5،7،9	45.55	91
Rhizoctonia solani Kuhn	1-4،7،9	28.21	32.14
Trichoderma harzianum	3،1	5.7	5.7
Stemphylium sp.	1،5-7	4.3	7.9
Torula sp.	6	6.6	6.6

*No. sample = numbers represent sample collection areas (Table 1).
%** repeat the fungus in the sample = (The number of fungus appeared in dishes\Total number of pieces used in the sample) x 100.

Figure 1. Some fungi isolated (the most frequent) from the roots of cowpea plants infected with root rot *M. phaseolina* on PDA medium and the fungus *F. solani* on PDA medium**.**

percentage of 92% in samples of the Al-Qasim area, and the fungus *M. phaseolina* in the Bidaa area reached 91%. The results are consistent with findings obtained by Rusuku *et al*. (1997) regarding the emergence and spread of soil fungi pathogenic, causing bean root rot diseases.

The examination results indicated the presence of many fungi associated with the roots of the cowpea plant, such as *Alternaria alternata, Aspergillus niger,* and *Stemphylium* sp. The presence of these types of fungi may be ascribable to the growth and penetration of their mycelium into the cells of decomposing tissues previously infected with the fungi, causing this condition. These provided them with protection from the action of the surface disinfectant, or they may include species with parasitic ability on plants (Matloob *et al.*, 2019). The fungus *T. harzianum* was also an isolate from a sample from the Al-Qasim area, with an occurrence rate of 5.8%. The presence of the fungus may have come as a result of its ability to parasitize the mycelium of pathogenic fungi inside the plant tissues, which made them immune to the action of the surface sterilizer (Harman, 1996). Another group of fungi reached isolation with less frequency (*Fusarium oxysporum, F. semitectum, Penicillium* spp., and *Torula* sp.). According to the priority of their isolation, the isolates received numbers next to the fungus symbol to distinguish them from other isolates.

Pathogenic isolates' detection using cabbage seeds

The results available in Figure 2 show that all isolates of the fungi *F. solani* and *M. phaseolina* caused a significant reduction in the percentage of germination of cabbage seeds. It was noteworthy that a difference in the pathogenicity of the fungal isolates existed, as the isolates Fu-7 were superior in their pathogenicity to others, which was clear in their effect on reducing the percentage of germination. Such treatments had 0% compared with the comparison treatment,

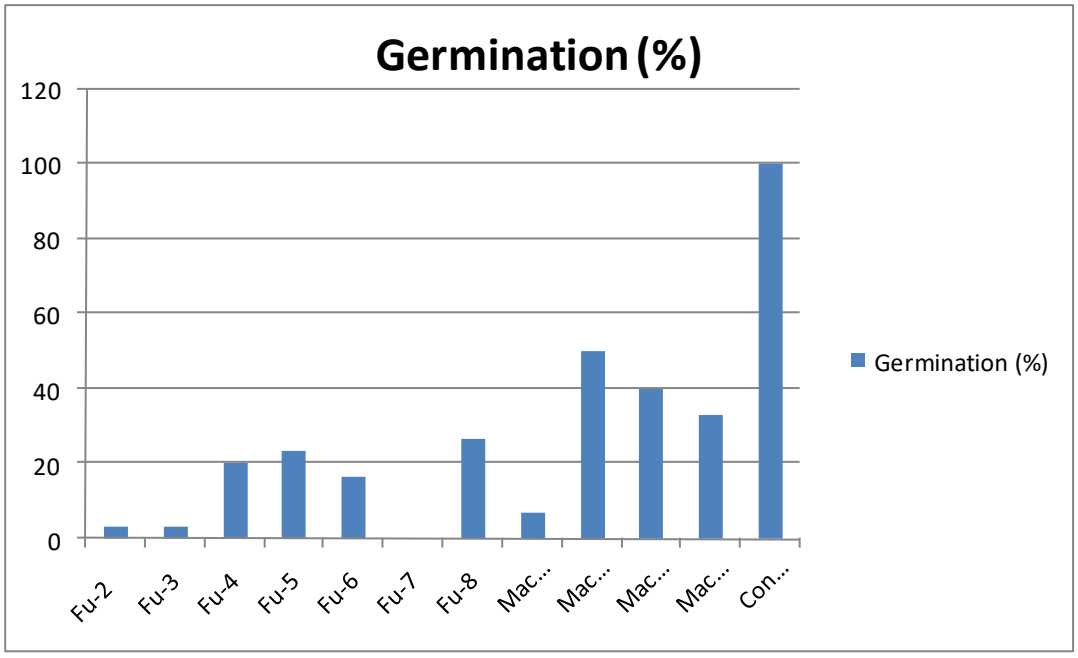

Figure 2. Detection of pathogenic isolates associated with the roots of infected cowpea plants using cabbage seeds**.**Each number represents an average of three replicates of L.S.D at a 5% level = 17.24; Fu = *Fusarium solani*; Mac = *Macrophomina phaseolina*; the number next to the symbol represents the isolate number**.**

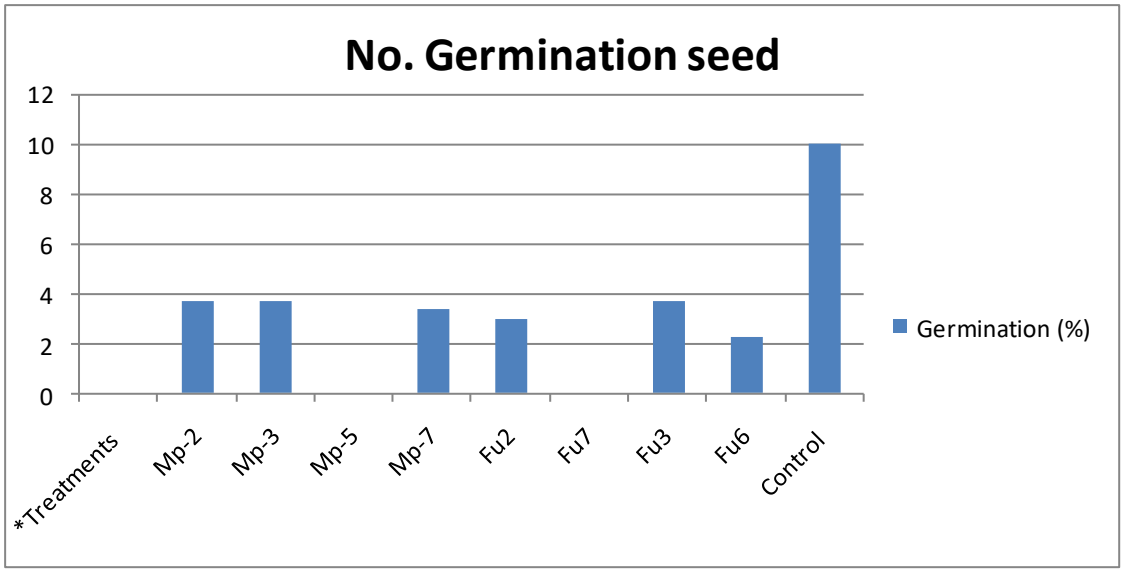

Figure 3. The effect of some pathogenic fungal isolates on the germination of cowpea seeds eight days after planting**.** Fu = *Fusarium solani;* Mp = *Macrophomina phaseolina***.**

wherein the germination rate was 100% (Figure 2). Meanwhile, the other isolates achieved a substantial reduction in the germination rate of cabbage seeds, which ranged in their treatments between 0% and 50%. Most tested fungi that cause root rot disease of the examined plants instigated a considerable decrease in the germination rate of cabbage seeds in the culture media compared with the control treatment. The reason for the effect of the isolates refers to the level of fungal secretions of toxic secondary metabolic compounds that kill embryos and the ability to produce hydrolytic enzymes. These are responsible for causing rot in the seeds and, thus, preventing them from germinating. The results agree with outcomes indicated by Matloob and Al-Baldawy (2020), where these fungi are among the critical and main causes of root rot disease and considerably one of the most important pathogens on many plant families.

Isolates effect cowpea seed germination

The findings (Figure 3) indicated that all tested isolates of the fungi *Fusarium solani* and *Macrophomina phaseolina* caused a significant reduction in the germination rate of cowpea seeds, as the isolates Fu-7 and Mac-5 completely prevented seed germination. These were in contrast with the control treatment without adding the pathogenic fungus, in which the germination rate was 10.0%. The isolates Mac 1-3 caused a significant reduction in germination rates of 3.67%, with similar results also showing for all isolates of the fungus *F. solani*. The tested results triggered a significant reduction in the seed germination rate, ranging from 3.0 to 3.67, which differed significantly from the comparison treatment without the addition of the pathogenic fungus. The outcomes (Figure 3) revealed that all isolates were pathogenic to the cowpea plant. *F. solani* is an opportunistic fungus that thrives monthly when the plant is in a state of stress, such as external and other conditions for growth (El-Mougy *et al.*, 2011).

Antagonistic ability of *Trichoderma* spp.

The antagonistic ability of four fungal isolates belonging to the fungus *Trichoderma* spp. obtained from the University of Karbala, succeeded testing after proving their antagonistic ability against a group of pathogenic fungi. They incurred trials against the pathogenic fungal isolates that cause the

Table 3. The effect of *Trichoderma* fungi on the growth of pathogenic fungi on the PDA medium.

Treatment	Colony diameter (cm)	Inhibition (%)
Trichoderma reesei+Fu	0.53	94.03
T. reesei+Mac	0.63	92.90
T. viride+Fu	0.467	98.07
T. viride+Mac	0.433	98.07
T. koningiopsis+Fu	0.63	92.93
T. koningiopsis+Mac	0.56	93.67
T. harzianum+Fu	0.167	95.13
T. harzianum+Mac	0.167	94.77
Control	9.0	0.0
LSD (0.05)	0.252	2.295

Fu = *Fusarium solani;* Mac = *Macrophomina phaseolina*.

selected cowpea root rot diseases, which are *Fusarium solani* and *Macrophomina phaseolina*. The isolates achieved the fungus *Trichoderma* spp. has a high antagonistic capacity against pathogenic fungi under laboratory conditions (Table 3). The *T. viride* isolate had the highest antagonistic capacity at level 1 against the pathogenic fungi *F. solani* and *M. phaseolina*, and a high antagonistic capacity was for the other isolates, *T. reesei* and *T. koningiopsis.* They also displayed the results of the antagonism ability of the fungus *Trichoderma* spp. against the pathogenic fungus *Fusarium solani*, where the highest antagonism ability was for the *T. viride* isolate with a score of 1. An antagonistic ability for the *Trichoderma koningiopsis* isolate against the fungus *M. phaseolina* also appeared. The results of the antagonistic ability for the isolates of the fungus *Trichoderma* spp. were at a high level, where four isolates of the fungus *Trichoderma* spp. achieved an antagonism of 1 for *T. viride, T. harzianum, T. koningiopsis*, and *T. reesei*. It is a fact that the fungus *Trichoderma* spp. can inhibit the growth of plant-pathogenic fungi using various mechanisms, such as fungal parasitism, competition for space and materials, and production of antifungal substances (Verma *et al*., 2007). The high antagonistic activity of the fungus *Trichoderma* spp. pathogens cause the formation of many secondary metabolic compounds, including antibiotics, such as gliotoxin, viridin, and Trichoderma. These have been proven to have antifungal activity to control various pathogens transmitted through soil, confirming *Trichoderma* species have an antagonistic property in controlling pathogens (lnovejas and Divina, 2018).

Chitosan role in inhibiting fungi growth

The results shown in Table 4 revealed that adding regular chitosan to the culture medium at all concentrations led to inhibiting the growth of the fungi *F. solani and M. phaseolina* on the PDA. The percentage of inhibition of the Fs1 isolate of the fungus *F. solani* reached 96.23%, 100.00%, 100.00%, and 100.00% for all concentrations of regular chitosan (5%, 10%, 15%, and 20%, respectively), compared with the 0.00% inhibition rate in the control treatment. The rate of inhibition of the growth of the fungus *M. phaseolina,* when adding the same concentrations of regular chitosan (5, 10, 15, and 20) to the culture medium, reached 95.86% (5%), while the concentrations of 10%, 15%, and 20% reached 100%. The results of the study showed the inhibitory effectiveness of chitosan on the growth of the fungi *F. solani* and *M. phaseolina* on the medium. This may be due to the effect of chitosan on the DNA of pathogenic fungi, such that it stopped the activity of some enzymes and proteins necessary for the growth of fungi. Younes and Rinaudo (2015) indicated that the mechanism of chitosan against the growth of pathogens increases the permeability of the cell membrane as a result of the interaction of the positively charged chitosan. With the negatively charged fungal membrane, it inhibits the synthesis of basic enzymes and proteins due to the change in DNA. The antifungal effectiveness of chitosan varies

Table 4. The effect of different concentrations of regular chitosan on inhibiting the growth of the fungi *Fusarium solani* and *Macrophomina phaseolina* on PDA culture media.

Treatment	Concentration	Colony diameter (cm)	Inhibition (%)
Control	0	9.00	0.00
Ch + Fu-7	5	0.33	96.23
	10	0.00	100.00
	15	0.00	100.00
	20	0.00	100.00
Control	0	9.00	0.00
Ch + Mac5	5	0.36	95.86
	10	0.00	100.00
	15	0.00	100.00
	20	0.00	100.00
LSD (0.05)	-	0.046	0.513

Each number represents the average of three replicates; Fu7 = *Fusarium solani*, Mac5 = *Macrophomina phaseolina.* The number near the symbol represents the isolate number**.**

depending on the inhibitory mechanism, which differs depending on the types of pathogens (Timofeeva *et al.*, 2022). The results are analogous to findings by Sattar and Matloob (2022), indicating that chitosan has an effect in hindering the growth of the pathogenic fungi *Fusarium solani* and *Macrophomina phaseolina***.**

CONCLUSIONS

The study concluded from the results that root rot disease, which affects cowpea plants in Iraq, is one of the critical and dangerous diseases that threatens the cultivation of the crop and its spread in the Babylon Governorate. Moreover, one of the most important main causes of this disease is pathogenic fungi that are present in the soil. *Fusarium solani* was the most abundant pathogenic fungus, followed by *Macrophomina phaseolina*. One also concludes from the results that some biological factors are ascribable to the fungus *Trichoderma* spp. and the chemical inducer chitosan in inhibiting the growth of the pathogenic fungi causing the root rot disease of cowpeas. The use of chitosan is one of the first studies in Iraq that tested and researched this influential factor.

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