



Identification of *Rhizoctonia solani* associated with Wheat (*Triticum aestivum* L.) Seeds and their Bio-Control in Lab conditions

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Abstract

A total of 22 fungal isolates were acquired and identified, with these isolates originating from wheat seeds, Sham-6 variety obtained from grain stores in Nineveh Governorate, 2024-2025 season, the fungus *Aspergillus niger* was the most frequently in wheat seeds with rate (31.81%) followed by *Aspergillus terreus* (27.27%) and *Rhizoctonia solani* (18.18%), while *A. flavus* and *Alternaria sp.* reached (13.63%), (9.09%) Consecutively. as well as the samples (Abaa-99 and Jihan) obtained from local markets were 100% contaminated with the *Rhizopus sp.* Bio-control strategies including fungal isolates, is an environment friendly strategy that supports the sustainable development goals of reducing the use of chemical pesticides that have a harmful effect on human health in the long term. The antagonistic activity assessed *in vitro* using the dual culture technique and Antibiosis activity by Using non-volatile metabolites to detect antifungal activity of *Trichoderma harzianum* against *Rhizoctonia solani* that showed statistically significant inhibition which reach scale 1 for dual culture technique and The inhibition percentage was reached 55.5% for Antibiosis activity. The morphological identification for the chosen pathogenic fungus *R. solani* confirmed by using polymerase chain reaction assay of ITS1, 5.8S, and ITS2 are parts of the internal transcribed spacer of the rDNA, The nucleotide sequences deposited in the Gene Bank. Based on the lineup process results, the local fungal isolate that was chosen had a nucleotide sequence that was of the type *R. solani* with the PV138271 registration number.

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1. Introduction

Wheat, (*Triticum aestivum*) L., belongs to the family Poaceae, is a staple food for more than one-third of the world's population. Various properties of grains, such as germination capacity, moisture content, and grain color, are affected by several biotic and abiotic factors during storage. Prolonged storage of grains increases their susceptibility to microorganisms, especially fungi, making them unsuitable for cultivation and unacceptable as human food or animal feed (Chattha, *et al.*, 2016)^[8]. Wheat grains are a major source of fungal growth (Chauhan, *et al.*, 2019). About 15-18 genera of fungi (*Rhizoctonia*, *Fusarium*, *Penicillium*, *Curvularia*, *Aspergillus*) have been identified as important fungal pathogens of cereals (Sadhasivam, *et al.*, 2017)^[23]. These fungi attack crops during cultivation or during harvest and transportation, and can remain alive for several years during storage. These fungi affect grain production, as some of them cause wheat root rot, especially the fungi in question. *Bipolaris sorkonin*, *Fusarium culmarum*, and *Rhizoctonia solani* (Al-Rashidi, 2019)^[2], and the fungus *R. solani* are some fungi that lead to significant losses in wheat production, as they infect agricultural crops in many regions around the world (2019, Barnett and hunter, 1972)^[4], and cause wheat root rot and reduce the plant's ability to absorb water and nutrients from the soil (Paulitz *et al.*, 2002)^[22].

The world's growing population has made food security a major concern in recent years. In the current situation, it is imperative to provide food for the expanding billions of people without upsetting the balance of the environment. Furthermore, these techniques have supplanted chemical-based approaches to pathogen control and pest limitation. One such important method that is currently at the forefront is the adoption of biological control (Pandit *et al*,2022)^[26] (Al-Dulaimi and Al-Tarjuman, 2024)^[27]. Biocontrol agents provide safe and environmentally friendly substitutes in addition to effective disease management (Saadallah *et al*, 2023a)^[24] (Mula *et al*, 2013; Saadallah *et al*, 2023b)^[25]. Antibodies are introduced into agricultural systems as part of the biocontrol concept. Continuous, non-chemical pathogen suppression can be accomplished by employing a living, reproducing biocontrol agent. On the other hand, chemical techniques might upset the microbiological community, which would allow advantageous organisms to flourish in a passive setting. (Anusha *et al*, 2019)^[1] the goals of this study:

1. Determine and separate the fungi that are contaminated wheat seeds existing in Iraqi seed stores and local marketplaces.
2. Confirming the diagnosis of harmful fungus *Fusarium solani* on wheat variety grain samples through PCR techniques.
3. Assess the ability of *Trichoderma harzianum* to inhibit the growth of the harmful fungus *Rhizoctonia solani* under laboratory conditions by using dual culture technique and Using non-volatile metabolites to detect antifungal activity

Materials and Methods Collecting samples

the local wheat variety sample namely Sham-6 was obtained from seed stores and two varieties (Abaa-99 and Jihan) from local market for the agricultural season 2024-2025. 1 kg for each variety stored in tightly closed paper bags.

Isolation of Seed-Borne Fungi

Seed-borne fungi were identified from some wheat seeds using the agar plate method according to the guidelines set by the International Seed Testing Association (I.T.S,1976). Seed-borne fungi were isolated in pure cultures to facilitate identification and further research.

The Agar Plate Technique

Potato Dextrose Agar (PDA) was the medium that dispensed into pre-sterilized petri dishes into pre-sterilized petri dishes, with each dish receiving 15 mL of the medium. In this experiment 100 surface-sterilized seeds were used, with 10 seeds placed in each petri dish. The plates were incubated at a temperature of $25 \pm 2^\circ\text{C}$. After seven days of incubation, the seeds were examined with light microscope to assess the presence of various fungal growths. Fungal slides were prepared for the identification and confirmation of seed borne fungi.

Morphological Diagnosis of Isolated Fungi

The isolated fungi were identified by examining their colony morphology through macroscopic features and observing their mycelia and asexual/sexual spores under a microscope (Ellis, 1971; Pitt and Hocking, 2009)^[10,21].

Diagnosis at the molecular level

Utilizing a Wizard Genomic DNA purification kit (Geneaid, Taiwan) modified in accordance with the manufacturer's instructions, the genomic DNA of an isolate of *R.solani*. was extracted. *R.solani* 's DNA purification was improved by employing the internal transcribed spacer (ITS) primer set, which included ITS-1 (5'-TCC GTA GGT GAA CCT GCG G- 3') and ITS-4 (5'-TCC TCC GCTTAT TGA TAT GC-3'). A 25 μL reaction mixture was used for PCR amplification reactions, which were conducted under PCR conditions. A single cycle of 95°C for five minutes was one of these conditions, which were followed by thirty cycles of denaturation at 95°C for thirty seconds, annealing at 52°C for thirty seconds, extension at 72°C for one minute, and a final extension cycle there for seven minutes. The resultant DNA fragments were electrophoresed and subsequently analyzed on a 1–5% agarose gel. A digital camera from Canon in the United States was used to take the pictures, and a UV transilluminator was used to view the fragments.

Identifying the Nitrogenous Base Sequence for the Products of Amplification

By submitting the amplification products to Psomagene/ USA for nitrogenous base sequencing analysis, the nitrogenous base sequence of the DNA bands from the gene under investigation was ascertained.

Fungal Isolates hesitation (Frequency)

Using the following formula^[15], the frequency of the fungal isolates was calculated: percent Frequency = ((Total number of fungal isolates / cultivar) / Total number of fungal isolates) $\times 100$.

Analysis of *Trichoderma Harzianum* Antagonistic Activity: Technique de Culture Duale

The adverse impact of *Trichoderma harzianum* isolate against the harmful fungus *R. solani in vitro* was assessed using the dual culture method. Separate cultures of *Trichoderma harzianum* and *R. solani* were maintained for seven days at 25°C on PDA medium. A disc with a diameter of 0.5 cm from the bio-control fungus was inoculated on the PDA medium surface on one side of the petri dish, and a disc with a diameter of 0.5 cm from *R. solani* was inoculated at an equal distance from the other side of the petri dish. Petri dishes were inoculated with a pathogenic fungus only as a control, and each replicate had three plates. The inoculation was incubated at 25°C for seven days, with the antagonistic degree determined in accordance with Bell *et al*. (1982) as shown in tab.1.

Table 1: The Bell scale is used to classify *Trichoderma* strains based on their antagonistic behaviors when co-cultured with plant pathogens.

Pattern of colonization	Rating scale for antagonism
The <i>Trichoderma</i> strain outcompetes harmful fungi by completely colonizing the culture medium.	1
At least roughly two-thirds of the surface area of the culture medium should be grown by the <i>Trichoderma</i> strain.	2
About half of the surface area of the culture medium is occupied by the <i>Trichoderma</i> strain and the pathogenic fungi, neither of which exhibits dominance over the other.	3
Pathogenic fungi show immunity to the <i>Trichoderma</i> strain and occupy at least two-thirds of the surface of the culture medium.	4
The entire surface of the culture medium is covered with pathogenic fungi, which only grow on the <i>Trichoderma</i> strain.	5

Using Non-Volatile Metabolites to Detect Antifungal Activity

Mycelial agar plugs (0.5 cm in diameter) were taken from the edges of a 7-day-old culture of *Trichoderma harzianum* to test the impact of non-volatile metabolites on the growth of mycelia in pathogen-poisoned food. were inoculated in 250 ml conical flasks with 100 ml of sterilized potato dextrose broth (PDB) and incubated for 10 days at 25±20C, shaking the flasks every two to three days. Sterile PDA plugs were used to inoculate the control conical flasks. Three conical flasks were used for each treatment. Following incubation, the culture was filtered through a Whitman No. First, one filter paper, followed by Millipore 0.22µ. Filtrates were adjusted in PDA to achieve a 50% concentration in petri plates. The center inoculated solidified agar plates with a pathogen mycelial disc that was 0.5 cm in diameter, and the plates were incubated at 25±2°C for 5 days. The control plates were plates that did not contain filtrate (Ibraheem, 2009) [14].

The ratio was calculated and had only a new part:

$$\text{Percentage of inhibitor} = \frac{(\text{The diameter of control colony} - \text{The diameter of treatment colony})}{\text{The diameter of control colony}} \times 100\%$$

Results and Discussion

Wheat seeds Recovered Fungi

As recommended by (I.T.S,1976), the Agar plate technique was used to detect seed-borne fungi, wheat seeds variety Sham-6 were surface sterilized with a 1% sodium hypochlorite solution. 100 seeds were analyzed in total. Table 2 shows that many fungal species from 3 different genera were isolated and identified from the seed samples, as well as the samples (Abaa-99 and Jihan) obtained from local markets were 100% contaminated with the *Rhizopus* sp. as shown in and fig.1 and 2.

Table 2: Fungi isolated from two types of wheat seeds on potato and dextrose medium

Seed samples/cultivar	Isolated fungal species	Isolation%	Fungal frequency
Sham- 6	<i>Aspergillus niger</i>	7	31.81
	<i>Aspergillus terrus</i>	6	27.27
	<i>Rhizoctonia solani</i>	4	18.18
	<i>A. flavus</i>	3	13.63
	<i>Alternaria</i> spp.	2	9.09
the total		22	

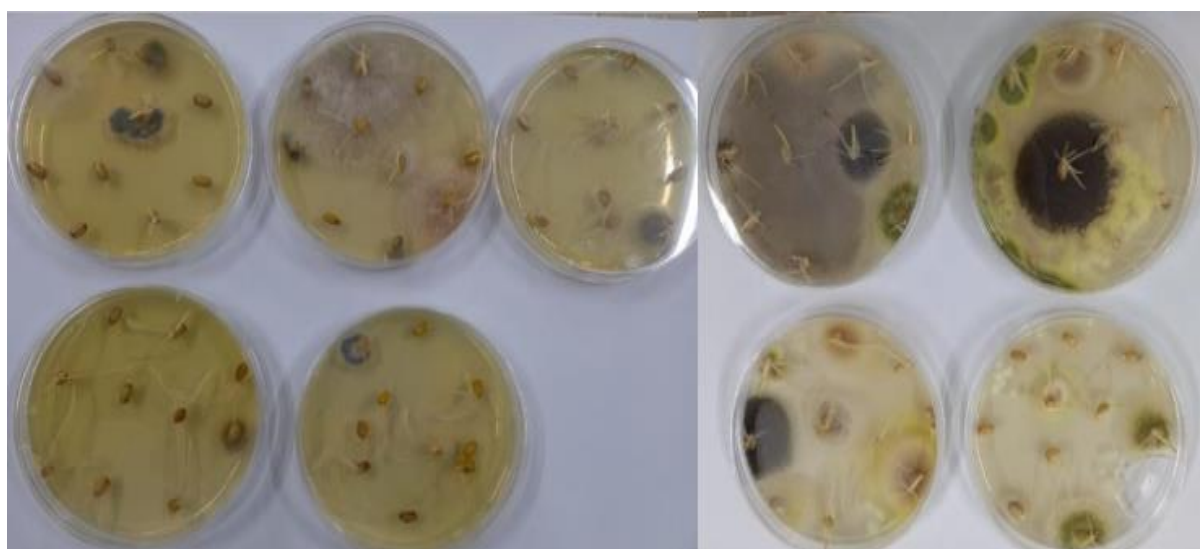


Fig 1: Fungal colonies associated with wheat seeds cultivar Sham- 6 on PDA

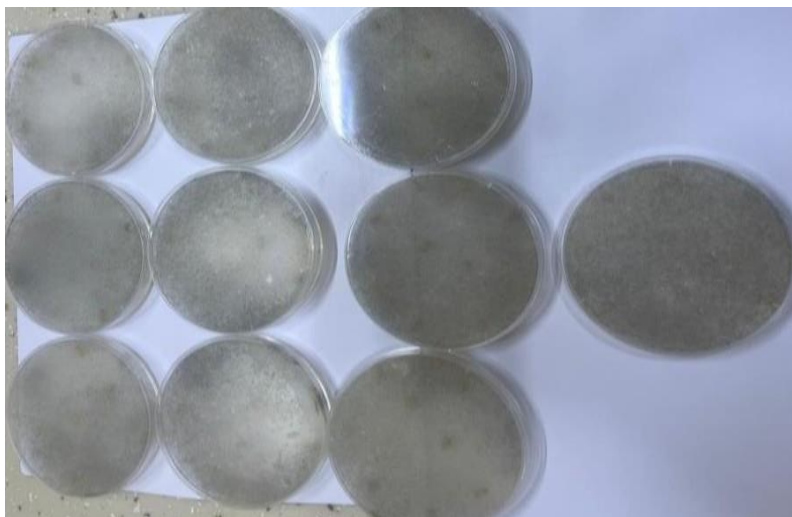


Fig 2: *Rhizopus* sp. colonies associated with wheat seeds cultivars Abaa-99 and Jihan on PDA

The Hesitation of Fungal Isolates

The most frequently isolated fungi from wheat seeds were *Aspergillus niger* (31.81%), followed by *Aspergillus terreus* (27.27%), *Rhizoctonia solani* (18.18%), *A. flavus* (13.63%),

and *Alternaria* spp. (9.09%), as shown in Table 2. And fig 3 and 4. Fungi can transmit by seeds to plants and lead to a decrease in their ability to germinate and grow (Ali *et al*, 2024)^[31].



Fig 3: *R. solani* isolated from wheat seeds at 40X magnification power

This research paralleled previous findings, indicating that the mycological analysis of wheat seeds identified a total of 16 fungal species, including *Alternaria alternata*, *Aspergillus candidus*, *Aspergillus flavus*, *A. niger*, *A. ochraceous*, *A. phoenicis*, *A. tamari*, *A. terreus*, *A. sydowi*, *Fusarium moniliforme*, *F. oxysporum*, *F. solani*, *P. glabrum*, and *Rhizopus nigricans* (Kumar *et al*, 2023)^[16]. Assessment conducted by (Momtaz *et al*, 2022)^[19] using blotters and agar plate techniques identified several key fungi linked to both healthy seeds and those exhibiting black point. These fungi include *Alternaria alternata*, *A. triticina*, *Bipolaris sorokiniana*, *B. tetramera*, *Curvularia lunata*, various *Fusarium* species, and certain *Aspergillus* species. (Khalaf *et al*, 2023)^[17] carried out a study in 2021 to identify the seed-borne fungi present in wheat cultivars at seed production facilities in Salah Al-Din Governorate, Iraq, Fungal identification was conducted both culturally and microscopically using established taxonomic keys. *A. flavus*

was found in all wheat seed samples, resulting in a 100% occurrence rate, alongside *A. niger*. Other species identified included *Alternaria* (*A. alternata*, *A. tenuissima*, *A. saponaria*) and *Aspergillus* (*A. flavus*, *A. fumigatus*, *A. terreus*, *A. niger*), as well as *Rhizopus oryzae*, *Rhizoctonia solani*, *Penicillium* spp., *Aureobasidium pullulans*, *Phoma* spp., *Curvularia lunata*, *Stemphylium* spp., and *Mucor* spp., each present at varying percentages. Additionally, a study was conducted to assess the impact of microwaves on fungal pathogens affecting different wheat varieties.

Amplifying the ITS region through PCR techniques:

In purified DNA samples, the target regions of ITS1, ITS2, and the 5.8S gene were symmetrically amplified using the primers ITS1 (5- TCCGTAGGTGA ACCTG CCG-3) and ITS4 (5- TCCTCCGCTTATTGATATGC-3). Following electrophoresis analysis on a 2 percent agarose gel, ethidium bromide staining, and UV visualization, the PCR products

showed bands corresponding to 330 bp (Fig. 4.) These findings are entirely consistent with the initial morphological

identification of isolates of *R. solani*.

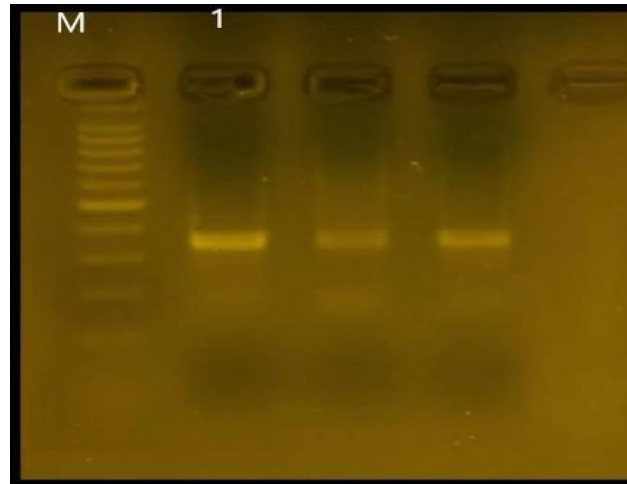


Fig 4: DNA amplification product of *R. solani* on agarose gel, M: indicates the volumetric marker pathway, 1: DNA bundle

Sequence of the Chosen Fungal Isolate's Nucleotides

Following the completion of the PCR reaction and the amplification of the intended target, the DNA bundle was cut from the agarose gel and placed in an Eppendorf tube, where the forward primer and deionized water were added. This was done using a specific extraction kit. It was sent to Psomagen/

USA in order to determine its nucleotide sequence. fig (5). The nucleotide sequences deposited in the Gene Bank. Based on the lineup process results, the local fungal isolate that was chosen had a nucleotide sequence that was of the type *R solani*. with the PV138271 registration number.

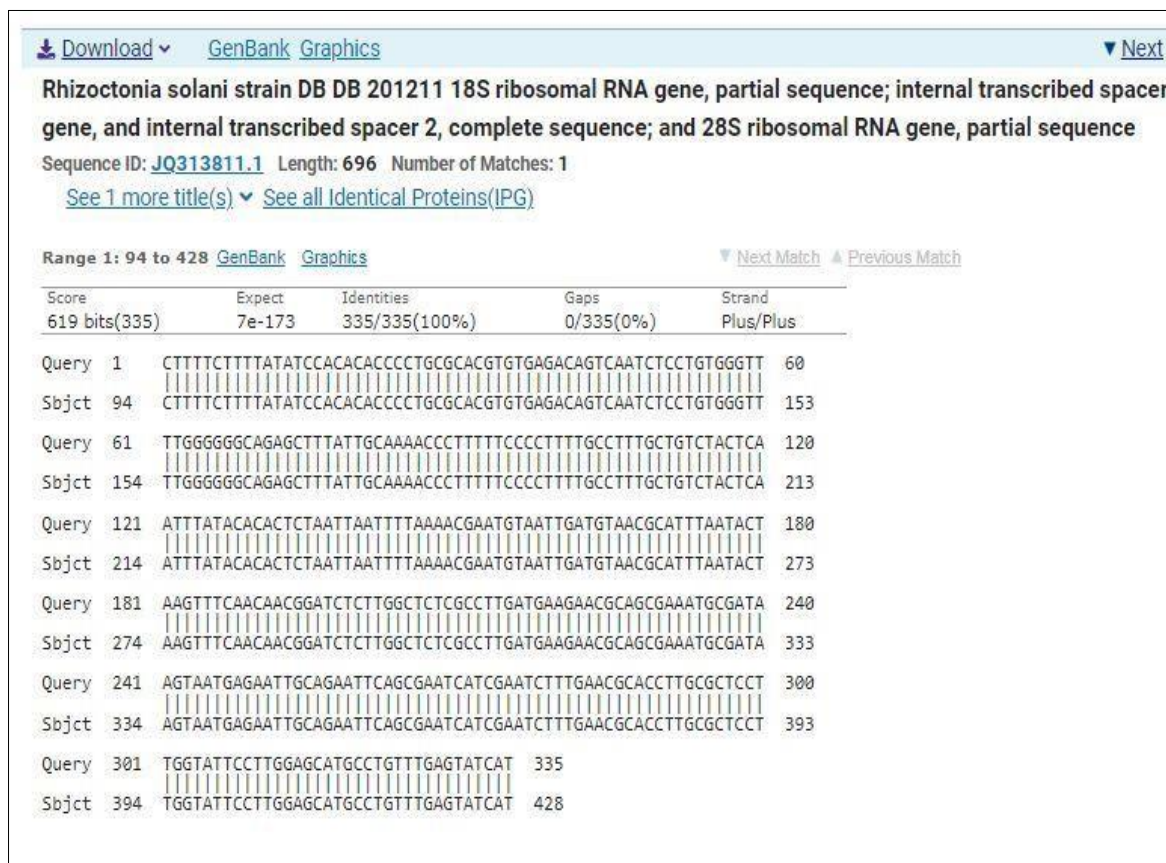


Fig 5: Nucleotide sequences of *R solani*

Technique de Culture Duale (The Antagonistic Activity):

The results of the dual culture test (fig. 6) demonstrated that the *T. harzianum* isolate inhibited the pathogen mycelial growth of *Rhizoctonia solani*, as seen in Tab.1, *T. harzianum*

exhibited statistically significant inhibition that reached 1 on the bell scale. The pathogenic fungus *Rhizoctonia solani* is outcompeted by the *T. harzianum* strain, which completely colonizes the culture medium.

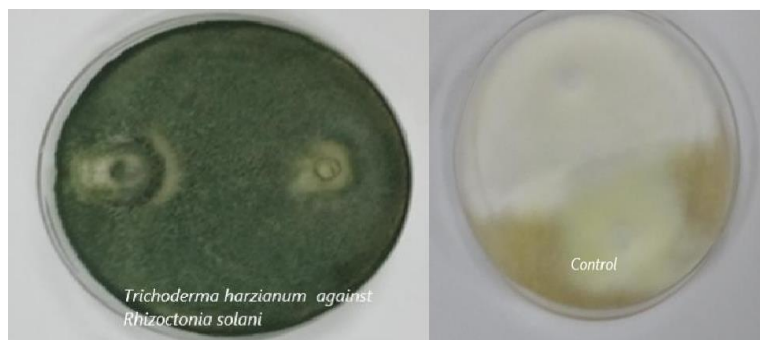


Fig 6: Antagonistic activity of *T. harzianum* against *Rhizoctonia solani*.

Trichoderma are saprophytic fungi that have a high degree of environmental adaptability and rapid mycelial growth (Ali *et al.*, 2024) [3]. It can capture the invasive portion of the pathogenic fungi in a plant's root, preventing the pathogen fungi from invading. Additionally, it has the ability to quickly absorb the nutrients needed for the pathogen fungi to grow, which can lead to nutrient deficiencies and prevent the pathogen fungi from growing and reproducing. (Guo *et al.*,

2019; Bazghaleh *et al.*, 2020; Halifu *et al.*, 2020) [11, 5, 13].

Using Non-Volatile Metabolites to Detect Antifungal Activity (Antibiosis Activity):

The cultural filtrate of *T. harzianum* exhibited antibiotic potential against *R.solani* by inhibiting its mycelial growth (Fig.7). The inhibition percentage was reached 55.5%.

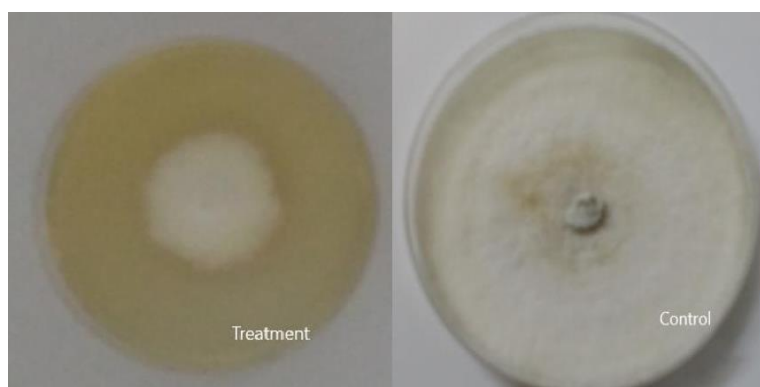


Fig 7: The Antibiosis activity of *T. harzianum* against *R.solani*

Mechanisms by which *Trichoderma* spp. are based on the development of lytic enzymes, antimicrobial compounds (Lorito *et al.*, 2010) [18]. Additionally, *Trichoderma* spp. are recognized to generate volatile organic compounds. (Crutcher *et al.*, 2013; Guo *et al.*, 2020) [7, 12].

Conclusions

The results of this study showed that the seeds of wheat, variety Sham 6, which were obtained from grain stores in Nineveh Governorate, were contaminated at a low rate with saprophytic fungi. As for pathogenic fungi *R.solani*, the contamination rate was very low, reaching 18.18 %, While seed samples obtained from local markets were 100% contaminated. *T. harzianum* inhibit the development of *R. solani* with High efficiency under *in vitro* conditions, when they grow in Petri dishes.

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