Evaluation the potential of indigenous biocontrol agent *Trichoderma harzianum* and its interactive effect with nanosized ZnO particles against the sunflower damping-off pathogen, *Rhizoctonia solani*

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Abstract. *Rhizoctonia solani* is a phytopathogenic fungus affecting a wide range of plants hosts including the sunflower causing various diseases such as damping-off. Current management approaches of this pathogen are inadequate. Aim of this study was to assess the potential of eco-friendly control methods, the indigenous biocontrol fungus *Trichoderma harzianum* and ZnO nanoparticles for controlling of the sunflower damping-off pathogen, *R. solani*. The biocontrol agent *T. harzianum* showed a high antagonism effect on *R. solani* growth. Additionally, growth of *R. solani* was significantly (p = 0.01) reduced gradually by presence of various concentrations of the ZnO NPs indicating to concentration-dependent toxicity effect. However, a similar impact was also observed on growth of *T. harzianum*. On the other hand, the percentage of seed germination and stem length of sunflower (Coban cv.) did not affect significantly by ZnO NPs. Conversely, the root lengths were significantly decreased. In the horticultural canopy trial, the best reduction to the sunflower damping-off incidence percentage was achieved by treatment of sunflower seedlings growing in compost inoculated with *T. harzianum* and *R. solani* 68.75% comparing to 100% in the control. In contrast, a significant reduction in severity percentage of damping-off symptoms was accomplished in most of the treatments. The best suppression was achieved in treatments of spraying the seedlings with ZnO NPs (15 mg/ml) in two days prior of planting that was 50% in compression with 97.50% in control. These findings can justify the application of the local biocontrol agent *T. harzianum* alone or in integration with ZnO NPs to be included with current management approaches of sunflower damping-off, which could lead to a diminution in the utilizing of fungicides.

1. Introduction

The sunflower (*Helianthus annuus* L.), which belongs to the Asteraceae family, is one of the most important oil crops in the world [1]. It provides the largest amount of oil per unit of cultivated area because its seeds contain a high percentage of oil up to 50%. It is widely used in the manufacture of high-quality edible oils and sometimes as a fertilizer or flammable source. Additionally, sunflower meal is a good component of animal feed because it contains a high quantity of proteins, lignocellulosic fibre and minerals. Furthermore, sunflower fields can be exploited for honeybee breeding to produce good...
quality honey, and the bees, in turn, lead to increased success in pollination [2]. However, the production of the sunflower crop in Iraq has declined significantly in the last decade [3]. Among many reasons for this, the aggravation and emergence of plant pathogens have caused new and more severe diseases which ultimately have affected the stability and yield of the sunflower crop. One of the most prevalent and destructive diseases of this crop is damping-off caused by numerous phytopathogens including *Rhizoctonia solani*. The symptoms of this disease include the development of dark brown and soft decaying lesions on the hypocotyl sides that extend rapidly to include the entire tissues of the hypocotyl and radicle of infected seedlings. These seedlings eventually die before or after emergence on the surface causing, a reduction in a number of seedlings and consequently an economic decline in the yield [4; 5; 6]. The conventional control approach for this disease is the intensive applications of synthetic fungicides. However, the effectiveness of these fungicides in several circumstances is deficient and there are also adverse negative consequences related to this practice, such as the development of pesticide-resistant phytopathogens and a direct threat to human and animal health. Consequently, public attention has been drawn to the need to improve sustainable and robust management approaches for plant disease, including damping-off [7].

These reasons and others have drawn attention to practicable alternatives to fungicides, such as biological control. Numerous microorganisms, for example, have been reported as biocontrol agents against plant pathosystems. Hence, they have been developed and employed successfully in disease management strategies in conventional and organic farming [8]. Several species of the genus *Trichoderma* such as *T. harzianum* have proven to be most effective biological control agents [9; 10]. These species have been used to inhibit several plant pathogens using various mechanisms such as hyperparasitism and producing diverse types of effective antibiotics and enzymes as well as competition for nutrients and space [11]. In addition, they are able to induce systemic resistance in plants against pathogenic microorganisms [12; 13].

Another alternative approach that has received considerable attention in the plant protection field is the application of Nano-particles (NPs). This is due to their small size (1-100nm) and large surface area that lead them to stimulate new reactive groups and unique chemical characteristics, which improve their positive impact on destructive microorganisms [14; 15]. Currently, many metal-based nanoparticles such as ZnO NPs have been applied in fertilizer which have demonstrated significant antimicrobial activity [16]. For example, the growth of several fungal causative agents of post-harvest fruit molds (*Penicillium expansum*, *Botrytis cinerea*, *Aspergillus niger* and *A. flavus*) were inhibited significantly due to the usage of ZnO NPs [17; 18]. Moreover, the ZnO NPs were found to be less toxic to plants and beneficial soil bacteria in comparison with other metal-NPs [19; 20]. Nevertheless, there is a lack of studies related to the effect of these nanoparticles on plant pathogens such as *R. solani* and the possibility of employing them in conjunction with biocontrol agents such as *T. harzianum* to control damping-off disease in sunflowers.

The aim of this study, therefore, was to assess the potential of the indigenous biocontrol agent *T. harzianum* and commercial ZnO NPs separately and in combination against the pathogen of sunflower damping off, *R. solani*.

2. Materials and methods

2.1 Isolation, identification and pathogenicity of the causative agent of sunflower damping-off

Samples of sunflower seedlings, exhibited sever damping-off symptoms were collected from sunflower fields located in the Agriculture college, University of Kerbala, Iraq during growth season of 2016-2017. The symptomatic seedlings were washed, cut into small sections (0.5-1 cm long), surface sterilized with sodium hypochlorite (2%), and washed again in distilled water. These sections were then sited on water agar media (WA) and incubated at 25±2. After three days, the fungal colony raised were purified on growth medium of potato dextrose agar (PDA) and incubated at 25±2 for 7 days. The identification of fungus associated with diseased seedlings was Rhizoctonia solani depending on its cultural and
morphological features [21; 22]. The pathogenicity of fungus isolated was assessed following the Petri plate technique described previously [23].

2.2 Effect of T. harzianum on R. solani
The efficacy of the biological control indigenous fungus T. harzianum, obtained from laboratory of biological control, Musiab Technical College, Al Furat Alawsat Technical University, Iraq, was evaluated following the Dual culture bioassay [24]. This bioassay was conducted in Petri dishes (8 cm diameter) comprising of PDA media. Pair of pure colony disks (0.5 cm diameter) of R. solani and T. harzianum were placed opposite each other at 1 cm distance from the dish edge. Simultaneously, others Petri dishes containing single disk of R. solani or T. harzianum separately were made as control. All dishes were then incubated at 25 °C in the darkness until the fungal growth of control filled the dishes. The antagonistic efficiency of T. harzianum against R. solani was estimated by following the five-grade scale [25] with some modifications. Growth rate of each control culture was measured at daily intervals starting three days after inoculation. The antagonistic ability of T. harzianum was estimated according to the five-grade scale as following: Grade 1 - The growth rate of the biocontrol fungus T. harzianum fills the whole dish of paired cultures which means the inhibition percentage is 100%. Grade 2 - the growth rate of the biocontrol T. harzianum fills ¾ of the dish while the remaining fills with growth of the pathogenic fungus R. solani and this equals an 75% inhibition percentage. Grade 3 – growth rate of T. harzianum fills half of the dish, whereas another half filled with growth of R. solani and this equals 50% inhibition percentage. Grade 4 – the growth rate of the pathogenic fungus R. solani fills ¾ of the dish while the remaining fills with growth of the biocontrol T. harzianum and this equals an 25% inhibition percentage. Grade 5 – The growth rate of the pathogen R. solani fills whole the dish of paired cultures and means the inhibition percentage is 0%. The biological agent is efficient once the antagonistic scale is 1 or 2.

2.3 Effect of ZnO NPs on R. solani and T. harzianum
Commercial ZnO NPs was purchased from MKnano, Missisauga, Canada. Based on information provided with the product, the size of nano-particles was 30 nm. This was confirmed through Fourier transform infrared spectroscopy (FTIR), Atomic force microscopy (AFM) and X-Ray Diffractometer examinations. The medium of potato dextrose agar media (PDA) was prepared according to instructions of manufacture (CDH Bioscience, India) and before autoclaving at 121 °C for 20 min, it was amended with diverse concentrations of ZnO NPs (0, 0.25, 0.5, 1, 2.5, 5, 10 and 15 g/l). After cooling to approximately 45 °C, the PDA was vigorously blended to disperse evenly the ZnO NPs product in the medium, before pouring it in the plates (about 20 ml per plate). Mycelial agar disks (0.5 cm in diameter) were taken separately from the edges of 7 days-old pure colonies of R. solani and T. harzianum and placed into the center of amended PDA plates. As well as, other PDA plates were amended with the fungicide Beltanol-L (1 ml/l) to be used as control. All plates prepared were incubated in darkness at 25+2 °C for 7 days. In order to assess adaptation of both fungi to ZnO NPs in PDA media, mycelial agar disks were cut from 7-days old colonies of both that were growing in plates containing 0.25, 0.5, 1 and 2.5 g ZnO NPs/l and relocated to fresh PDA (free of ZnO NPs) plates for 7 days at 25+2 °C. The inhibition percentage of fungal growth was measured by following the equation below:

\[
\text{Inhibition} \% = \frac{C - T}{C} \times 100
\]

T is the average of fungal growth colony in diameter in the amended plates, while C is the average fungal growth colony in diameter in the (0 g ZnO/l) plates.

2.4 Evaluation the influence of effective concentrations of ZnO NPs on seeds and seedlings of sunflower
The influence of the most effective ZnO NPs (2.5, 5, 10 and 15 mg/ml) on the pathogenic fungus R. solani was assessed on seeds and seedlings of sunflower (Coban cv.) plants. The seeds were surface-sterilized (sodium hypochlorite 2%), washed thoroughly with distilled water and placed on sterilized
Whatman® filter paper on a plate (10 seeds per plate). Subsequently, they were watered with the selected concentrations of ZnO NPs every two days. In contrast, seeds on control plates were watered with distilled water only. All treatments were conducted in quadruplicate and incubated at 21 °C and 16 h light/8 h dark in a growth chamber. After 14 days, the percentage of seeds which had germinated was calculated using the following formula:

\[
\text{Germination\%} = \frac{\text{Number of seeds germinated}}{\text{Total number of seeds}} \times 100
\]

Additionally, the lengths of the hypocotyls and radicles of the sunflower seedlings were determined in addition to the dry weight of seedlings after dehydration at 50 °C for three days.

2.5 The interactive influence of *T. harzianum* and ZnO NPs in the protection of sunflower seeds and seedlings from *Rhizoctonia solani* causing damping-off

An experiment was conducted to determine the effectiveness of *T. harzianum*, ZnO NPs and the integration between them in controlling sunflower damping-off caused by *R. solani*. The pathogen inoculum was prepared by blending the contents of five pure *R. solani* plates in 1L of distilled water for 2 min which were then added to the autoclaved compost (50 ml per container) [26]. At the same time, the biocontrol agent *T. harzianum* inoculum was prepared by collecting spores of pure 10 day-old colonies of *T. harzianum* via distilled water. They were then diluted and mixed with the autoclaved compost (1x10^7 spore/g). The inoculated compost was utilized after three days of incubation at 25±2 °C [27]. The sunflower (Coban cv.) seeds were surface-sterilized using sodium hypochlorite 2% and washed with distilled water and used in the following treatments:

1) The sunflower seeds were soaked for two days in ZnO NPs (15 mg/ml) and then sowed in the *R. solani* inoculated compost.
2) The sunflower seedling roots were soaked for two days in ZnO NPs (15 mg/ml) before transferal to the *R. solani* inoculated compost.
3) The sunflower seedlings were sprayed with ZnO NPs (15 mg/ml) two days after transfer to the *R. solani* inoculated compost.
4) The sunflower seedlings were planted in *R. solani* and *T. harzianum* inoculated compost.
5) The sunflower seeds were soaked for two days in ZnO NPs (15 mg/ml) and sowed in the *R. solani* and *T. harzianum* inoculated compost.
6) The sunflower seeds were soaked for two days in ZnO NPs (15 mg/ml) and sowed in the *R. solani* and *T. harzianum* inoculated compost and the seedlings were then sprayed with ZnO NPs (15 mg/ml).
7) The seedlings were planted in *R. solani* and *T. harzianum* inoculated compost and sprayed with ZnO NPs (15 mg/ml).
8) The seedlings planted in *R. solani* inoculated compost were treated with the fungicide Beltanol-L (1ml/l).
9) The seedlings were planted in *R. solani* inoculated compost only as a negative control. Each treatment was quadruplicate with 4 seeds or seedlings in each pot.

After two weeks of planting, the incidence of sunflower damping-off was calculated using the following equation:

\[
\text{Disease incidence\%} = \frac{\text{Number of diseased seedlings}}{\text{Total number of seedlings}} \times 100
\]

After four weeks of planting, the severity of sunflower damping-off was evaluated employing a standard disease index (Lahuf et al. 2019) consisting of five indices (0 : no symptoms appear on the crown and root of seedlings; 1 : discoloration with softness on crown and root (1-25%); 2 : small brown lesion on crown and root rot (26-50%); 3 : wilting of seedlings and large dark lesions on crown and root rot (51-75%); and 4 : dead seedlings). The disease index was then calculated by applying the following equation:

\[
\text{Disease severity\%} = \frac{\Sigma (\text{Score of disease} \times \text{Number of plants})}{\text{Total number of seedlings} \times 5} \times 100
\]
2.6 Statistical analysis
All data were analyzed by operating one-way ANOVA (SPSS version 13.0) at a significance level of \( p < 0.01 \).

3. Results and Discussions

3.1 Isolation, identification and pathogenicity of the causative agent of sunflower damping-off
The fungus associated with diseased sunflower seedlings showed symptoms of severing damping-off including wilt, yellowing converting to dark browning and rot of the crown and area roots were isolated and purified. Its colonies have thick light brown fungal mycelia which turn darker with age and produce some sclerotia that were of irregular spherical shape and black in colour (Fig. 1A). The microscopic investigation revealed that the fungus did not produce spores and the mycelia were divided with septa or partitions inside. At the beginning of a new hyphal branches, there was a slightly narrower region with a septum and these new branches formed perpendicular angles with the main branches (Fig. 1B). These unique morphological features are consistent with previous descriptions referring to the fungus as \( R. solani \) [21; 22; 28]. The pathogenicity test of the isolated fungus showed (Fig. 1C-D) its high ability to cause the infection and death of all seeds examined (100%) compared with the control (0%). This fungus it is well-known to infects a wide range of plant hosts including sunflowers [29; 30; 7].

![Fig. 1 Morphological and microscopic features and pathogenicity of fungus \( R. solani \) associated with sunflower seedlings showed damping off symptoms. (A) cultural characteristics (left upper surface, while right is underneath the surface of the fungal colony). (B) microscopic features, (C) healthy germinated seeds, and (D) seeds infected with \( R. solani \).](image)

3.2 Antagonistic effect of \( T. harzianum \) on \( R. solani \)
The results of the experiment proved that the isolation of indigenous \( T. harzianum \) samples exhibited a high antagonistic ability against \( R. solani \) causing a significant reduction in its growth that was less than 2 according scale [25] with inhibition percent between 75-100% (Fig. 2). It is worth mentioning that the
mycelia of the biological agent covered the growth of the fungal pathogen, which indicates a case of interaction between the mycelia of both fungi as a result of the parasitism of the biological agent on the pathogen. Where it is known that mycelia of *T. harzianum* are relatively smaller in diameter, and they spiral around the fungal filaments of the pathogen forming compressive structures and producing enzymes such as Cellulases, Chitinases, Proteases and β-glucanases leading to the death of the pathogen [31]. It has also been found that the biocontrol agent *T. harzianum* produces different antibiotic compounds, such as Trichorzanines, Alamethicine, Trichodermi, Peptaibols and Alkylpyrones, that were found to inhibit or kill the pathogens [13; 32].

3.3 Effect of ZnO NPs on *R. solani* and *T. harzianum*

The growth of both fungi *R. solani* and *T. harzianum* was challenged by diverse concentrations of nanosized ZnO particles. The effect of the nanoparticles represented significant (p = 0.01) dosage-dependent inhibition of both fungal species (Fig. 3). A concentration of 15 mg/ml caused full inhibition (100%) of *R. solani* and *T. harzianum* growth and this was comparable to the effect of the bacterial fungicide Beltanol® which was used as a control. However, lower concentrations of ZnO NPs (10, 5 and 2.5 mg/ml) led to lower levels of inhibition at 83.21, 71.03 and 57% respectively of *R. solani*. Conversely, these same concentrations completely inhibited (100%) the growth of *T. harzianum*. On the other hand, lower ZnO NPs concentrations of 1, 0.5, and 0.25 mg/ml had the least inhibitory effect on the growth of *R. solani* (43.1, 46.2 and 0% respectively) and on the growth of *T. harzianum* (61, 27 and 0% respectively). No inhibition was observed in the control plates with fungus only. Nanosized ZnO particles have been found to affect various microorganisms, including some fungi, by preventing them producing conidia and conidiophores, ultimately leading to death [17]. The mechanism of the influence of ZnO NPs on microorganisms has been suggested to be a result of the production of free radicals on the surfaces of nano-particles and the formation of toxic compounds such as hydroxyls and superoxides which damage the lipid compounds in the cell membrane of microorganisms, leading to leakage and the collapse of cell membranes and the inhibition of their functions through the transmission of high energy levels from the inside to the outside of affected cells. This ends with cell death [33].

Additionally, the effect of ZnO NPs on both fungi was found to involve (fungistatic) inhibition rather than being lethal (fungicidal). Normal growth rates could be restored when they were transferred from a modified PDA medium with lower concentrations of ZnO NPs to unmodified PDA medium. This finding is in agreement with previous results concerning the influence of the same nanoparticles on the fungal plant pathogen *Fusarium graminearum* which exhibited a similar mode of action [20].
3.4 Evaluation of the influence of effective concentrations of ZnO NPs on the seeds and seedlings of sunflower

The influence of the most effective ZnO NPs concentrations (2.5, 5, 10 and 15 g/l) on the pathogen R. solani was assessed on the seeds and seedlings of sunflower (Coban cv.) plants (Fig. 3). All sunflower seeds germinated successfully (100%) in all ZnO NPs concentrations tested and without significant differences compared to controls. This can indicate the possibility of applying ZnO NPs to seeds for later use in disease management. Furthermore, although the average length of seedling hypocotyls was reduced in all ZnO NPs treatments, there were no significant differences between their length and the average hypocotyl length in controls. The concentration with the least affect was 2.5 g/l (5.28 cm) followed by 5 (4.52 cm) then 10 and 15 (3.95 cm for both concentrations). Conversely, the average length of sunflower seedling radicals dropped significantly (p>0.01), particularly in the concentrations 5 and 2.5 g/l (0.13 and 0.39 cm respectively), whereas in concentrations of 10 and 15 g/l, the average lengths were 0.62 and 0.60 cm respectively. The results obtained in this experiment are consistent with those found in previous studies. For instance, the effectiveness of ZnO nano-particles has been assessed in terms of the percentage seed germination and root length of cucumber and corn crops. It was found that concentration of these nanoparticles of 1g/ l led to significantly reduced root lengths of cucumber to 51% and corn to 17%, yet the percentage seed germination was not affected in either host [34]. Moreover, study of the impact of various concentrations of ZnO NPs on various growth parameters in the sweet leaf plant Stevia rebaudiana revealed a positive effect on growth compared to controls [35].
Most treatments did not reduce the incidence of damping-off disease in sunflower seedlings (Fig. 5). However, treatments 4 and 8 decreased it significantly (p > 0.05) to 68.75 and 93.75% respectively compared with 100% in control treatments.

On the other hand, most treatments caused a significant decline (p > 0.05) in disease severity (Fig. 6). The highest reduction was achieved for treatment 3 (50%) followed by treatments 6 (56.25%) and 4 (59.37%) respectively. Whereas, for treatments 2 and 8, disease severity was reduced to 70.31 and 76.56% respectively. However, the rest of the treatments failed in the protection of sunflower seedlings from the infection by *R. solani*.

These results reveal that the application of the indigenous biocontrol agent, *T. harzianum* alone or in specific combinations with ZnO NPs supplied more protection to the seedlings of sunflower from the damping-off disease caused by *R. solani*.

These finding are consistent with those of previous studies that have shown the efficacy of *T. harzianum*, which is known to be an effective biological agent because of its high antagonism and competitiveness and for the production of various antibiotics and effective enzymes that negatively affect many phytopathogens [12; 13]. Additionally, this biocontrol agent can stimulate plant defences that can participate positively in the control of many plant pathogens. It also has positive consequences for the growth and development of plants [36]. Moreover, the efficacy of ZnO NPs observed in this study is similar to that identified in previous investigations which have shown its effectiveness against many fungal plant pathogens such as *Fusarium graminearum*, *F. oxysporum*, *Botrytis cinerea*, *Alternaria alternate*, *Penicillium expansum* and *Rhizopus stolonifer* [17; 37]. Furthermore, the integration of control approaches has demonstrated high efficiency in the management of various pests, including phytopathogens [29]. Despite the fact that biocontrol microorganisms and nanoparticles can provide high capability in controlling plant pathogens, investigations related to the possibility of their integration against plant pathogens are limited. Thus, according to the data produced in this research, the application

![Graph showing the average length of sunflower seedlings hypocotyls and radicles affected by different concentrations of ZnO NPs.](image)

**Fig. 3**: The average length of sunflower seedlings hypocotyls and radicles affected by different concentrations of ZnO NPs

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of *T. harzianum* alone and in integration with ZnO NPs could represent a promising strategy to control damping-off disease and to increase sunflower crop yields and develop the greater reliability of biological control agents in various climatic environments. Consequently, they could be used in approaches to manage damping-off disease in sunflower crops in Iraq.

![Graph showing disease incidence percentage for different treatments](image)

**Fig. 4:** Effect of different treatments on disease incidence of damping-off on sunflower crop. (T.1) The sunflower seedling roots were soaked for two days in ZnO NPs (15 mg/ml) before transferal to the *R. solani* inoculated compost; (T.2) Seedlings planted in *R. solani* inoculated compost were treated with the fungicide Beltanol-L (1ml/l); (T.3) sunflower seedlings were sprayed with ZnO NPs (15 mg/ml) two days before transferring them to the *R. solani* inoculated compost; (T.4) sunflower seedlings were planted in *R. solani* and *T. harzianum* inoculated compost; (T.5) The sunflower seeds were soaked for two days in ZnO NPs (15 mg/ml) and sown in the *R. solani* inoculated compost; (T.6) sunflower seeds were soaked for two days in ZnO NPs (15 mg/ml) and sown in the *R. solani* and *T. harzianum* inoculated compost; (T.7) sunflower seeds were soaked for two days in ZnO NPs (15 mg/ml) and sowed in the *R. solani* and *T. harzianum* inoculated compost and the seedlings were then sprayed with ZnO NPs (15 mg/ml); (T.8) seedlings were planted in *R. solani* and *T. harzianum* inoculated compost and sprayed with ZnO NPs (15 mg/ml); (T.9) seedlings were planted in *R. solani* inoculated compost only as a negative control.
Fig. 5: Effect of different treatments on the disease severity of damping off on sunflower crop. Treatments are the same as in Fig.4.

4. Conclusion
The outcome of this study can validate the application of the indigenous biocontrol agent *T. harzianum* alone or in integration with ZnO NPs to be involved with current strategies of management the sunflower damping-off pathogen, which is possibly leading to decrease the application of fungicides.

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