Evaluation of enzyme production and biocontrol agent of zinc nanoparticles from *Gonatorrhodiella parasitica*

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Background and objectives
Biosynthesis of zinc nanoparticles (Zn NPs) is considered one of the new approaches for enzyme production and good biofertilizer and biocontrol agent for pathogenic disease of plant. The selected fungal strain *Gonatorrhodiella parasitica* was used for biosynthesis of Zn NPs. Determination of enzyme activity in fermentation medium containing olive mill wastes (OMW) as substrate for production of protease, pectinase, and chitinase at different pH values (6) was done to produce detailed information on the progress of reduction of zinc nitrate on the nanosecond times (6.0, 8.0, and 10.0). Absorbance ultraviolet–visible spectra of Zn NPs at pH 6.0 were at λ_{max} 280 nm. Transmission electron spectroscopy measurements indicate that extracellular biosynthesis of Zn NPs by *G. parasitica* was in the size of 16 nm. Composting of OMW inoculated with *G. parasitica* for 15–30 days led to decrease in total phenol, flavonoids, 2,2 diphenyl-1-picrylhydrazyl (DPPH), and aflatoxins found in OMW. Comparison of *G. parasitica* free and nano zinc particles as biocontrol agent of soil-borne fungi causing preemergence and postemergence damping off of green bean plants (seed treatment) under field conditions measured from 7 to 60 days and its effect of growth parameters confirm the use of Zn NPs for enzymes production and biocontrol application.
Materials and methods
The local fungal strain *G. parasitica* used in this study was isolated from soil and identified at the Department of Chemistry of Natural and Microbial Products at the National Research Center. Determination of enzymes activity (pectinase, chitinase and protease) was done. Determination of major phytochemicals in prepared extracts (total phenolic content, total flavonoid content, DPPH scavenging activity, and aflatoxin) was done. A field experiment was conducted during the 2018 season at Kafer-Eldawar (Behira) Governorate, in a heavily naturally infested field, to study the effect of free and Zn NPs of *G. parasitica* on soil-borne fungi causing preemergence and postemergence damping off of green bean plants (seed treatment) under field conditions.
Results and conclusion
Composting of OMW inoculated with *G. parasitica* for 15–30 days led to decrease in total phenol, flavonoids, DPPH, and aflatoxins found in OMW. Moreover, suppressive soil-borne fungi caused preemergence and postemergence root rot of green bean plants (seed treatment) under field conditions.

Keywords:
biocontrol, biofertilizers, enzymes, *G*onatorrhodiella *parasitica*, olive mill wastes, zinc nanoparticles

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Introduction

Nanoparticles are the building blocks of nanotechnology. Metal nanoparticles have various functions, which are not observed in bulk phases. These nanoparticles are studied for their specific catalytic and antimicrobial functions [1].

Olive mill waste water (OMW) is a major environmental issue in several olive growing countries in the world, which causes enormous pollution to the land and air [2].

Zinc oxide nanoparticles (ZnO NPs) are one of the inorganic multifunctional nanoparticles with effective antimicrobial activity [3].

The main advantages of using ZnO NPs is the stability and long shelf life compared with organic antimicrobial agents [4].

In the nanoscale form, ZnO NPs have a strong toxicity toward a wide range of microorganisms including fungi, bacteria, algae, fish, and plants [5].

Biotechnological approach toward the synthesis of nanoparticles has many advantages, such as possibility of easily covering large surface areas, suitable growth of the mycelia, economic viability, and its green chemistry nature, provided the microorganism medium is safe [6].

ZnO NPs are able to inhibit the mycelia growth of aflatoxigenic molds, especially inhibition of aflatoxin production .The ability of ZnO NPs to inhibit the growth of all types of mycotoxigenic molds and to prevent production of their respective mycotoxins on liquid medium has been illustrated [7,8].

Nanoparticles and nanocapsules provide an efficient means to distribute fertilizers and pesticides in a controlled fashion with high site specificity while reducing damage. Farm application of nanotechnology is gaining attention through controlling and releasing of pesticides, herbicides, and fertilizers [9].

The main purpose of this work was the synthesis of zinc nanoparticles (Zn NPs) from local fungal strain *Gonatorrhodiella parasitica* using OMW as substrate and its role in enzyme production, biofertilizer, and biocontrol applications.

Materials and methods

Microorganism

The local fungal strain *G. parasitica* used in this study was isolated from soil and identified at the Department of Chemistry of Natural and Microbial Products at the National Research Centre, Dokki, Cairo, Egypt. The strain was kept on potato dextrose medium and stored at 4°C.

Fermentation condition

Fermentation was carried out in a 250-ml Erlenmeyer flask, containing 50 ml of fermentation medium, which consists of (in g/l) OMW (20.0), zinc nitrate (2.0), K_2HPO_4 (1.0), $MgSO_4$ (0.5) KCl (0.5), and $FeSO_4$ (0.01). It was autoclaved at 121°C for 15 min at different pH values 6.0, 8.0, and 10.0 for comparison. One ml of 10^6 spore suspension of the selected fungal strain was inoculated in each flask and incubated at 28–30°C for 7 days at 200 rpm.

Determination of enzyme activity

Pectinase assay

Pectinase activity was determined using citrus pectin as substrate. The reaction mixture, containing equal amounts of 1% pectin prepared in sodium acetate buffer (0.05 M; pH 5.5) and suitably diluted crude enzyme, was incubated at 50°C in water bath for 30 min. The reaction was stopped with 1.0 ml of dinitrosalicylic acid solution [10], after which the mixture was boiled for 10 min and cooled. The color was read at 540 nm using a spectrophotometer. A standard graph was generated using standard glucose solution. One unit of pectinase activity was defined as the amount of enzyme that liberated 1 μm of glucose per min.

Chitinase assay

Colloidal chitin was prepared from chitin powder (Sigma Co., Sigma Aldrich St. Louis, Missouri, United States). The colloidal chitin suspension was adjusted to pH 7.0 with 1 N NaOH and recentrifuged. The pelleted colloidal chitin was stored at 4°C until used.

Determination of enzyme activity was carried out according to the method of Reid and Ogryd-Ziak [11]. Overall, 1 ml of 1% colloidal chitin in citrate phosphate buffer (pH 6.6) was taken in test tubes. One milliliter of culture filtrate was added and mixed by shaking. Tubes were incubated in a water bath at 37°C for 60 min, then cooled and centrifuged before assaying. Reducing sugars were determined in 1 ml of the supernatant by 3,5-dinitrosalysilic acid. Optical density was measured at 540 nm.

Protease assay

Protease activity in the culture supernatant was determined according to the method of Tsuchida *et al.* [12] using casein as a substrate. A mixture of 500 μl of 1% (w/v) of casein in phosphate buffer (pH 7.0) and 200 μl crude enzyme extract was incubated in a water bath at 40°C for 20 min. After 20 min, the enzyme reaction was terminated by the addition of 1 ml of 10% (w/v) trichloroacetic acid and was kept at room temperature for 15 min. Then, the reaction mixture was centrifuged to separate the unreacted casein at 10 000 rpm for 5 min. The supernatant was mixed with 2.5 ml of 0.4 M Na_2CO_3, and then 1 ml of Folin-Ciocalteu's phenol reagent was added. The resulting solution was incubated at room temperature in the dark for 30 min, and the absorbance of the blue color developed was measured at 660 nm against a reagent blank using a tyrosine standard. One unit of protease was defined as the amount of enzyme that releases 1 μg of

tyrosine per milliliter per minute under the standard conditions of supernatant solution.

Characterization of zinc nanoparticles by ultraviolet–visible spectral analysis

The bioreduction of zinc in suspension was observed by ultraviolet–visible spectroscopy (UV–Vis) of the solution between 200 and 500 nm by using Perkin-Elmer, Water Corporation Ser Green, Beaconsfield, BucksHP9 2FX, England, LAMBDA 35 UV–Vis spectrophotometer (USA).

Transmission electron microscopy

For the confirmation of size and shape, transmission electron microscopy (TEM) measurements were carried out using drop-coating method in which a drop of solution containing nanoparticles was placed on the carbon-coated copper grids and kept under vacuum desiccation till dryness. TEM and high-resolution TEM micrographs of the sample were taken using the JEM-2100F TEM instrument. The instrument was operated at an accelerating voltage of 200 kV.

Determination of major phytochemicals in the prepared extracts

Determination of total phenolic content

The total phenolic content was determined according to the Folin-Ciocalteu's procedure [13]. In brief, the extract (100 µl) was transferred into a test tube and the volume adjusted to 3.5 ml with distilled water and oxidized with the addition of 250 µl of Folin-Ciocalteu's reagent. After 5 min, the mixture was neutralized with 1.25 ml of 20% aqueous sodium carbonate (Na_2CO_3) solution. After 40 min, the absorbance was measured at 725 nm against the solvent blank. The total phenolic content was determined by means of a calibration curve prepared with gallic acid and expressed as milligrams of gallic acid equivalent per gram of sample. Additional dilution was done if the absorbance value measured was over the linear range of the standard curve.

Determination of total flavonoid content

The total flavonoid content was determined according to Zilic *et al.* [13] using aluminum chloride colorimetric assay. In brief, 300 µl of 5% sodium nitrite was mixed with 100 µl of extract. After 6 min, 300 µl of a 10% aluminum chloride solution was added, and the volume was adjusted to 2.5 ml using distilled water. After 7 min, 1.5 ml of 1 M NaOH was added, and the mixture was centrifuged at 5000 g for 10 min. Absorbance of the supernatant was measured at 510 nm against the solvent blank. The total flavonoid content was determined by means of a calibration curve prepared with catechize, and expressed as milligrams of catching equivalent per gram of sample. Additional dilution was done if the absorbance value measured was over the linear range of the standard curve.

Determination of radical 2,2 diphenyl-1-picrylhydrazyl scavenging activity

Free radical scavenging capacity of extracts was determined using the stable 2,2 diphenyl-1-picrylhydrazyl (DPPH) according to Hwang and Do Thi [14]. The final concentration was 200 µM for DPPH, and the final reaction volume was 3.0 ml. The absorbance was measured at 517 nm against a blank of pure methanol after 60 min of incubation in a dark condition. Percent inhibition of the DPPH free radical was calculated by the following equation:

$$\text{Inhibition}(\%) = 100 \times [(\text{A control} - \text{A sample})/\text{A control}]$$

.

where: A control is the absorbance of the control reaction (containing all reagents except the test compound). A sample is the absorbance with the test compound.

Extract concentration of sample providing 50% inhibition was calculated using linear regression analysis.

Determination of aflatoxins

Derivatization

The derivatives of samples and standard were done as follows: 100 µl of trifluoroacetic acid was added to samples and mixed well for 30 s and the mixture stand for 15 min. Then, 900 µl of water : acetonitrile (9 : 1 v/v) was added and mixed well by vortex for 30 s, and the mixture was used for HPLC analysis. The HPLC system consisted of Waters Binary Pump Model 1525, a Model Waters 1500 Rheodyne manual injector, a Waters 2475 Multi-Wavelength Fluorescence Detector, and a data workstation with software Breeze 2. A phenomenex C18 (250×4.6 mm i.d.) column, 5 µm, from Waters Corporation (USA), was used. An isocratic system with water : methanol : acetonitrile 240 : 120 : 40 was used. The separation was performed at ambient temperature at a flow rate of 1.0 ml/min. The injection volume was 20 µl for both standard solutions and sample extracts. The fluorescence detector was operated at wavelength of 360 nm for excision and 440 nm for emission. Samples were prepared by incubating the media that contained dry OMW residue with the tested fungus *G. parasitica* [15].

Field experiments

A field experiment was conducted during the 2018 season at Kafer-Eldawar (Behira) Governorate in a heavily naturally infested field, to study the effect of effect of free and Zn NPs of *G. parasitica* on soil-borne fungi causing preemergence and postemergence damping off of green bean plants (seed treatment) under field conditions.

The field was divided into plots (3×3.5 m). Each pot consists of five rows, and each row contained 20 hills on the eastern side. Three seeds/hill of green bean Valintino cv. which were conducted in randomly complete block design with three replicates (plots) for each particular treatment as well as control (check treatment). Green bean seeds (Valintino cv.) were mixed with each one of the treatments individually, and then sown in the soil at the rate of three seeds per pit.

Cultivated plots received the traditional agricultural practices. Percentages of root rot disease incidence at preemergence and postemergence stages of green bean plants were recorded after 7, 15, 30, 45, and 60 days from sowing date. The beneficial effects of different seed treatments on vegetative growth and yield quality as well as early and total green pods yield was investigated during season 2018.

A representative sample of eight plants was taken randomly 45 days after sowing (flowering stage) from each experimental plot for measuring the plant growth characteristics, including plant height from soil surface to the highest point of the plant, number of leaves, and branches per plant, using the standard methods as illustrated by AOAC [16].

At harvest stage (60 days from seed sowing), the total green pods from each plot were collected along the harvesting, and the average number of pods per plant, pods weight (g), and total green pods (ton/fadden) were calculated.

Statistical analysis

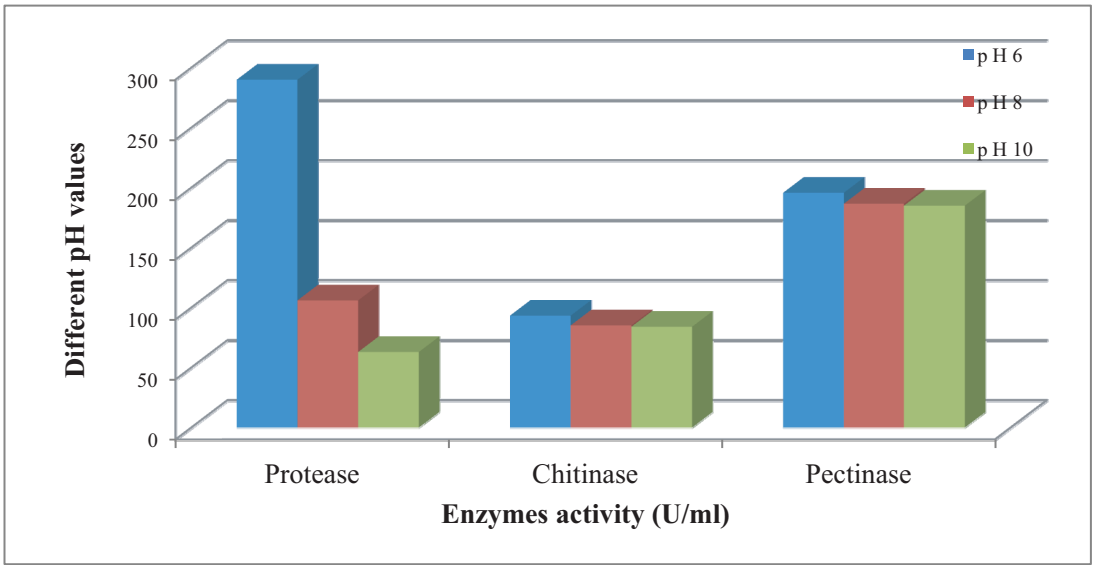
All experiments were set up in a complete randomized design. One-way analysis of variance was used to analyze differences between antagonistic inhibitor effect and linear growth of pathogenic fungi in vitro. A general linear model option of the analysis system SAS (SAS Institute Inc., 1996) was used to perform the analysis of variance. Duncan's multiple range tests at P value less than 0.05 level was used for means separation [17].

Results and discussion

Effect of zinc nanoparticles on enzymes production from *Gonatorrhodiella* parasitica

G. parasitica was grown on media containing OMW as carbon source in presence of zinc nitrate for biosynthesis of Zn NPs under different pH values (6.0, 8.0, and 10.0). Results in Fig. 1 showed that different pH values affected on enzymes production, especially pH 6.0, which produce maximum activity for all enzymes (290.10 U/ml for protease, 93.04 U/ml for chitinase, and 197.78 U/ml for pectinase), followed by pH 8.0 and 10.0. Effect of addition of Zn NPs in

Figure 1



Effect of zinc nanoparticles on enzymes production from.

fermentation media containing OMW as carbon source is shown in Fig. 1. Fungi have the ability to make accumulations of metals for biological production of metallic nanoparticles [18]. There is reduction of extracellular and intracellular enzymes by production of metal nanoparticles from fungi [19,20]. OMW can be used as substrate for enzyme production from different fungal strains in the fermentation media inoculated with *Cryptococcus albidus* var. *albidus* IMAT-4735 [21].

Gonatorrhodiella parasitica

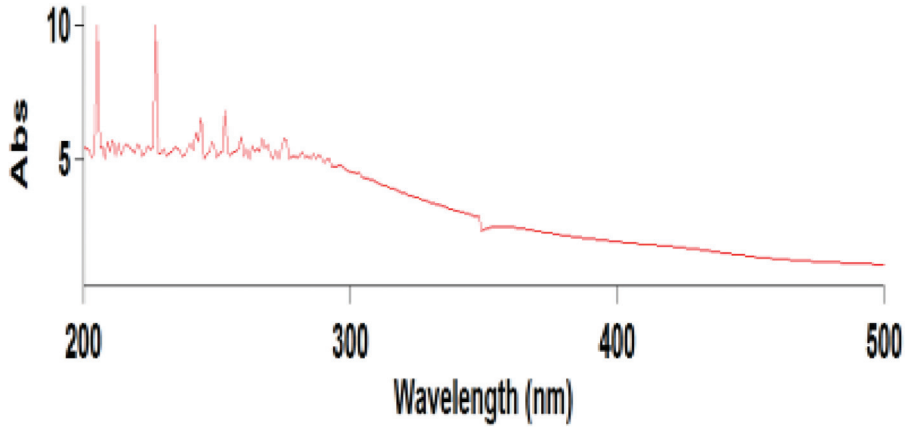
Characterization of zinc nanoparticles ultraviolet–visible spectral analysis

UV–Vis spectroscopy was used to follow up with the reaction process. The spectra recorded from *G. parasitica* at pH 6.0 are reported in Fig. 2. The strong surface was at λ_{max} 280 nm. The biosynthesis of metal nanoparticle by the fungal strain *Aspergillus fumigatus* TFR-8 through extracellular enzyme production was owing to the conversion of the metal salt of macroscale and microscale to nanoscale diameter by catalytic effect [20]. The biological synthesis of Zn NPs is safe and cheap, because they are the capping agents of natural fungal proteins [22]. Synthesis of extracellular production by nanoparticles from *F*usarium *oxysporum* has been conducted, with production of different forms and size variety [19].

Transmission electron microscopy

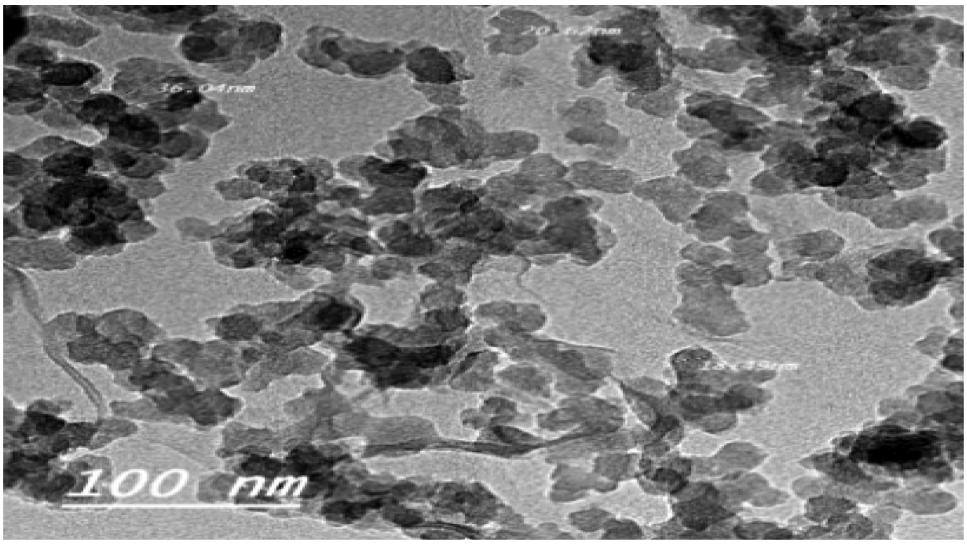
The particle size of prepared Zn NPs was 16 nm. The results in Fig. 3 revealed that Zn NPs with granular and

Figure 2



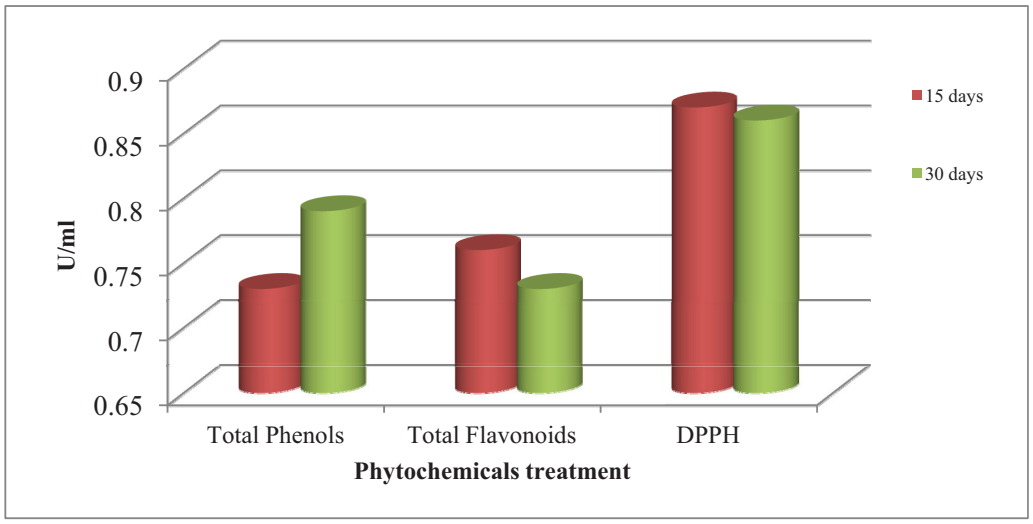
UV–visible spectral analysis. UV, ultraviolet.

Figure 3



Transmission electron microscopy.

Figure 4



Determination of total phenols, flavonoids, and DPPH. DPPH, 2,2 diphenyl-1-picrylhydrazyl.

spherical morphology had unique distribution. TEM of ZnO nanostructures is shown in Fig. 3. Zn NPs are one of the main subjects of the nowadays research. ZnO NPs are free and durable and have great effect on the soil fertility [23].

Determination of major phytochemicals in prepared extracts

Results in Fig. 4 showed that after composting of *G. parasitica* on OMW for 15–30 days, all phytochemicals decreased in their phenol, flavonoids, and DPPH. Phytochemicals after composting of *G. parasitica* on OMW are shown in Fig. 4. Phenols are considered one of the main agents responsible for the toxic effect of wastes on plant health [24]. According to Ben Hamman *et al.* [25], Phenolic content of OMW detoxified by P. flavido-alba decreased. P. flavido-alba also dephenolizes the semisolid olive residue in solid-state cultures, and polyphenol oxidases catalyze the oxidation of phenols to quinones, which is a natural compound that contributes in the defense mechanisms of plants against attacks by bacteria of the genus *Pseudomonas syringae* [26,27].

Determination of aflatoxins

Results in Table 1 show that increasing composting date led to decrease in aflatoxin in OMW.

Aflatoxins of OMW (Table 1) proved to be rich in polyphenol compounds and could be used as low-cost edible natural antioxidants for protection against aflatoxicosis in animals, and human aflatoxin B1 was the most prevalent aflatoxin usually found in cases of aflatoxicosis, and was responsible for chronic toxicity, acute toxicity, teratogenicity, carcinogenicity, hepatotoxicity, genotoxicity, teratogenicity, and immune-toxicity [15]. The dry OMW residue has toxic components against microorganism growth and plants [28].

Table 1 Determination of aflatoxins

Treatment (days)	AFG1	AFG2	AFB1	AFB2
15	0.16	0.18	0.19	0.24
30	0.11	0.9	0.10	0.09

In vivo effect of free and zinc nanoparticles ($ZnNO_3$) of Gonatorrhodiella parasitica on soil-borne fungi causing preemergence and postemergence damping off of green bean plants (seed treatment) under field conditions

Treatment of seeds with *G. parasitica* after being grown on OMW free or with $ZnNO_3$ nanoparticles before sowing bean plants resulted in reducing root rot disease under field conditions. Results in Table 2 showed that all applied treatments reduced the incidence of preemergency and postemergency caused by *Fusarium solani* and *Rhizoctonia solani*. The highest reduction in disease was observed with the treatment of *G. parasitica*+Zn NPs followed by *G. parasitica*+Zn NPs and control. Moreover, the same trend on the highest % of survival plants was 97.78, 77.78, and 61.11%, respectively. For those features Zn-compounds being the bests with $ZnSO_4$ and Zn $(ClO_4)_2$ treatments, which had stronger anti mycotoxins properties. The modifications of fungal hyphae were observed. Figures with the same letter are not significantly different (P=0.05).

Table 2 Effect of free and zinc nanoparticles of *Gonatorrhodiella parasitica* on soil-born fungi causing preemergence and postemergence damping off of green bean plants (seed treatment) under field conditions

	% Preemergence damping-off	% Postemergence damping-off			Survival plants (%)
Treatment	After 7 days	After 15 days	After 30 days	After 45 days	After 60 days
Gonatorrhodiella parasitica+Zn NPs	0.00c	0.00b	1.11c	2.22c	97.78a
Gonatorrhodiella parasitica free	18.89b	0.00b	3.33b	3.33b	77.78b
Control	30.00a	3.33a	5.56a	8.89a	61.11c

Zn NPs, zinc nanoparticles. Figures with the same letter are not significantly different (P=0.05).

Table 3 Effect of free and zinc nanoparticles of *Gonatorrhodiella parasitica* on crop parameter of green bean plants under field conditions

	Crop parameters							
Treatment	A	B	C	D	E	F	G	H
Gonatorrhodiella parasitica+Zn NPs	63.3	10.5	25.5	21.3	45.3	55.5	6.5	44.4
Gonatorrhodiella parasitica free	53.3	8.8	18.6	17.2	37.5	48.3	5.3	17.7
Control	50.0	7.7	15.0	15.9	35.0	42.6	4.5	–

A: plant height (cm), B: number of branches/plant, C: number of leaves/plant, D: number of pods/plant, E: number of pods/plant, F: number fees/plant, G: total green pods yield (t/fed), H: increase of control. Zn NPs, zinc nanoparticles.

Effect of free and zinc nanoparticles of Gonatorrhodiella parasitica on crop parameter of green bean plants under field conditions

Data in Table 3 show that the average plant height was 63.3 cm in plants treated with biocontrol *G. parasitica* +Zn NPs formulated on OMW, which was followed by plants treated with *G. parasitica* free or control formulated on OMW (53.3 and 50.0 cm).

Not only plant height was highest but also number. of branches, number of bods, and number of seeds were highest in treatment *G. parasitica*+Zn NPs formulated on OMW (10.5, 25.5, 21.3, and 45.3, respectively) followed by plants treated with *G. parasitica* free or control formulated on OMW (7.7, 15.0, 15.9, 35.0, and 42.6, respectively).

The yield takes the same trend. The most significant increases in yield with treatment by biocontrol *G. parasitica*+Zn NPs formulated on OMW (6.5 ton/fed), followed by treatment with *G. parasitica* free formulated on OMW (5.3 ton/fed) compared with control (4.5 ton/fed).

Utilization of *G. parasitica*+Zn NPs formulated on OMW in order to increase the productivity may be a viable alternative to organic fertilizers which also helps in reducing the pollution and preserving the environment in spirit of an ecological agriculture. It can be a promising source for plant growth-promoting agent in agriculture inoculants for improving the yield and the growth of agricultural crops.

In vivo (Tables 2 and 3), the Zn-compound applications impeded in the cellular metabolism of the fungi and led to morphological alterations, hyphae, conidial, and reduction mortality [29,30].

Amendment of soil with bulk ZnO caused an increase in shoot and root biomass by 225 and 10%, respectively [31].

Zn NPs and their ionic salts may be toxic during growth and early development stages in cabbage and maize, as reported by Pokhrel and Dubey [32]. These authors studied the histology and anatomy and the primary root morphology using light metal biouptake, microscopy, rate of germination, moisture content, and root elongation. Significant changes in the cellular morphology of vital structures of young plants were also observed, such as cellular alterations in the apical meristem and zone of elongation in maize primary root cells.

Conclusion

The selected fungal strain *G. parasitica* was used for biosynthesis of Zn NPs. Determination of enzymes activity in fermentation medium containing OMW as substrate for production of protease, pectinase, and chitinase at different pH values (6.0, 8.0, and 10.0) was done. Absorbance UV–Vis spectra of Zn NPs at pH 6.0 were at λ_{max} 280 nm. Transmission electron spectroscopy showed size of 16 nm. Composting of OMW inoculated with *G. parasitica* for 15–30 days led to decrease in total phenol, flavonoids, DPPH, and aflatoxins found in OMW. Comparison of *G. parasitica* free and nanoparticles for biocontrol agent of soil-borne fungi causing preemergence and postemergence damping off of green bean plants (seed treatment) under field

conditions measured from 7 to 60 days and its effect of growth parameters confirm the use of Zn NPs for enzymes production and biocontrol application.

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Conflicts of interest

There are no conflicts of interest.

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