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Biosynthesis of Selenium nanoparticles by bioagents and their fungicidal activity against soil-borne diseases of fennel plants

Haggag, W. M.[1*], Zaher, E.[2], Abada, K. A.[2], Sara, M. Z.[1] and Elgamal, N. G.[1]

[1]Plant Pathology Department, National Research Centre, Giza, Egypt; [2]Plant Pathology. Department Faculty Agric., Cairo Univ., Egypt.

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Abstract The microorganisms use as the biological control of plant pathogens have the ability to biosynthesize nanoparticles such as nano-selenium which have applied in various fields, including agriculture. Biosynthesis selenium nanoparticles (SeNPs) is done by some bio-agents i.e. *Trichoderma harzianum*, *Chaetomium globosum*, *Streptomyces griseus*, *Pseudomonas putida* and *Bacillus subtilis* and evaluated their fungicidal activities against soil-borne diseases in fennel plants (*Foeniculum vulgare* L.) caused by *Macrophomina phaseolina*, *Fusarium solani*, and fungus like *Pythium aphanidermatum*. In vitro experiments, all tested treatments were significantly reduced growth of pathogenic fungi i.e. *M. phaseolina*, *F. solani* and *P. aphanidermatum* as compared with untreated control. Morover, under greenhouse conditions, results indicated that all tested treatments were significantly reduced damping-off and root-rot diseases of fennel plants. SeNPs synthesis by *S. gresius* were the higher effect than other treatments. Also these treatments were found to increase the activity of oxidative enzymes i.e. peroxidase, polyphenoloxidase, chitinase and soluble proteins. Furthermore, under field conditions, fennel seeds treated with the selected SeNPs synthesis by bioagents at a concentration of 250 ppm were affected to reduce damping-off and root-rot diseases of fennel plants. The commercial biocide (*Bacillus subtilis*) was also used in the same manner. A concentration of 0.5 ml/L was applied for comparison and the untreated seeds were used as control treatment. Results indicated that SeNPs synthesis of S. griseus showed the most effective treatment. They showed the highest increase in all growth parameters / plant i.e. plant height, number of branches, dry weight of inflorescences, dry weight of seeds, dry weight of 1000 seeds, plant yield, percentage of volatile oil and its frequency during the two successive growing seasons.

Keywords: Fennel, Soil-borne diseases, Bioagents, Selenium nanoparticles (SeNPs), Enzymes

Introduction

Fennel (*Foeniculum vulgare* L.) is the most important medicinal crops in the world contain essential oil which is used as flavouring agent in

[*] **Corresponding Author:** Haggag, W. M.; **Email:** wafaa_haggag@yahoo.com

manufacturing (Abdellaoui *et al*., 2020 and Dahmani *et al*., 2022). Fennel crop suffer from many diseases by pathogenic fungi, including damping off and root rot of seedlings cause by *Macrophomina phaseolina*, *Fusarium solani*, and *Pythium aphanidermatum* (Khare *et al*., 2014, Khalequzzaman, 2020).

The use of biological control and its nanoparticles are represents one of the strategies to control and combat harmful pathogens naturally and represents less harm due to its high nature of sustainability and its outstanding activity as biocides in the required doses (Kumar, 2022). Microorganisms were considered bio-nano factories to provide a clean and promising alternative process for nanoparticle fabrication (Akl *et al*., 2020, El-Saadony *et al*., 2020a, Reda *et al*., 2020 and Sheiha *et al*., 2020). Biosynthesized nanoparticles have become super-growth promoters as well as antifungal agents (Haggag, 2022). Nanotechnology were used in controlling plant disease (Haggag, 2022 and Song *et al*., 2022). Nanotechnology is a biofertilizer factory used in agriculture, controlling agrochemical usage, enhancing plant resistance against disease, and efficient nutrient utilization and enhanced plant growth, crop yield quality and quantity. The biological method involves nanoparticle synthesis by microorganisms, enzymes, plants, and plant extracts have merit over the alternative chemical and physical methods because the former has been suggested possible clean, non- toxic, and eco-friendly in nature (Bhattacharjee *et al*., 2019 and kumar and Prasad, 2021).

Selenium nanoparticle has a high scale of absorption relative to selenium element. So it is all-important to improve new techniques to promote the transportation of selenium substance (selenoenzymes, etc.) by reproducible their bioactivity and controlled release. Se-NPs have special attention regarding their application as food additives and therapeutic agents. Selenium nanoparticle has biomedical and pharmaceutical uses due to its antioxidant and antimicrobial effects (Kumar and Prasad 2021). Synthesis of Se-NPs of 10− 50 nm size range using *Bacillus licheniformis* for the reduction of selenium dioxide (Khiralla and El-Deeb, 2015). Similarly, *Bacillus pumilus* sp. BAB-3706 was used for the reduction of sodium selenite to prepare biogenic selenium nanoparticle (Prasad *et al*., 2015). Two strains of Actinomycetes L-155 and Actinomycetes L-156 were used to synthesize Se-NPs by reduction of selenium dioxide (Ratnakomala *et al*., 2018). *Pseudomonas* sp. CA5 bacteria was used to reduce selenite and selenate into elemental red selenium nanoparticles (Hunter and Manter, 2009). The synthesis of selenium nanoparticle were done using different fungal species (Hariharan *et al*., 2012; Zhang *et al*., 2019a, 2019b) inculding culture filtrate of Alternaria alternata. The cell-free extract of fungi *Gliocladium roseum* was used for the reduction of sodium selenite to synthesize selenium nanoparticle (Kumar and Prasad 2021).

Selenium nanoparticles (Se-NPs) synthesized biologically by Bacillus megaterium exhibited influential antifungal activity against *Rhizoctonia solani* RCMB 031001 in vitro and in vivo and improved healthy of Vicia faba cv. Giza 716 seed germination, morphological, metabolic indicators, and yield by facilitating the uptake of macromolecules needed to increase resistance to plant diseases and promote growth (Nair *et al*., 2010, Kaur, 2018 and Hashem *et al.*, 2021). Se-NPs derived from *Trichoderma* have recently been demonstrated to control pearl millet downy mildew disease and improve plant growth under greenhouse conditions (Nandini *et al.,* 2017) due to inhibit the sporulation of pathogens on infected host leaves. It can be postulated that Se-NPs can be a potential tool in integrated plant disease management. *Trichoderma* is a free living, asexually reproducing, root colonizing fungus that is known to possess mycoparasitic and antimycotic activities against fungal pathogens (Jogaiah *et al.,* 2018). The biocontrol agent (Trichoderma) triggers systemic and localized resistance in plants against a large number of biotic stresses (Jogaiah *et al*., 2018.) This induction of disease resistance is mainly attributed to the presence of ABC transporters that are involved in the secretion of antibiotics and cell wall degrading enzymes to synthesize Se-NPs from *Trichoderma* and establish the broad spectrum activity against several fungal plant pathogens. Xia (2007) and Vrcek (2018) tested and establish the broadspectrum antifungal activity of mycogenic selenium nanoparticles (Se-NPs) synthesized from *Trichoderma atroviride*, which displayed excellent in vitro antifungal activity against *Pyricularia grisea* and inhibited the infection of *Colletotrichum capsici* and *Alternaria solani* on chili and tomato leaves at concentrations of 50 and 100 ppm, respectively. Selenium is found to be less toxic and more biologically active (Xia, 2007 and Vrcek, 2018) in its reduced nano-form when compared to its other chemical forms such as sodium selenite and selenium sulphide. Biosynthesis is an eloquent, safe, biocompatible, eco-friendly, and recyclable way of preparing selenium nanomaterials (El-Ramady *et al*., 2016). Selenium is a valuable element for immunity and appropriate health in animals and increases plant growth (Bunglavan *et al.,* 2014, Ragavan *et al.,* 2017 and Qiu *et al.,* 2018). Se-NPs are being explored explicitly for their anti-microbial (Vrcek, 2018), antioxidant and anti-inflammatory properties (Khiralla and El-Deep 2015). Se-NPs are widely used in nutritional supplements, medical apparatus, and nanotherapeutics. The use of biological systems, that the micronutrient is the main component of selenozyme enzymes, including glutathione peroxidase and others, which play a role in defense such as antioxidants, detoxification, and metabolism (Forootanfar *et al.,* 2014). The biological activities and good adsorptive ability of Se-NPs can be attributed to the interactions between the

nanoparticles and functional groups present in proteins such as NH, C=O, COO–, and C–N (Wang *et al.*, 2007).

Thus, the aim of this study was to evaluate the ability of antagonistic bacteria (*S. griseus*, *P. putida*, and *B. subtilis*) and antagonistic fungi (*T. harzianum* and *C. globosum*) to produce nano selenium against soil-borne fungi (*F. solani*, *M. phaseolina* and *P. aphanidermatum*) *in vitro* and *vivo*.

Materials and methods

Isolation of pathogenic fennel fungi

The infected fennel plant roots were used to isolate soil-borne patgogens. The isolated pathogens were purified using hypha tip technique and identified at the Plant Pathology Department, National Research Center, Dokki, Egypt, according to Booth (1977) and Domsch *et al.* (1980). Pure cultures were maintained at 5 ℃ on PDA inclines and restored once a month for other experiments.

Laboratory experiments

Biosynthesis of selenium nanoparticles (SeNPS)

Trichoderma harzianum, *C. globosum*, *S. griseus*, *P. putida*, and *B. subtilis* as bioagent isolates were used to create nanoparticles. These fungi and bacteria pre-inoculum was added to 90 mL of Potato Dextrose broth (PDB), Starch nitrate (StN broth) media, King's medium (KM broth), and Nutrient glucose broth (NG broth) medium, and incubated for 72 hours at 31 ℃ under shaking conditions (200 rpm). The fungal and bacterial biomass were harvested and washed with autoclaved water under sterile conditions during the log phase of the growth cycle. The harvested fungal and bacterial biomass (1gm wet weight) were then resuspended in a 100 mL aqueous solution containing 0.2 g sodium selenite and kept anaerobically on a shaker (200 rpm) at 31℃. The fungal and bacterial biomass was subjected to a 48-hour reaction with sodium selenite. After centrifugation at 5000 rpm for 10 minutes to separate the fungal and bacterial biomass from the reaction medium, the reaction products were collected. The harvested bacterial biomass was resuspended in sterilize deionized water in the absence of sodium selenite in a control experiment, and the obtained product was characterised by the presence of SNePs.

Nanoparticles characterizations by (TEM)

The obtained biotransformed products were analyzed and characterized by transmission electron microscopy (TEM). The nanoparticles were produced by drop coating of the isolated solution and suspended through the carbon-

coated copper grids. The measurements were made using a TEM microscope (Faculty of Agriculture, Cairo University) on a JEOL 1200EX instrument having an acceleration voltage of 120 kV.

Antifungal effect of SeNPs on three pathogenic fungi

The antifungal effect of SeNPs obtained by fungi and bacteria were evaluated against the growth of the three pathogenic fungi *i.e., F. solani, M. phaseolina*, and *P. aphanidermatum*. Each treatment was taken under sterilized conditions and added to sterilized PDA medium before pouring into sterilized Petri dishes to give 150, 200, and 250 ppm. Three replicates were used for each treatment. All dishes were inoculated with 4 mm discs, 7-day-old of tested fungi. Plates containing PDA medium with 4mm discs, and 7-day-old of tested fungi only were used as control. The plate was incubated at 25 ± 2 ℃ for 5 days. The linear growth (mm) of the three pathogenic tested fungi was measured when the growth of the pathogenic fungi of control treatment completely covered the plate (Abo-Shady *et al*., 2007). The percentage of reduction in pathogenic fungal mycelial growth was calculated using the formula: R = (G2- G1/G2) × 100 according to (Singh *et al*., 2021 and Fokkema, 1973), \where R = % reduction in the growth of pathogenic fungus.

Greenhouse experiment

Plant materials

Fennel seeds (local variety obtained from Hort. Res. Inst., Medici. and Arom. Plants) were planted in 25cm diameter plastic pots (10 seeds per pot contained the autoclaved soil clay/sand 2:1) and grown at 22 to 25 ℃ under a 16-h photoperiod. Under greenhouse conditions, plants were irrigated with water to keep moisture at field capacity. The greenhouse experiment was designed as a completely randomize block. All greenhouse experiments were conducted at the National Research Center's greenhouse (NRC).

Inoculation of fennel plants with soil-borne fungi

All three pathogenic fungi tested were grown for 15 days at 25 ° C on sterilised corn/sand medium. Inoculation was done at a rate of three grammes of each inoculum per one kilogramme of soil (clay/sand 2:1) and was mixed and irrigated for seven days before sowing. Seeds were surface sterilised for three minutes with 0.1% sodium hypochlorite, then washed three times with sterilised water. The seeds were immersed in various concentrations of SeNPs.

Effect of SeNPs on damping off and root rot diseases in fennel plants

Under artificial infection conditions, the ability of antagonistic bacteria (*S. griseus*, *P. putida*, and *B. subtilis*) and the antagonistic fungi (*T. harzianum*

and *C. globosum*) to produce nano selenium at concentrations of 200 and 250 ppm to control soil-borne fungi (*F. solani*, *M. phaseolina*, and fungus like *P. aphanidermatum*) was tested.

Assessment of diseases

Pre- and post-emergence damping off was observed 15 and 30 days after sowing, respectively. Root rot plants were evaluated 45 days after sowing (Abada *et al*., 2016 and Khalequzzaman, 2020).

Enzyme analysis

One gram of leaf samples were collected from infested and non-infested fennel plants. Leaf samples were collected to test the activity of peroxidase, polyphenoloxidase, chitinase, and soluble proteins. Three leaves per plant were collected and frozen for 36 hours. The leaves were then dried and powdered before being homogenized with 10 mL of phosphate buffer and centrifuged. The enzyme activity was measured using the clear supernatant (Kar and Mishra, 1976).

Polyphenoloxidase (PPO) determination

PPO activity was determined by measuring the rise in absorbance at 420 nm with a spectrophotometer. The activity was measured in 3 mL of reaction, a mixture of 1 mL of substrate (0.02 M catechol + distilled water), 100 l of enzyme extract, and 1.9 mL of phosphate buffer (pH 6.5) in a 1cm light path quartz cuvette, increase in absorbance at 420 nm, the quantity of enzyme that results in an increase in absorbance of 0.001 minutes is considered one unit of PPO activity Shi *et al*. (2002).

Determination of peroxidase (P)

A 3 ml reaction mixture was used to measure the amount of peroxidase (P) activity. The reaction mixture contained the following ingredients: 1 ml of 0.2 mol/m3 potassium phosphate buffer with pH = 7.6, 0.1 ml of 2 mM (NADPH), 0.5 ml of 3 mM DTNB, and 0.1 ml of enzyme extract. Distilled water was used to achieve a final volume of 2.9 mL. To begin the reaction, one unit of (P) activity is added. A spectrophotometer was used to measure the change in absorbance at 412 nm at 25℃ over a five-minute period. The Bradford (1976) method was used to determine the amount of peroxidase extracted.

Determination of chitinase

Colloidal chitin was prepared from chitin powder for the chitinase assay. The test tubes addes one millilitre of 1% colloidal chitin in citrate phosphate

buffer (pH 6.6) and 0.18 millilitres of sample homogenate, incubated for one hour in a water bath at 37 ℃, then cooled and centrifuged before assaying. 3.5-dinitrosalysilic acid (DNS) which was used to determine the reducing sugars in 1 ml of supernatant. UV spectrophotometer was used to measure absorbance at 530 nm alongside the substrate and enzyme blanks (Vahed *et al*., 2013).

Determination of soluble proteins' activity

100 ml of 85% (w/v) phosphoric acid was added to the Bradford reagent. When the dye is completely dissolved, diluted to 1 litre and filtered through Whatman No. 1 paper just before use. It is best to use a light brown Bradford reagent. Filtering was repeated to completely purge the reagent of blue components. Despite the fact that the dye lots used in the Bio-Rad concentrate was screened for maximum effectiveness. Spectrophotometer was acclimated before use. The sample in at least one assay tube was diluted with a 100l sample to contain between 5 and 100 g of protein. Then, each sample was added an equal volume of 1 M NaOH and vortex. If this option is chosen, NaOH should be included in the standards as well. The standards in a 100-liter volume was prepared with protein concentrations ranging from 5 to 100 micrograms (albumin or gamma globulin are recommended). After adding 5 ml of the dye reagent, then incubated for 5 minutes. Absorbance at 595 nm is measured. Histidine, lysine, tyrosine, tryptophan, and phenylalanine residues are less than arginine residues are affected by the dye reagent's reaction. The assay is less accurated for basic or acidic proteins. The Bradford assay detected bovine serum albumin about two times more sensitively than "average" proteins. Immunoglobulin G is the most widely used protein standard (also known as IgG-gamma globulin). Stoscheck (1990) suggested adding 1 M NaOH to enable membrane protein solubilization and reduce protein-to-protein variation in colour yield.

Field experiment

During two consecutive winter growing seasons (2020/2021 and 2021/2022), the experiments were conducted in a field withhigh infestation with pathogenic soil-borne fungi at El Qanater El-Khairia (Bahada location), Qaluobiya governorate. Each plot was measured 3 x 3.5 m. Fennel seeds obtained from (Hort. Res. Inst., Medici. and Arom). Each ridge received twenty-five seeds, and four ridges/plots (100 plants/plot) resembled each other. For each treatment, a Complete Randomized design (CRD) was used. Fennel seeds were sterilised and then treated with nano selenium synthesis by some bioagents at a concentration of 250 ppm, as well as the commercial biocide (*Bacillus subtilis*). For comparison, a concentration of 0.5 mL/L was used. The

nontreated seeds were used as control treatment. In addition, the foliar spray was carried out on the fennel leaves 30 and 60 days after sowing with the same treatments.

Disease assessment

The pre- and post-damping-off percentages were calculated 15 and 30 days after sowing, respectively. Root rot disease was detected 45 days after sowing and survival plants at the end of growth.

Crop parameters

Crop parameters was measured as number of branches, plant height (cm), dry weight of inflorescences (g), dry weight of seeds (g), the weight of 1000 seeds (g), and total yield/plant (g) which determined at the end of each growing season for the tested treatments and the control (Khalequzzaman, 2020, Ahmed *et al*., 2016 and Gebily, 2016).

Essential oils assay content

Content

According to the Egyptian Pharmacopoeia (1984), the percentage of essential oils in each sample was determined by hydro-distillation for 3 hours at Clevenger-type apparatus using 100 g of each sample. Each treatment's essential oil was dehydrated separately with anhydrous sodium sulphate and stored in the deep freezer until GC-MS analyses.

Frequency

The essential oil samples were analysed using gas chromatography-mass spectrometry (GC-Ms) instrument stands at the Department of Medici and Aromatic Plants Research, National Researh Center and TRACE GC Super Gas Chromatograph (THERMO Scientific Corp., USA), equipped with a THERMO mass spectrometer detector. A TG-WAX MS column (30 m 0.25 mm, 0.25 m film thickness) was used to connect the system to the GC-MS device. The analyses were carried out with helium gas, and the mass spectra of the compounds were obtained through electron ionization (EI) at 70 eV and a spectral range of m/z 40-450. Using mass spectra, the presence and proportions of the compounds were determined (Original Chemicals, Wiley Spectral Library Collection and NSIT Library).

Statistical analysis

SAS version 9.4 was used to analyse the experimental data, which were presented as means and standard deviations (SAS Institute, Cary, NC). Duncan's Multiple Range Test was used to make comparisons.

Results

Evaluation of selenium nanoparticles (SeNPs) synthesized by different bio-agents in reducing the linear growth of the tested fungi in vitro

Two antagonistic fungi *i.e. T. harzianum* and *C. globosum* and three antagonistic bacteria *i.e. S. griseus*, *P. putida* and *B. subtilis* were used for synthesis selenium nanoparticles that were tested against the linear growth of three pathogenic fungi *i.e., F. solani, M. phaseolina* and *P. aphanidermatum*. Results indicated that all bioagents synthesis selenium nanoparticles significantly reduced the mycelial growth of all the tested pathogenic fungi as seen in Figure 1 and 2. SeNPs is synthesized by *S. griseus* showed the most effective against the linear growth of the tested fungi which decreased the linear growth of *M. phaseolina* by 2.66 mm with 96.96 % reduction, *F. solani by* 2.66 mm with 98.80 % of reduction and *P. aphanidermatum* by 4.00 mm equal 95.46 % reduction when compared with untreated control (90.00 mm), followed by SeNPs synthesized by *B. subtilis* which reduced the linear growth of *M. phaseolina* by 5.66 mm with 93.63 % reduction, *F. solani* (1.00 mm) equal to 96.96 % of reduction and *P. aphanidermatum* (4.66 mm) equal to 90.73 % reduction when compared with untreated control (90.00 mm), Meanwhile SeNPs synthesized by *C. globosum* caused moderate inhibition of fungal linear growth of *M. phaseolina* (5.60 mm) with 93.77 % reduction, *F. solani* (2.56 mm) equal to 97.06 % of reduction and *P. aphanidermatum* (1.00 mm) with 98.80 % of reduction when compared with untreated control. Therefore, increasing the concentration led to decrease the linear growth and increasing reduction percentage.

Transmission electron microscopy (TEM) image of selenium nanoparticles obtained from some antagonistic fungi and bacteria

Transmission electron microscopy (TEM) examination provided further insight into the morphology and size details of synthesized selenium nanoparticles (Se-NPs). Large variation in particle size were observed average diameter ranging from 6.78 to 19.0nm. Positive result was observed with nano selenium by *Streptomyces griseus* that produced the small size of SeNPs from 6.78 to 11.1nm as shown in Figure 3-7. Moreover, SeNPs that synthesized by *B. subtilis* produced average particle size from 12.2 to 18.1 nm. However, SeNPs is synthesized by *C. globosum* 11.2 to 14.1 nm.

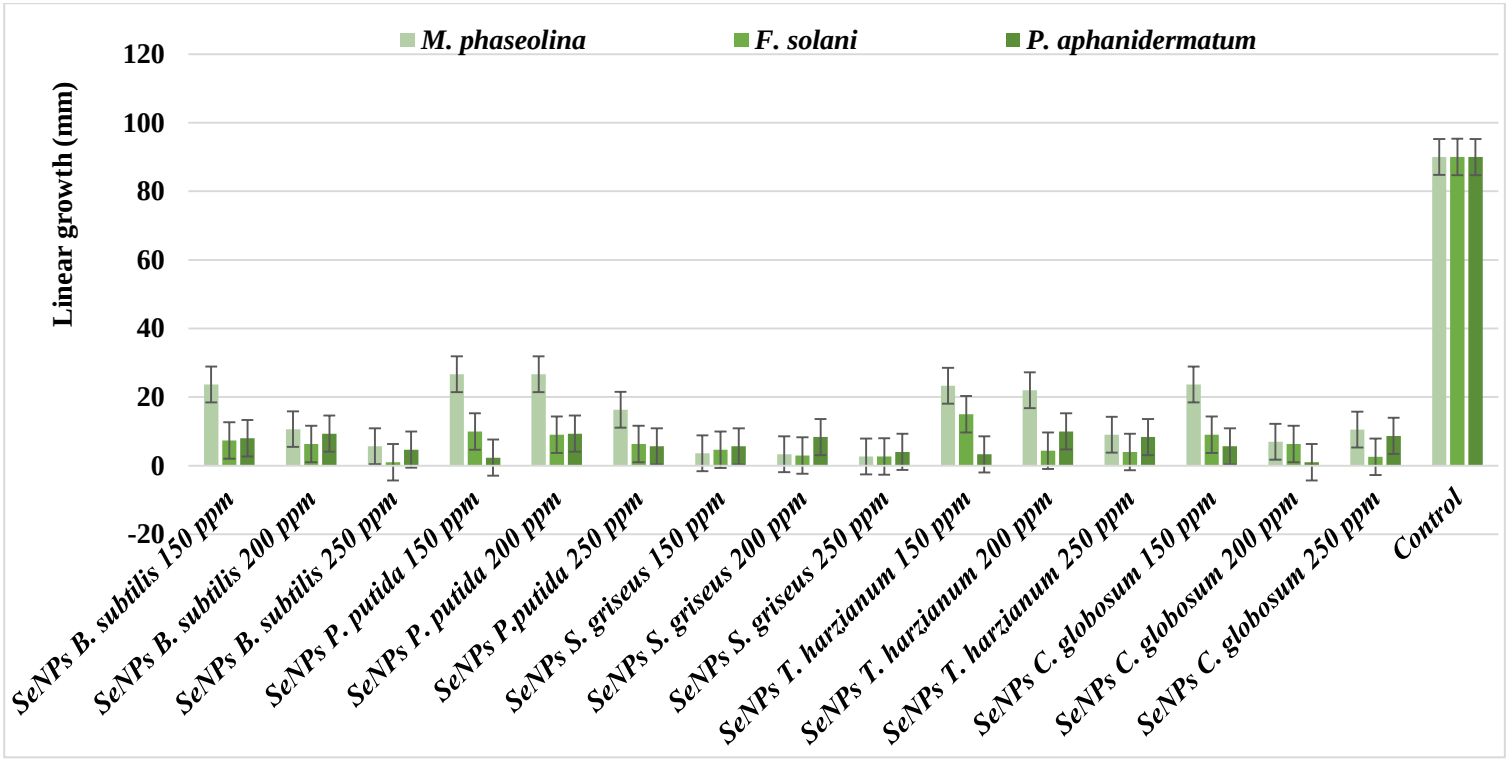

Figure 1. Evaluation of selenium nanoparticles (SeNPs*)* synthesis by different bioagents in reducing the linear growth of the tested fungi *in vitro*

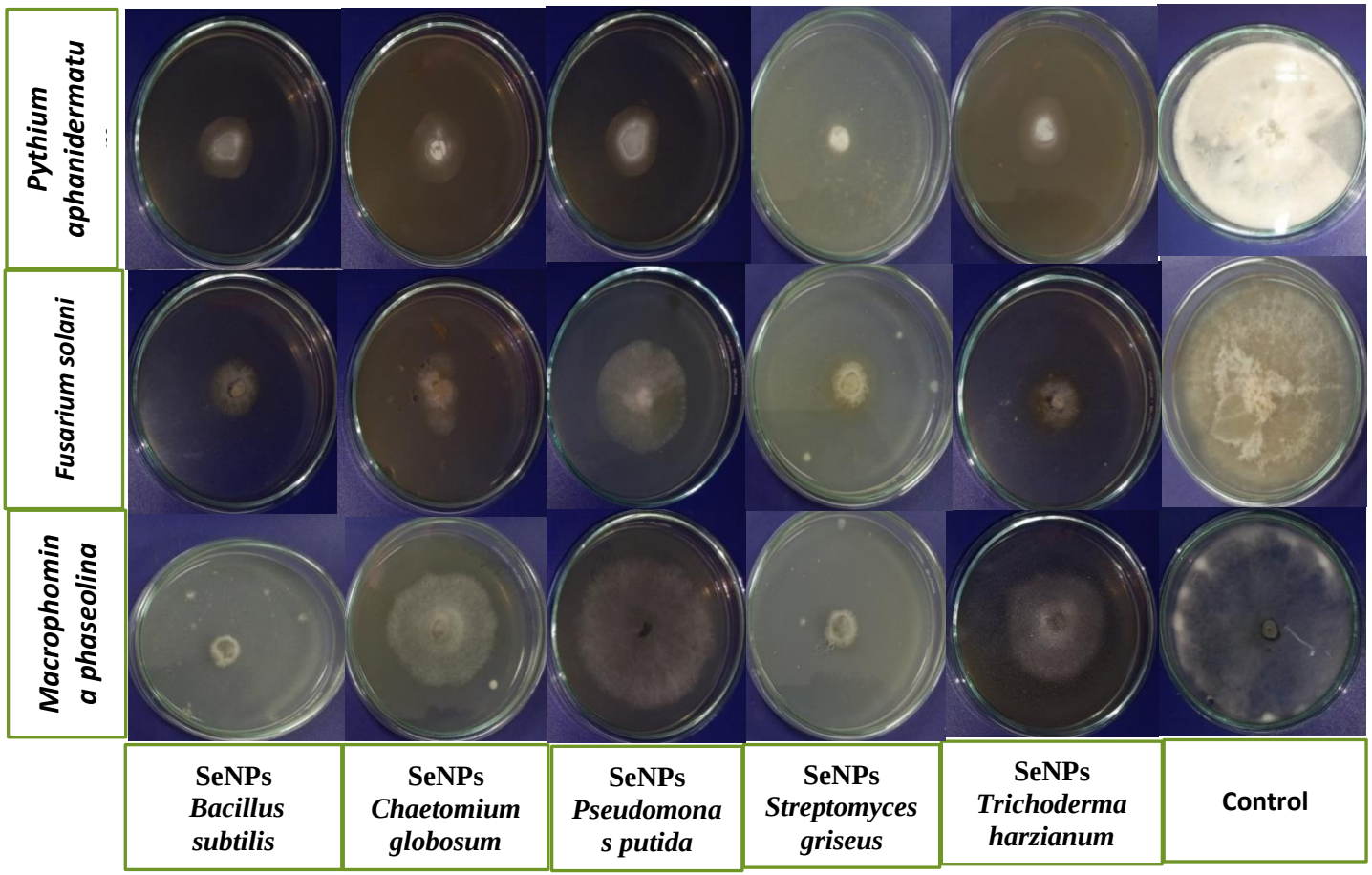

Figure 2. Evaluation of selenium nanoparticles synthesis by different bioagents in reducing the linear growth of tested fungi *in vitro*

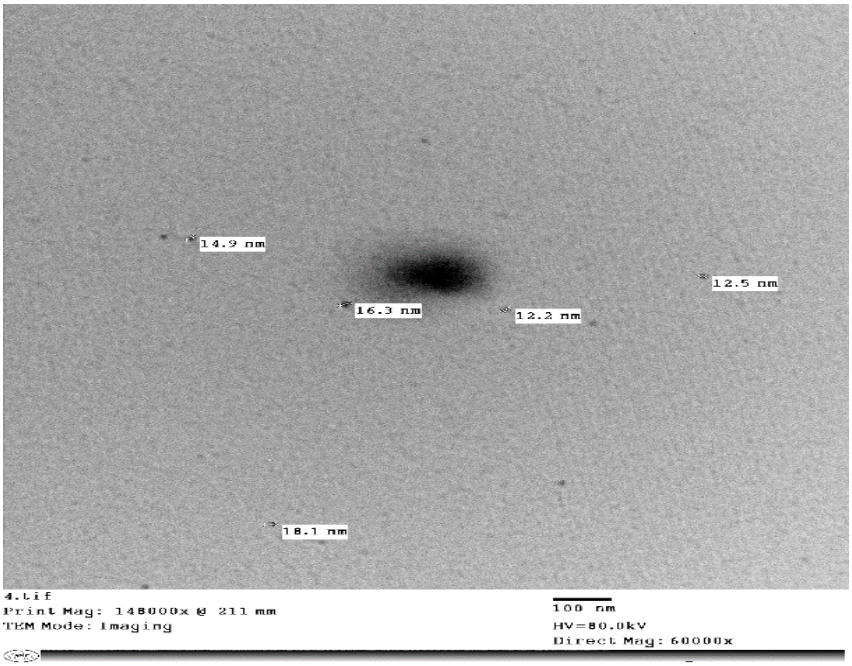

Figure 3. Transmission electron microscopy (TEM) image of selenium nanoparticles (Se-NPs) *synthesis* by *Bacillus subtillis*

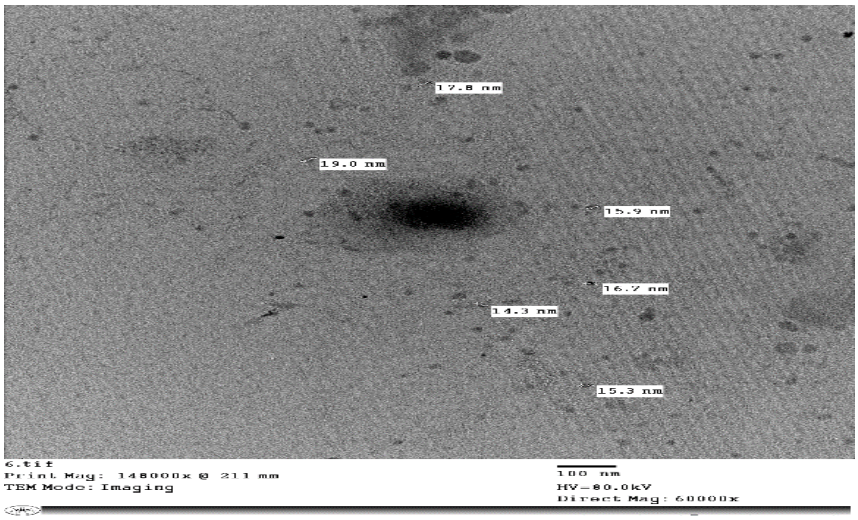

Figure 4. Transmission electron microscopy (TEM) image of selenium nanoparticles (Se-NPs) synthesis by *Pseudomonas putida*

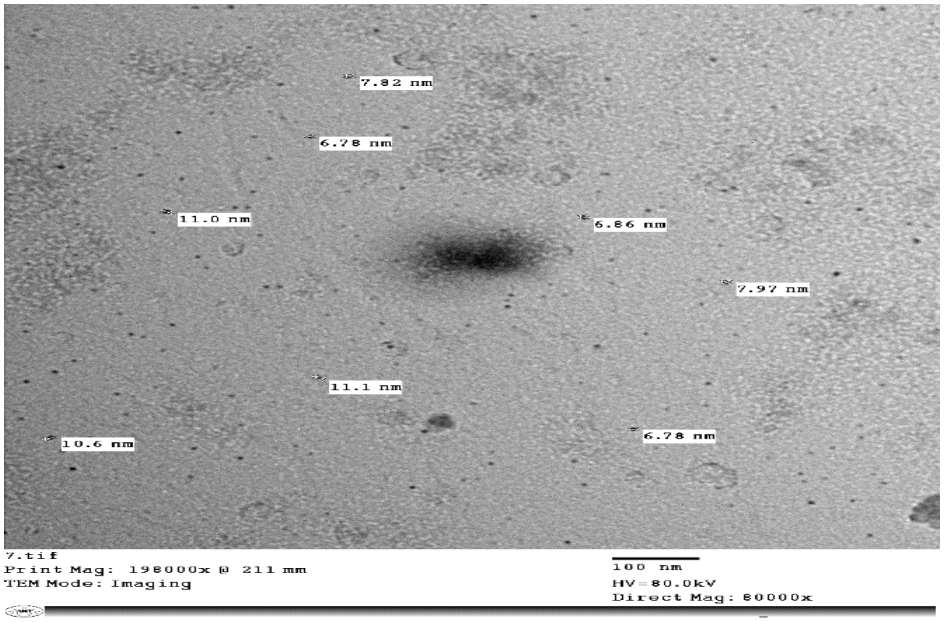

Figure 5. Transmission electron microscopy (TEM) image of selenium nanoparticles (Se-NPs) synthesis by *Streptomyces griseus*

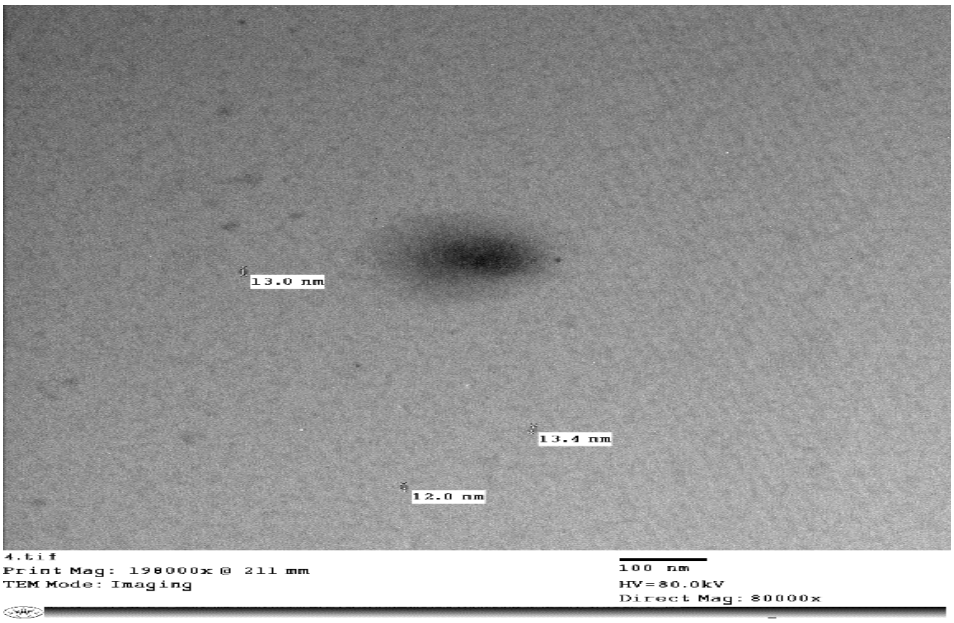

Figure 6. Transmission electron microscopy (TEM) image of selenium nanoparticles (Se-NPs) synthesis by *Trichoderma harzianum*

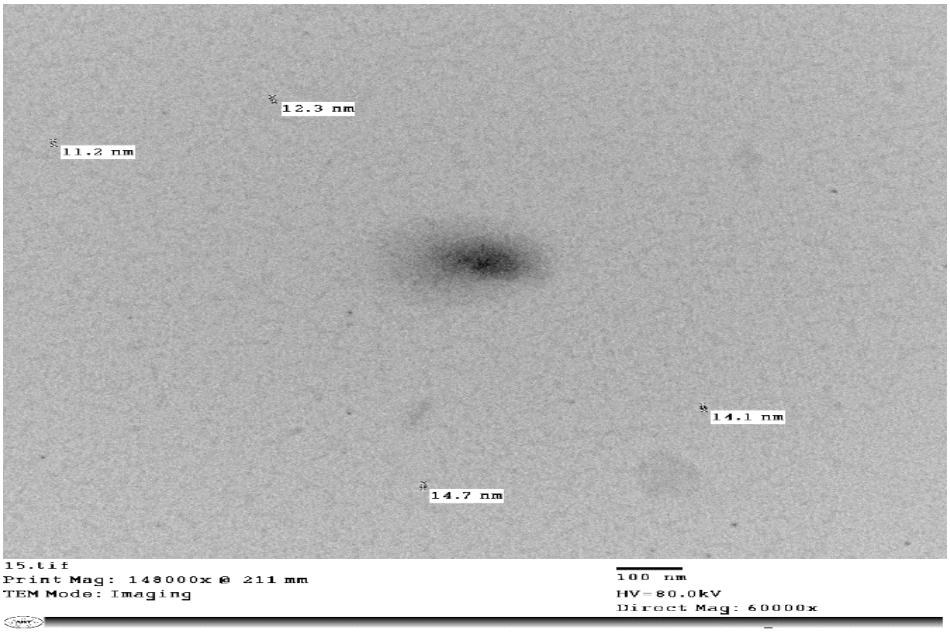

Figure 7. Transmission electron microscopy (TEM) image of selenium nanoparticles (Se-NPs) synthesis by *Chaetomium globosum*

Greenhouse experiments

Efficacy of selenium nanoparticles in controlling Fusarium solani, Macrophomina phaseolina and fungus like Pythium aphanidermatum under greenhouse conditions during 2019-2020 growing season

Two antagonistic fungi (*T. harzianum* and *C. globosum*) and three antagonistic bacteria (*S. griseus, P. putida* and *B. subtilis*) were used in synthesized selenium nanoparticles for controlling damping off and root-rot diseases caused by *F. solani, M. phaseolina* and *P. aphanidermatum* under artificial inoculation conditions in greenhouse. Result revealed that SeNPs synthesized by *S. griseus* was the most effective against all tested fungi (Table 1). It decreased damping off caused by *M. phaseolina* at 6.0 %, whereas reduced root-rot of 2.0 % and increased the survived plants of 92.0 %, While decrease damping off caused by *F. solani* at 4.0 %, root-rot of 2.0 % which increased plants survival of 94.0 %. Moreover reduced damping of caused by *P. aphanidermatum,* of 2.0 %, decreased root-rot at 2.0 %, while increased survival plants of 96.0 % under greenhouse conditions which followed by SeNPs synthesized by *B. subtilis*, *C. globosum* and *T. harzianum* showed moderately effective in reducing damping off and root-rot against the three tested fungi and plants survival. *B. subtilis* increased survival plants of 66.0 % in the infected control treatment at 94.0 %, followed by SeNPs synthesis by *C.*

globosum showed moderate effect which decreased damping off caused by *F. solani* at 6.0 %, root-rot at 4.0 % and increased plants survival of 90.0 %, while decreased damping off caused by *M. phaseolina* at 8.0 %, root-rot 4.0 % and increased plant survivals of 88.0 %. However it educed damping of caused by *P. aphanidermatum* at 6.0 %, decreased root-rot of 2.0 %, while increased plants survival of 92.0 % when compared with infected control under greenhouse conditions. Meanwhile, *P. putida* was the least effective in reducing damping off and root rot.

Table 1. Efficacy of selenium nanoparticles in controlling *Fusarium solani, Macrophomina phaseolina* and fungus like *Pythium aphanidermatum* under greenhouse conditions during 2019-2020 growing season

Treatments	Conc. ppm	%Soil borne disease								
		F. solani			*M. phaseolina*			Fungus like *P. aphanidermatum*		
		%Damping off	%Root rot	%Plants survival	%Damping off	%Root rot	%Plants survival	%Damping off	%Root rot	%Plants survival
SeNPs *B. subtilis*	200	8.0±0.21^{bcd}	4.0±0.16^{bc}	88.0±0.36^{bc}	10.0±0.40^{b}	6.0±0.24^{bc}	84.0±0.65^{abc}	6.0±0.24^{ab}	2.0±0.08^{cd}	92.0±0.32^{abc}
	250	6.0±0.20^{ab}	2.0±0.03^{c}	92.0±0.20^{abc}	8.0±0.20^{bc}	2.0±0.09^{cd}	90.0±0.12^{ab}	4.0±0.07^{bc}	2.0±0.14^{de}	94.0±0.17^{abc}
SeNPs *P. putida*	200	12.0±0.23^{bc}	6.0±0.14^{b}	82.0±0.36^{c}	12.0±0.35^{b}	6.0±0.17^{bc}	82.0±1.14^{cd}	10.0±0.27^{b}	6.0±0.25^{ab}	84.0±0.30^{bc}
	250	10.0±0.42^{a}	2.0±0.03^{c}	88.0±0.41^{bc}	12.0±0.23^{b}	4.0±0.04^{cd}	84.0±1.34^{cd}	8.0±0.13^{ab}	4.0±0.04^{cd}	88.0±1.19^{c}
SeNPs *S. grisus*	200	4.0±0.06^{cd}	4.0±0.03^{c}	92.0±0.08^{ab}	6.0±0.10^{bc}	4.0±0.10^{bcd}	90.0±0.15^{ab}	4.0±0.20^{bc}	2.0±0.44^{cd}	94.0±0.21^{abc}
	250	4.0±0.13^{ab}	2.0±0.04^{c}	94.0±0.13^{abc}	6.0±0.17^{bc}	2.0±0.06^{cd}	92.0±0.20^{ab}	2.0±0.09^{b}	2.0±0.04^{cd}	96.0±0.10^{ab}
SeNPs *T. harzianum*	200	8.0±0.32^{bcd}	6.0±0.24^{b}	86.0±0.24^{bc}	10.0±0.41^{b}	8.0±0.32^{b}	82.0±0.73^{abc}	8.0±0.32^{ab}	4.0±0.16^{de}	88.0±0.48^{abc}
	250	6.0±0.26^{ab}	6.0±0.19^{b}	88.0±0.38^{bc}	8.0±0.14^{bc}	8.0±0.13^{b}	84.0±0.24^{abc}	8.0±0.15^{ab}	6.0±8.60^{bc}	86.0±0.28^{abc}
SeNPs *C. globosum*	200	8.0±0.29^{bcd}	4.0±0.11^{bc}	88.0±0.28^{bcd}	10.0±0.37^{b}	4.0±0.07^{bcd}	86.0±0.45^{ab}	8.0±0.20^{ab}	2.0±0.04^{cd}	90.0±0.29^{abc}
	250	6.0±0.18^{bcd}	4.0±0.10^{bc}	90.0±0.25^{abc}	8.0±0.23^{bc}	4.0±0.12^{bcd}	88.0±0.28^{ab}	6.0±0.18^{bc}	2.0±0.05^{cd}	92.0±0.21^{abc}
Inoculated control		34.0±0.44^{a}	20.0±0.28^{a}	46.0±0.72^{d}	46.0±0.39^{a}	24.0±0.28^{a}	30.0±0.46^{c}	22.0±0.68^{a}	12.0±0.32^{a}	66.0±0.98^{d}
Untreated, control		0.00±0.00^{d}	0.00±0.00^{d}	100.0±0.00^{a}	0.00±0.00^{d}	0.00±0.00^{d}	100.0±0.00^{a}	0.00±0.00^{c}	0.00±0.00^{d}	100.0±0.00^{a}

* Data with the same letter are not significantly different at P > 0.0001, according to Duncan's Multiple Range Test (Duncan, 1955).

* The recorded values are the percentage ± standard deviations.

Effect of selenium nanoparticles synthesized by bio-agents on polyphenoloxidase, peroxidase, chitinase and soluble protiens activity of fennel plants grown under artificial inoculation with the three tested fungi

Result indicated that both of selenium nanoparticles obtained by *S. griseus* and *B. subtilis* recorded the highest increase in the activity of polyphenoloxidase in plants infected with each of *M. phaseolina, F. solani* and *P. aphanidermatum* which increased polyphenoloxidase activity to 0.042, 0.051 and 0.064 mg-1min, respectively, followed by selenium nanoparticles synthesis by *B. subtilis* at 0.034, 0.043 and 0.049 mg-1min respectively. On the contrary, treatments with *M. phaseolina, F. solani* and *P. aphanidermatum* showed the lowest increase in polyphenoloxidase activity of 0.014, 0.013 and 0.011 mg-1min, respectively. Meanwhile, un-inoculated control recorded 0.017 mg-1min.

Both of selenium nanoparticles obtained by *Streptomyces griseus* and *Bacillus subtilis* increased the activity of peroxidase in plants infected with each of *M. phaseolina, F. solani* and *P. aphanidermatum*. The highest increased in peroxidase was obtained with selenium nanoparticles synthesized by *S. griseus*, which increased peroxidase activity to 0.304, 0.273 and 0.225 mg-1min, respectively, followed by selenium nanoparticles synthesis by *B. subtilis* of 0.150, 0.158 and 0.104 mg-1min, respectively. On the contrary, treatments with each of *M. phaseolina, F. solani* and *P. aphanidermatum* showed the lowest increase in peroxidase activity of 0.193, 0.176 and 0.131 mg-1min, respectively. Meanwhile un-inoculated control was 0.0854 mg-1min.

Result showed that both of selenium nanoparticles obtained by *S. griseus* and *B. subtilis* increased the activity of chitinase in plants infected with each of *M. phaseolina, F. solani* and *P. aphanidermatum* (Table 2). The highest increased in chitinase was obtained with selenium nanoparticles synthesized by *S. griseus* which increased chitinase activity of 1598.30, 1421.20 and 1037.48 ppm, respectively, and followed by selenium nanoparticles synthesized by *B. subtilis* at 978.45, 870.22 and 840.70 ppm, respectively. On the contrary, treatments with each of *M. phaseolina, F. solani* and *P. aphanidermatum,* showed the lowest increase in chitinase activity of 592.5, 561.7 and 542.8 ppm respectively. Meanwhile, non inoculated control was 513.8 ppm (glucose).

Both of selenium nanoparticles obtained by *S. griseus* and *B. subtilis* increased the activity of soluble proteins in plants infected with each of *M. phaseolina, F. solani* and *P. aphanidermatum*. The highest increased soluble protein was obtained with selenium nanoparticles synthesized by *S. griseus*, which increased soluble proteins activity of 52.51, 52.20 and 67.86 mg-1ml, respectively and followed by selenium nanoparticles synthesized by *B. subtilis*

at 46.40, 46.51 and 49.24 mg-1ml, respectively. On the contrary, treatments with each of *M. phaseolina, F. solani* and *P. aphanidermatum* showed the lowest increase in soluble proteins activity of 40.70, 40.20 and 39.70 mg-1ml, respectively. Meanwhile, the un-inoculated control was 39.80 mg-1ml.

Table 2. Effect of selenium nanoparticles synthesized by some bioagents on polyphenoloxidase, peroxidase, chitinase activity and soluble protiens of fennel plants grown under artificial inoculation with the three tested fungi

Treatments	Soil-borne fungi	Enzyme activitiy			
		Polyphenol-oxidase mg-1min	Peroxidase mg-1min	Chitinase u/ml	Soluble protiens mg-1ml
SeNPs synthesized by *B. subtilis*	*M. phaseolina*	0.034	0.150	978.45	46.40
	F. solani	0.043	0.158	870.22	46.51
	p. aphanidermatum	0.049	0.104	840.70	49.24
SeNPs synthesized by *S. griseus*	*M. phaseolina*	0.042	0.304	1598.30	52.51
	F. solani	0.051	0.273	1421.20	52.20
	p. aphanidermatum	0.064	0.225	1037.48	67.86
Inoculated Control	*M. phaseolina*	0.014	0.193	592.50	40.70
	F. solani	0.013	0.176	561.70	40.20
	p. aphanidermatum	0.011	0.131	542.80	39.70
Un-inoculated Control		0.017	0.0854	513.80	39.8

Field experiments

The following experiments were ensured that antagonistic fungi and bacteria, selenium nanoparticles synthesized by bio-agent can work effectively under field conditions to replace chemical fungicides used to protect fennel plants. Only treatments that exhibited an impact in disease control under greenhouse conditions were chosen for field experiments.

Effect of selenium nanoparticles in controlling fennel soil borne diseases under field conditions during 2020/ 2021 and 2021/2022 growing seasons

Result indicated that the antagonistic fungi (*T. harzianum* and *C. globosum*) and the antagonistic bacteria (*S. griseus*, *P. putida* and *B. subtilis*) were used in synthesis selenium nanoparticles and tested against damping off and root-rot diseases under natural infection (Figure 8). Selenium nanoparticles were used as seed soaking for controlling soil borne diseases of fennel under field conditions. The survived plants were increased when treated by seed

soaking. Data showed that SeNPs synthesized by *S. griseus* were the most effective to reduce disease incidence of 4.0 and 6.0 % during the two successive growing seasons respectively, in comparison with control and suppressed damping off and root-rot diseases, and followed by SeNPs synthesized by *B. subtilis* which decreased disease incidence over two consecutive growing seasons when compared to the control and the commercial biocide and increased survived plants from 61.0 to 94.0 % during the first season from 58.0 to 92.0 % during the second season.

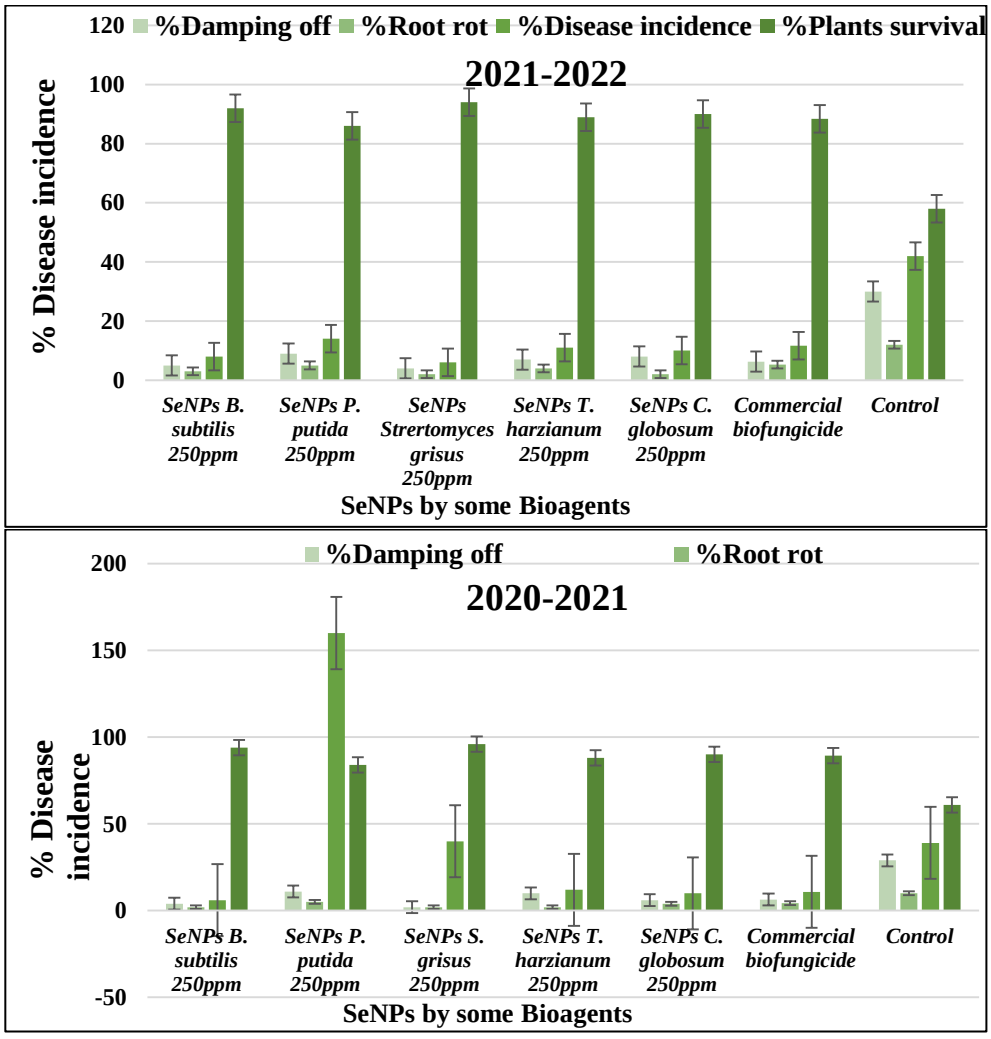

Figure 8. Efficacy of selenium nanoparticles synthesized by some bio-agents in controlling fennel damping off and root rot diseases under field conditions during the two successive seasons

Whereas, SeNPs obtained from *C. globosum* had moderate effect in decreasing disease incidence throughout both seasons when compared to the control and the commercial biofungicide in order to control damping off disease from 29.0 and 30.0% to 6.0 and 8.0 %, respectively during 2021 and 2022 growing seasons. SeNPs obtained from *C. globosum* were found to be a moderate effect on root-rot disease control during the two successive seasons from 10.0 and 12.0 % in control treatment to 4.0, 2.0 % respectively. The other treatments showed a moderate impact.

Efficacy of selenium nanoparticles on growth parameters and yield plant under field conditions during 2020/ 2021 and 2021/2022 growing seasons

Effect of selenium nanoparticles synthesized by bio-agents on the growth parameters and plant yield under field conditions. Result showed that applying SeNPs synthesized by *S. griseus* for treating fennel seeds increased yield in both seasons 2020/2021 and 2021/2022 growing seasons (Table 3). It was the most effective treatment which increased plant height, number of branches, dry weight of inflorescences, dry weight of seeds, dry weight of 1000 seeds, and plant yield during the two successive growing seasons were 225.0 cm, 9.6, 683.0 g, 449.0 g, 33.5 g, 597.0 g, respectively in 2020/2021growing season and gave 230.0 cm, 34.0 g, 442.1 g, 254.6 g, 32.3 g, 357.1 g, respectively in 2021/ 2022 growing season in comparison with the control.

Effect of selenium nanoparticles on volatile oil percent in fennel seeds

It showed that both selenium nanoparticles synthesized by *S. griseus* and *B. subtilis* increased oil percentage in fennel plants compared with treated commercial biocide and untreated control (Figure 9). The highest oil percentage was found in plants treated with SeNPs synthesized by *S. griseus*, which increased from 0.18 to 1.8 %. Also, SeNPs synthesized by *B. subtilis* increased oil percentage from 0.18 in control treatment to 1.4 % and the commercial biocide gave moderate effect (0.95%).

Effect of SeNPs synthesized by different bio-agents on percentage of oil compounds

The chemical components of fennel oil were analyzed by GC/MS. The results showed that fourteen compounds were identified; all treatments increased the active substances. Using SeNPs from *S. griseus* and SeNPs from *B. subtilis* resulted to increase in Estragole 88.29 and 84.52% respectively, when compared to the control (77.17%) and the commercial biocide (86.76%)

while SeNPs from *T. harzianum* and *C. globosum* showed moderate effect (83.44 and 81.91 %) as shown in Table 4. Anethole expressed in different manner more than Estragole. There was not noticeable increased when the measurements were done whereas the oil was richer in D- limonene and L-Fenchone than the other.

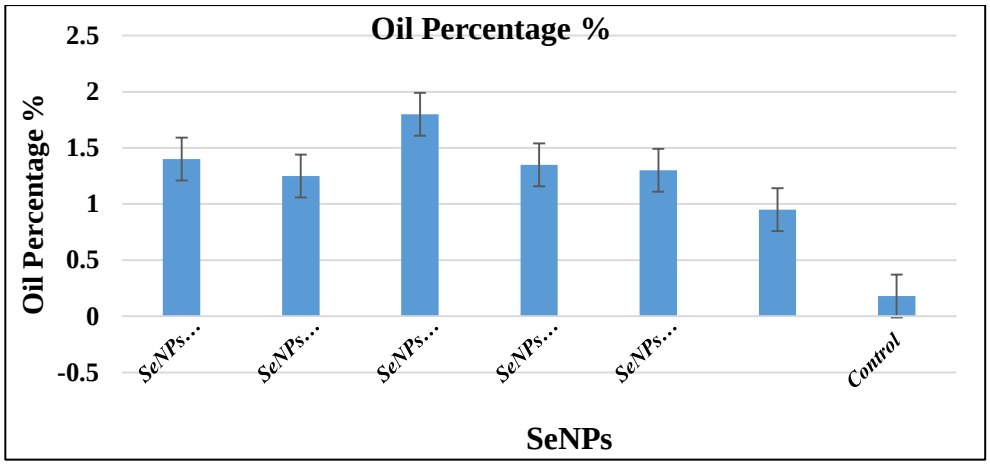

Figure 9. Effect of selenium nanoparticles on volatile oil percent of fennel seeds

Discussion

Nanotechnology has been given a great attention in recent years because of its various applications and has appeared as promising agents for plant growth as fertilizer and an alternative solution for controlling plant pathogens. The nanoparticle application in various crops enhanced crop quality and quantity. Among nanoparticles, Selenium is a valuable element for immunity and increases plant growth. The biosynthesis of selenium nanoparticles (SeNPs) from two antagonistic fungi *i.e. T. harzianum* and *C. globosum* and three antagonistic bacteria *i.e. S. griseus*, *P. putida* and *B. subtilis* was investigated as eco-friendly biofungicides for fungal root diseases of fennel *in vitro* and *in vivo*. *In vitro* evaluation of selenium nanoparticles (SeNPs) synthesis by antagonistic microorganisms exhibited antifungal activity against the growth of the tested fungi. SeNPs synthesis by *S. griseus* was the most effective against the linear growth of tested fungi, followed by SeNPs synthesis by *B. subtilis* and SeNPs of *C. globosum.*

Table 3. Efficacy of selenium nanoparticles on growth parameters and yield / plant under field conditions during 2020/ 2021 and 2021/2022 growing seasons

Treatment	2020/2021 season						2021/2022 season					
	Plant height (cm)	No. of branches	Dry weight of inflorescences (g)	Dry weight of seeds (g)	Weight of 1000 seeds (g)	Yield / plant (g)	Plant height (cm)	No. of branches	Dry weight of inflorescences (g)	Dry weight of seeds (g)	Weight of 1000 seeds (g)	Yield / plant (g)
SeNPs *B. subtilis*	215.0±30.1^{ab}	7.3±2.2^{c}	323.0±65.3^{b} ^{cd}	396.0±75.1^{a}	28.5±1.7^{ab}	359.1±18.9^{abc}	219.0±24.0^{ab}	24.5±2.9^{abc}	358.1±67.6^{a} ^{b}	192.8±1.9^{b}	27.0±3.0^{ab}	325.1±69.0^{abc}
SeNPs *P. putida*	200.3±11.3^{a}	6.2±1.5^{ab}	373.0±38.1^{a} ^{bc}	140.0±20.8^{cd}	27.0±10.2^{cd}	245.0±29.6^{cde}	206.0±53.9^{bc}	20.5±8.2	237.6±68.0^{c} ^{d}	122.9±9.1^{c}	25.7±3.2^{e}	149.8±30.3^{de}
SeNPs *S. grisus*	225.0±53.2^{ab}	9.6±2.5^{a}	683.0±48.0^{a}	449.0±57.4^{a}	33.5±7.2^{a}	597.0±72.0^{ab}	230.0±34.0^{a}	34.0±2.6^{a}	442.1±20.8^{a}	254.6±35.0^{a}	32.3±4.4^{ab}	357.1±57.3^{abc}
SeNPs *T. harzianum*	205.0±18.0^{a}	6.6±1.2^{ab}	381.0±50.6^{a} ^{b}	208.0±10.3^{bc}	27.3±4.3^{bc}	346.6±96.5^{bcd}	209.0±61.1^{bc}	21.4±0.8^{e}	363.2±59.7^{a} ^{bc}	126.9±12.2^{cd}	25.9±4.4^{de}	221.3±7.2^{c} ^{de}
SeNPs *C. globosum*	212.0±15.6^{a}	7.4±2.1^{c}	381.0±157.7^{ab}	221.0±41.4^{b}	27.3±2.1^{bc}	279.7±15.0^{cd}	147.3±63.7^{e}	25.0±2.8^{ab}	338.8 ±12.2^{bc}	156.0±10.7	26.8±23.2^{cd}	309.8±9.4^{b} ^{cd}
Commercial biofungicide (*B. subtilis*)	183.0±15.6^{c}	6.3±1.1^{ab}	170.3±10.8^{d} ^{e}	140.0±14.6^{cd}	23.6±5.6^{cde}	248.9±34.4^{de}	180.0±28.9^{cd}	23.8±2.8^{cd}	201.7±19.7^{d} ^{e}	126.6±9.6^{cde}	26.7±1.8^{abc}	288.5±36.2
Control	150.0±25.1^{b}	3.6±0.8^{b}	156.0±28.0^{f}	96.0±7.8^{c}	21.7±1.4^{e}	75.0±11.8^{a}	171.0±20.5^{cd}	8.0±1.5^{f}	156.2±26.7^{e}	108.9±6.0^{de}	22.0±1.5^{ef}	110.0±3.7^{e} ^{f}

* Data with the same letter are not significantly different at P > 0.0001, according to Duncan's Multiple Range Test (Duncan, 1955).
* The recorded values are the means ± standard deviations.

Table 4. Effect of SeNPs synthesized by different bio-agents on percentage of oil compounds

Compounds	Area %						
	Control	Commercial biofungicide (*B. subtilis*)	SeNPs *B. subtilis*	SeNPs *P. putida*	SeNPs *S. griseus*	SeNPs *T. harzianum*	SeNPs *C. globosum*
α-Pinene	0.28	0.31	0.64	0.27	94.0	0.42	0.48
à-Phellandrene	0.15	0.17	0.25	0.30	0.32	0.16	0.22
D-Limonene	4.59	4.81	9.20	5.47	9.56	6.52	7.25
Eucalyptol	0.11	0.123	0.37	0.21	0.49	0.21	0.19
trans- à–Ocimene	0.15	0.26	0.33	0.19	0.51	0.25	0.33
ç-Terpinene	0.11	0.19	0.29	0.11	0.32	0.15	0.28
L-Fenchone	6.12	6.41	7.87	3.79	8.96	6.12	6.17
Limonene oxide	0.05	0.05	0.11	0.04	0.13	0.09	0.09
Trans-Limonene Oxide	-	-	0.06	-	0.08	0.04	0.04
Camphor	-	-	0.03	-	0.07	-	-
Estragole	77.17	86.76	84.52	81.17	88.29	83.44	81.91
Fenchyl acetate	0.07	0.09	0.17	0.07	0.19	0.09	0.13
d-Carvone	-	-	0.03	-	0.09	-	-
Anethole	1.35	1.53	1.37	1.36	1.39	1.36	1.36

Xia (2007) and Vrcek (2018) who established the broadspectrum antifungal activity of mycogenic selenium nanoparticles (SeNPs) synthesized from *Trichoderma atroviride*, the synthesized nanoparticles displayed excellent *in vitro* for antifungal activity against *Pyricularia grisea* and inhibited the infection of *Colletotrichum capsici* and *Alternaria solani* on chili and tomato at concentrations of 50 and 100 ppm, respectively. Anyasi *et al*. (2017) and El-Saadony *et al*. (2020b) reported that the antimicrobial mechanisms of nanoparticles are briefed by DNA damage and cell wall disruption, electrostatic interactions between nanoparticles and the cell wall or cell membrane cause cell wall disruption, resulting causing hypha malformation and cell death.

Under greenhouse condition, *T. harzianum*, *C. globosum*, *S. griseus*, *P. putida* and *B. subtilis* were used to synthesize selenium nanoparticles found to be effective against all tested fungi *i.e. F. solani*, *M. phaseolina* and *P. aphanidermatum*. SeNPs synthesized by *S. griseus* was the most effective followed by SeNPs synthesis by *B. subtilis* and SeNPs synthesized by *C. globosum* in compare with the inoculated control and untreated control. Efficacy of selenium nanoparticles synthesized by bio-agents expressed poly phenyl oxidase, peroxidase, chitinase and soluble protiens activity of fennel plants grown under artificial inoculation with the three tested fungi in both of SeNPs obtained by *S. griseus* and *B. subtilis* which found to increase the activity of polyphenol oxidase, peroxidase, chitinase and soluble protiens in plants infected with *M. phaseolina*, *F. solani* and *P. aphanidermatum* as compared with the untreated control.

Withthis, Nandini *et al*. (2017) recorded that SeNPs derived from *Trichoderma* have recently been demonstrated to control pearl millet downy mildew disease and improved plant growth under greenhouse conditions. Nagaraju *et al*. (2012) and Jogaiah *et al*. (2018) reported that the biocontrol agent (*Trichoderma*) was triggered systemic and localized resistance in plants against a large number of biotic stresses. The same results were reported by Forootanfar *et al*. (2014) and Messarah *et al*. (2012) who stated that in biological systems, the micronutrient metalloid is reported to be the main component of selenoenzymes such as glutathione peroxidase, iodothyronine deiodinase, and thioredoxin reductase, which are involved in antioxidant defense, detoxification, and metabolism, respectively. The biological activities and good adsorptive ability of SeNPs can be attributed to the interactions between the nanoparticles and functional groups presented in proteins such as NH, C=O, COO– and C–N. SeNPs act as potential tool in integrated plant disease management.

Efficacy of selenium nanoparticles in controlling fennel soil borne diseases under field conditions during 2020/2021 and 2021/2022 growing

seasons indicated that the antagonistic fungi which were used in synthesis selenium nanoparticles against damping off and root-rot diseases were increased in survival plants of fennel comparing with the commercial biocide and untreated control under field conditions. *S, griseus* was the highest effect treatment to reduce disease incidence and followed by *B. subtilis.*

According to Bunglavan *et al*. (2014) and Ragavan *et al.* (2017) who reported that reported that nanotechnology acts as a bio-fertilizer factory used in agriculture, controlling agrochemical usage, enhancing plant resistance against disease, and efficient nutrient utilization and enhanced plant growth. Kaur (2018) and Hashem *et al.* (2021) stated that SeNPs exhibited antifungal activity against *R. solani in vitro* and *in vivo* which able to decrease the pre-and post-emergence damping-off and minimized the severity of root rot disease, improved healthy *Vicia faba* cv. Giza 716 seed germination, morphological, metabolic indicators, and yield when soaking and spraying. Xia (2007) and Vrcek (2018) concluded that the practical application of SeNPs to manage plant diseases in an eco-friendly manner, due to their mycogenic synthesis and broad spectrum antifungal activity against different phytopathogens. Hashem *et al.* (2021) reported that the application of nanoparticles in agriculture is beneficial for improving the growth and yield of crops as well as inhibiting plant pathogens by facilitating the uptake of macromolecules needed to increase resistance to plant diseases and promote growth.

Also, applying SeNPs synthesis by *S. griseus, B. subtilis, P. putida, T. harzianum* and *C. globosum* to fennel seeds increased growth parameters, yield percentage of volatile oil and frequency in both growing seasons of 2020/2021 and 2021/2022. SeNPs synthesis of *S. griseus* was the most effective treatment. It showed the highest increase all growth parameters / plant *i.e.* plant height, number of branches, dry weight of inflorescences, dry weight of seeds, dry weight of 1000 seeds, plant yield, percentage of volatile oil and frequency during the two successive growing seasons. Bunglavan *et al*. (2014) and Ragavan *et al*. (2017) who stated that nanotechnology used in agriculture could be limited agrochemical usage, improved plant resistance to disease, and nutrient utilisation and plant growth. The biological method involves nanoparticle synthesis by microorganisms, enzymes, plants, antioxidants and plant extracts. Biological methods have merit over the alternative chemical and physical methods which suggested possible to clean, non- toxic, and eco-friendly in nature. According to El-Ramady *et al.* (2016) and Kumar and Prasad (2021) who reported that biosynthesis is an eloquent, safe, biocompatible, eco-friendly, and recyclable way for preparing selenium nanomaterials. Vrcek (2018) concluded that SeNPs may provide a more secure and effective

approach against plant pathogens because of their lower toxicity, higher bioavailability and antioxidant properties.

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