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ORIGINAL ARTICLE / ORIGINALBEITRAG

Biocontrol Potential of *Trichoderma harzianum* and Zinc Nanoparticles to Mitigate Gray Mold Disease of Tomato

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Abstract

Botrytis cinerea is a destructive phytopathogenic ascomycete causing severe pre- and postharvest yield losses in tomato-growing areas worldwide. Due to fungicide resistance development in *B. cinerea* strains, its chemical control has become a serious challenge for tomato growers. In the present investigation, 47 fungal isolates were obtained and screened for their biocontrol potency against *B. cinerea*, and 12 isolates showed significant biocontrol efficacy. In 12 fungal bioagents, *Trichoderma harzianum* isolate Tr-3, identified by internal transcribed spacer (ITS) region sequence analysis, significantly suppressed the in vitro mycelial growth of *B. cinerea*. Furthermore, different concentrations (10, 25, 50, and 100 ppm) of zinc nanoparticles (ZnO-NPs) demonstrated remarkable suppression of in vitro mycelial growth. At higher concentrations (100 ppm) of ZnO-NPs, 88% mycelial growth inhibition of the pathogen was recorded. Moreover, foliar applications of *T. harzianum* suspension and ZnO-NPs in the greenhouse provided a promising control of *B. cinerea* infection in tomato plants, and a significant reduction in disease severity (68.5 and 83.4%, respectively) was recorded. While the foliar applications attenuate disease intensity, a significant increase in plant biomass was also recorded, which demonstrated the plant growth-promoting potential of indigenous *T. harzianum* and ZnO-NPs. Additionally, the antioxidant and phytochemical analysis of treated tomato leaves demonstrated higher levels of catalase (CAT) and peroxidase (PO) activity in ZnO-NP-treated plants followed by *T. harzianum*-treated plants. Thus, these results suggested that ZnO-NPs and indigenous *T. harzianum* as biocontrol could suppress *B. cinerea* infection in the greenhouse, either directly or indirectly as resistance inducers. Therefore, ZnO-NPs and *T. harzianum* may be applied as an alternative to fungicides to alleviate gray mold disease in tomato caused by the resistance problems in *B. cinerea*.

Keywords *Botrytis cinerea* · Yield · Resistance · *Trichoderma* · Plant biomarkers

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Bioregulatorpotenzial von *Trichoderma harzianum* und Zink-Nanopartikeln zur Eindämmung von Grauschimmel bei Tomaten

Zusammenfassung

Botrytis cinerea ist ein zerstörerischer phytopathogener Ascomycet, der in Tomatenanbaugebieten weltweit schwere Ertragseinbußen vor und nach der Ernte verursacht. Aufgrund der Entwicklung von Fungizidresistenzen bei *B.-cinerea*-Stämmen ist seine chemische Bekämpfung zu einer ernsten Herausforderung für Tomatenanbauer geworden. In der vorliegenden Untersuchung wurden 47 Pilzisolate gewonnen und auf ihre Bioregulatorwirksamkeit gegen *B.-cinerea*-Stämme untersucht, wobei 12 Isolate eine signifikante Bioregulatorwirksamkeit zeigten. Von den 12 Pilz-Bioagenzien unterdrückte das Isolat Tr-3 von *Trichoderma harzianum*, das durch ITS-Sequenzierung („internal transcribed spacer“) identifiziert wurde, das In-vitro-Myzelwachstum von *B. cinerea* erheblich. Außerdem zeigten verschiedene Konzentrationen (10, 25, 50 und 100 ppm) von Zink-Nanopartikeln (ZnO-NP) eine bemerkenswerte Unterdrückung des In-vitro-Myzelwachstums. Bei höheren Konzentrationen (100 ppm) von ZnO-NP wurde eine 88%ige Hemmung des Myzelwachstums des Erregers festgestellt. Die Blattanwendungen von *T.-harzianum*-Suspension und ZnO-NP im Gewächshaus führten zu einer vielversprechenden Eindämmung von *B.-cinerea*-Infektionen auf Tomatenpflanzen, und es wurde eine signifikante Reduzierung der Krankheitsschwere (68,5 % bzw. 83,4 %) festgestellt. Obwohl die Blattanwendungen die Krankheitsintensität abschwächten, wurde auch ein signifikanter Anstieg der Pflanzenbiomasse festgestellt, was das pflanzenwachstumsfördernde Potenzial von *T. harzianum* und ZnO-NP belegt. Darüber hinaus zeigte die antioxidative und phytochemische Analyse der behandelten Tomatenblätter eine höhere Aktivität von Katalase (CAT) und Peroxidase (PO) bei den mit ZnO-NP behandelten Pflanzen, gefolgt von den mit *T. harzianum* behandelten Pflanzen. Diese Ergebnisse deuten darauf hin, dass ZnO-NP und *T. harzianum* als Bioregulator die *B.-cinerea*-Infektion im Gewächshaus entweder direkt oder indirekt als Resistenzinduktoren unterdrücken können. Daher können sie als Fungizidalternative eingesetzt werden, um die Resistenzprobleme bei Grauschimmel an Tomaten durch *B. cinerea* zu lindern.

Schlüsselwörter *Botrytis cinerea* · Ertrag · Resistenz · *Trichoderma* · Pflanzliche Biomarker

Abbreviations

ANOVA	Analysis of variance
CAT	Catalase
FRAC	Fungicide Resistance Action Committee
ITS	Internal transcribed spacer
PDA	Potato dextrose agar
PO	Peroxidase
ZnO-NPs	Zinc oxide nanoparticles

Introduction

Tomato (*Solanum lycopersicum* Mill.) is the most significant and widely grown crop around the world (Zhang et al. 2020) and also ranked first vegetable in terms of area and production cultivated in Egypt and Saudi Arabia (Morci et al. 2020; Imran et al. 2022a). Higher production of tomato is obtained in the greenhouse as compared to the field, although various fungal and bacterial phytopathogens attack the different growth stages of plants in the greenhouse. Among fungal diseases, gray mold disease caused by the pathogen *Botrytis cinerea* is most destructive and causes substantial pre- and postharvest yield losses (Zhang et al. 2020). Various site-specific synthetic fungicides with different modes of action have been extensively used to control *B. cinerea* infection, but persistent application of fungicides has engendered resistance (Wang et al. 2021), which is mainly associated with point mutations in the target genes of *B. cinerea* (Imran et al. 2021). The development of resistance to fungicides has become a serious and threatening challenge for the management of gray mold disease in the greenhouse as well as in the field, because mutations leading to fungicide resistance have already been reported in this pathogen (Zhang et al. 2020) and *B. cinerea* has been categorized as a high-risk pathogen by the Fungicide Resistance Action Committee (FRAC) due to the existence of resistance in the pathogen to various fungicides. Control of gray mold disease is mainly based on integrated management strategies including physical factors, cultivar resistance, and reduction of inoculum, but the application of fungicides to mitigate phytopathogenic diseases remains a key approach due to the rapid action of chemicals for controlling phytodiseases (Fan et al. 2017; Yin et al. 2018).

Biological control is an emerging alternative to control various fungal plant pathogens because the use of indigenous fungal and bacterial microorganisms as bioagents against plant pathogens can be considered an ecofriendly approach compared to fungicides. Various indigenous *Trichoderma* species have been widely used as promising antagonists against different phytopathogenic fungi (Dukare et al. 2018; Singh et al. 2019; Wonglom et al. 2021), because *Trichoderma* species as biocontrol plays an important

role in the formulation of biopesticides. However, the antagonistic efficiency of the elements in *Trichoderma* may vary among strains and inhabitants (López Bucio et al. 2015; Imran et al. 2022b) because their antagonistic potency is influenced by various environmental factors. Various researchers have reported *Trichoderma harzianum* (in vivo and in vitro) to be an effective biocontrol agent for gray mold disease of strawberries and anthracnose decay disease of tomato (Xiangming et al. 2010; Hassine et al. 2022). As biocontrol antagonists, *Trichoderma* species compete for nutrients and space and/or inhibit the pathogen by producing highly fungi-toxic antibiotics, volatile compounds (Vinale et al. 2014), and extracellular cell wall-degrading enzymes (Qualhato et al. 2013) under in vitro conditions. Divergent mechanisms are involved in the action of *Trichoderma*, including production of antioxidant enzymes and volatile and non-volatile compounds against various pathogens, which are receiving significant attention in agriculture to control phytopathogens (Ghazanfar et al. 2018; Srivastava 2021). As biostimulators, many *Trichoderma* species induce resistance in plants by producing phytochemical compounds that protect plants from different microbes and also play a significant role in sustaining the physiological traits as well as growth and development of plants (Shoresh et al. 2010).

Currently, the use of nanotechnology in agriculture fields is an emerging key tool receiving significant attention for management of plant diseases by nanoagrochemical formulation with nanomaterial (Chen and Yada 2011; Thiruvengadam et al. 2018). Zinc (Zn) is an essential microelement with significant functions. Involved in different enzyme activities and as a cofactor, it regulates the synthesis of protein (Sharma et al. 2013). Zinc deficiency in plants causes diminution of metabolic processes, i.e., plant growth and physiology, which subsequently affects yield (Gurmani et al. 2012). Various types of nanoparticles (NPs) have been reported and used for control of fungal plant diseases (Abdel-Hafez et al. 2016; Rossi et al. 2019) because their applications stimulate the antioxidant system and the photosynthetic machinery in tomato plants (Faizan et al. 2021). Application of zinc oxide nanoparticles (ZnO-NPs) significantly improves growth of plants by regulating the photosynthetic process and photogeneration of active oxygen species such as hydroxide anion and superoxide as well as the performance of antioxidative enzymes (Venkatachalam et al. 2017; Garcia-Lopez et al. 2019). Various studies have reported the positive impact of nanoparticle-based fertilizers on physiological parameters and the induction of oxidative stress in plants (Nhan et al. 2015). To the author's knowledge, no study has been conducted for the control of *B. cinerea* using indigenous *Trichoderma* along with ZnO-NPs in this region. Therefore, the present study aimed to isolate and identify native *Trichoderma* species from the rhizosphere of healthy tomato fields; establish a conventional approach for control of gray mold disease by evaluating the biocontrolling potential of native *Trichoderma* along with ZnO-NPs; conduct an in vivo evaluation of native *Trichoderma* as biocontrol agents against *B. cinerea* strain; and asses the influence of *Trichoderma* and ZnO-NPs on plant biomass and enzymatic markers responsible for resistance.

Materials and Methods

Botrytis cinerea Strain and Growth Conditions

Highly virulent *Botrytis cinerea* strain BC-1101 previously isolated by Imran et al. (2021) was obtained from the fungal stock culture of the laboratory of Plant Pathology, King Abdulaziz University, Jeddah, Saudi Arabia. *B. cinerea* strain was subcultured on potato dextrose agar (PDA) plates at 20 ± 2 °C for 3 days. Actively grown fungal mycelial culture of the strain was further plated on PDA plates to use throughout the study.

Isolation of Indigenous *Trichoderma*

To isolate *Trichoderma* species from the rhizosphere of healthy tomato field of Assiut University Experimental Station, Assiut, Egypt, soil samples were collected (5 inches depth around the roots) randomly from tomato field from different locations, packed into sterile polythene bags, and directly brought to the laboratory of Plant Pathology of Assiut University and stored at 4 °C. Impurities and root debris were removed and fungal colonies were isolated using the dilution-plate method as reported by Abdel-Gawad et al. (2021). Briefly, a soil sample (2 g) from each sample was subjected to 10 ml sterile double-distilled water. Tubes were gently mixed by vortex and placed at room temperature for 30 min to settle the precipitate. Different dilutions, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, and 10^{-9}, were prepared from the supernatant (Bin et al. 2020). To obtain fungal cultures, 500 µl of each dilution was spread over the surface of PDA plates supplemented with streptomycin (to inhibit bacterial growth; 20 µg L^{-1}). Plates were incubated at 27 °C for 48 h. Germinated fungal colonies were observed and purified by the single-spore transfer method (Dou et al. 2019). Fugal isolates were preliminarily identified (data not shown) using morphological and microscopic characteristics (Myung-Soo et al. 2005; Kumar et al. 2016). Purified fungal cultures were maintained in PDA glass tubes incubated at 4 °C for 4 days and then preserved at 4 °C for further use.

Evaluation of the Antagonistic Potential of *Trichoderma*

The in vitro antagonistic potential of isolated *Trichoderma* against *B. cinerea* strain BC-1101 was evaluated with the dual-culture method described by Abdel-Rahim and Abo-Elyousr (2018). A mycelial disc (5-mm diameter) from a 3-day-old *B. cinerea* colony was excised and placed face down near the edge of a PDA plate. Subsequently, a mycelial disc (identical diameter) from 3-day-old *Trichoderma* colonies was placed on the opposite side of the plate to the pathogen at an equal distance from the plate margin. A mycelial disc of pathogen (identical diameter) placed at the same portion was a control. Plates were incubated at 20 ± 2 °C for 5 days. Colony diameter was measured, and the growth inhibition of pathogen by *Trichoderma* was recorded.

Molecular Identification of Selected *Trichoderma* Antagonists

The *Trichoderma* isolates exhibiting strong in vitro antagonistic potential were screened and selected for identification by amplification of internal transcribed spacer (ITS) regions analysis using polymerase chain reaction (PCR). Genomic DNA of selected isolates was extracted by cetyltrimethylammonium bromide (CTAB) lysis and the phenol/chloroform extraction method. For amplification of ribosomal ITS regions of the DNA of selected *Trichoderma* isolates, the universal primer pair ITS5 (5′GGAAGTAAAAGTCGTAACAAGG3′) and ITS4 (5′TCCTCCGCTTATTGATATGC3′) was used (Jiang et al. 2016), and a standard PCR reaction performed in a thermal cycler (iCycler™; Bio-Rad, Hercules, CA, USA) with final volume of 50 µl reaction mixture containing 5 µl 10X standard Taq reaction buffer, 4 µl 10 mM dNTPs, 1 µl of 10 µM forward primer, 1 µl of 10 µM reverse primer, 0.5 µl Taq DNA polymerase enzyme, 1 µl template DNA, and 37.5 µl nuclease-free water. The PCR reaction was performed with the following conditions: initial denaturation at 94 °C for 3 min followed by 30 cycles of denaturation at 94 °C for 1 min, 56 °C for 30 s, 72 °C for 30 s and a final extension at 72 °C for 10 min followed by 10 min cooling at 4 °C. PCR products were visualized in an AlphaImager™ (ProteinSimple; Santa Clara, CA, USA) gel imager system in 2% agarose gel after staining with 0.5 µg ethidium bromide solution for 10 min. Products were sequenced (Macrogen, Seoul, South Korea) and the obtained sequences compared with the sequence available in the public domain of the National Center for Biotechnology Information (NCBI) library using the Basic Local Alignment Search Tool (BLAST). Isolates were identified by their high similarity (%) to previously reported sequences and the identified sequences were submitted to NCBI under a specific accession number. The phylogenetic tree of a selected isolate was constructed using MEGA 6X package (Pennsylvania State University, State College, PA, USA) by the neighbor-joining (NJ) method (Kim et al. 1993) and topology of the phylogenetic tree was evaluated as described (Felsenstein 1985).

In Vitro Application of Zinc Oxide Nanoparticles

The zinc oxide nanoparticles (ZnO-NPs) used in present study were obtained from Sisco Research Laboratories (SRL) Pvt. Ltd (Mumbai, India). From the initial stock solution, different concentrations, namely 10, 25, 50, and 100 ppm, were prepared in sterile double-distilled water. The in vitro effect of these concentrations on mycelial growth inhibition of pathogen was studied. PDA plates amended with the abovementioned ZnO-NP concentrations were used and identical discs (previously used to evaluate the *Trichoderma* isolates) of 3-day-old *B. cinerea* colony were excised and placed face down on the mesial surface of ZnO-NP-amended plates. The PDA plates lacking nanoparticles but with pathogenic mycelial discs served as a control. Plates were incubated at 20 ± 2 °C for 5–7 days. The experiment was performed in triplicate for each of the concentrations and five plates were used for each replicate. Mycelial growth inhibition (%) in ZnO-NP-treated plates was measured and compared to the mycelial growth of control plates.

Establishment of a Conventional Approach by Nanoparticles and Bioagents

Based on the in vitro mycelial growth inhibition by fungal bioagents, selected bioagents along with ZnO-NPs were used to investigate their influence on disease severity (%) and plant biomass of a tomato variety, Doucen, under greenhouse conditions. Seedlings (15–20 seeds/pot) were grown in 18-cm plastic pots containing peat moss (1:3), and at the 3–4-leaf stage, seedlings were transplanted to new pots (one seedling/pot). Cell suspension of the selected bioagent grown priorly on PDA for 5 days at 27 °C was prepared by crushing the colony in 20 ml sterile double-distilled water. The suspension was filtered with three layers of cheesecloth to remove the mycelial fragments and spores were adjusted (6×10^8 conidia/ml). Then, 20-day-old tomato plants were sprayed with ZnO-NPs (10 mg/L; 30 ml/plant) and the cell suspension of the selected fungal bioagent (30 ml plant^{-1}) with a compression sprayer (Blue Stallion Co., Ltd, India; Wintgens 2009; Abo-Elyousr et al. 2014; Singh et al. 2019). Foliar application of the treatments was in the morning and plants were sprayed till dripping point.

A cell suspension of highly virulent *B. cinerea* strain grown priorly on PDA was sprayed (10^4 spores/ml; 50 ml pot^{-1}) 24 h after spraying of bioagents and nanopar-

ticles with a compression sprayer (Blue Stallion Co., Ltd, India). Plants sprayed (at the same time as pathogen inoculation) with sterile distilled water were used as a healthy control treatment, infected control plants were treated with the suspension of pathogen. After pathogen inoculation, plants were covered with sterile plastic polythene bags for 72 h to initiate the infection by pathogen. Plants were irrigated as per requirements and standard agronomic practices were carried out throughout the experiment. Temperature (20 ± 2 °C) and humidity (up to 85%) inside the greenhouse was maintained. A complete randomized design was used to perform the greenhouse experiments and 16 plants were used for each treatment with four replicates. Plants were monitored regularly, and the symptoms developed by tomato plants were observed after 20 days of pathogen inoculation. Disease severity on tomato plants was measured with a 0–4 disease-rating scale: 0 = no disease, 1 = 0.1–5%, 2 = 5.1–20%, 3 = 20.1–40%, and 4 = 40.1–100% (Lee et al. 2006), and disease severity (%) was calculated as:

$$\text{Disease severity (\%)} = \frac{\sum (\text{the number of diseased leaves} \times \text{disease severity index})}{4 \times \text{the number of leaves rated}} \times 100$$

After observing disease severity, all plants in all treatments were harvested to study the effect of treatments on physiological parameters, i.e., plant height and fresh and dry weight of roots and shoots. After determining the fresh weight, plants were placed in a moisture dryer at 60 °C for 4–6 days and dry weight was calculated. Means of all effective treatments were observed and compared with controls.

Estimation of Antioxidant Enzymes

Preparation of Leaf Aliquots

Different antioxidant enzymes, namely catalase activity (CAT) and peroxidase (PO), were measured in treated tomato leaves according to Chance and Maehly (1956). After 5 days of foliar application of ZnO-NPs and bioagents, leaf samples were randomly collected from each treatment (12 plants were sampled from each treatment) and stored at –80 °C. Then, leaf tissues (0.5 g/10 ml) were homogenized in 50 mM phosphate buffer (pH 5.2) containing 1% polyvinylpyrrolidone. The homogenate was centrifuged at 15,000 rpm for 10 min (4 °C). The pellet was discarded and the supernatant transferred to new tubes. Tubes were placed at –20 °C to measure the antioxidant compounds in supernatant.

Peroxidase Activity

The peroxidase activity (PO) of the supernatant was determined using guaiacol as a substrate by adding 0.2 ml of supernatant sample into the mixture containing 1 ml of 0.1 M sodium acetate buffer (pH 5.2), 0.2 ml of 1% guaiacol, and 2.2 ml of 1% hydrogen peroxide (H_2O_2). The reaction was incubated at 25 °C for 5 min and PO activity was measured at 436 nm absorbance with a spectrophotometer (Thermo Fisher Scientific; Waltham, MA, USA) for 2 min with intervals of 20 s. The reaction mixture lacking enzyme extract but including extraction buffer only was used as a blank. Each extracted sample was measured thrice for each replicate. Four replicates per analysis were used and PO activity was expressed as μmol/min mg^{-1} protein.

Catalase Activity

The catalase activity (CAT) of the supernatant was measured by adding 0.5 ml of supernatant sample extract to 3 ml of Sörensen phosphate buffer (pH 7) and 200 μl of 30% hydrogen peroxide (H_2O_2). The reaction was incubated at 25 °C for 1 min. The CAT activity was measured at 240 nm absorbance with a spectrophotometer (Thermo Fisher Scientific) for 2 min with intervals of 20 s. Each extracted sample was measured thrice for each replicate. Four replicates per analysis were used and CAT activity was expressed as μmol/min mg^{-1} protein.

Statistical Data Analysis

A completely randomized design (CRD) was used to perform the greenhouse experiments. Four replicates were used for each treatment and four plants were used for a replicate. The significance of the difference between the mean values was calculated and one-way analysis of variance (ANOVA) was performed. The significance of differences among the treatments was determined according to Fisher's least significant difference (LSD) test (Gomez and Gomez 1984).

Results

Isolation and In Vitro Screening of Antagonists

A total of 47 fungal isolates were obtained from the healthy rhizosphere of tomato field. All the isolates were grown on PDA to determine the morphological and microscopic characteristics. Morphological characteristics, i.e., average colony growth diameter (8.1–8.7 cm), colony color (green/yellow, dark green/green), conidia size (3.1–5.3 μm), and shape (globose to subglobose), and the sizes (3.0 × 2.4–11.5 × 2) and shape of phialide (slender,

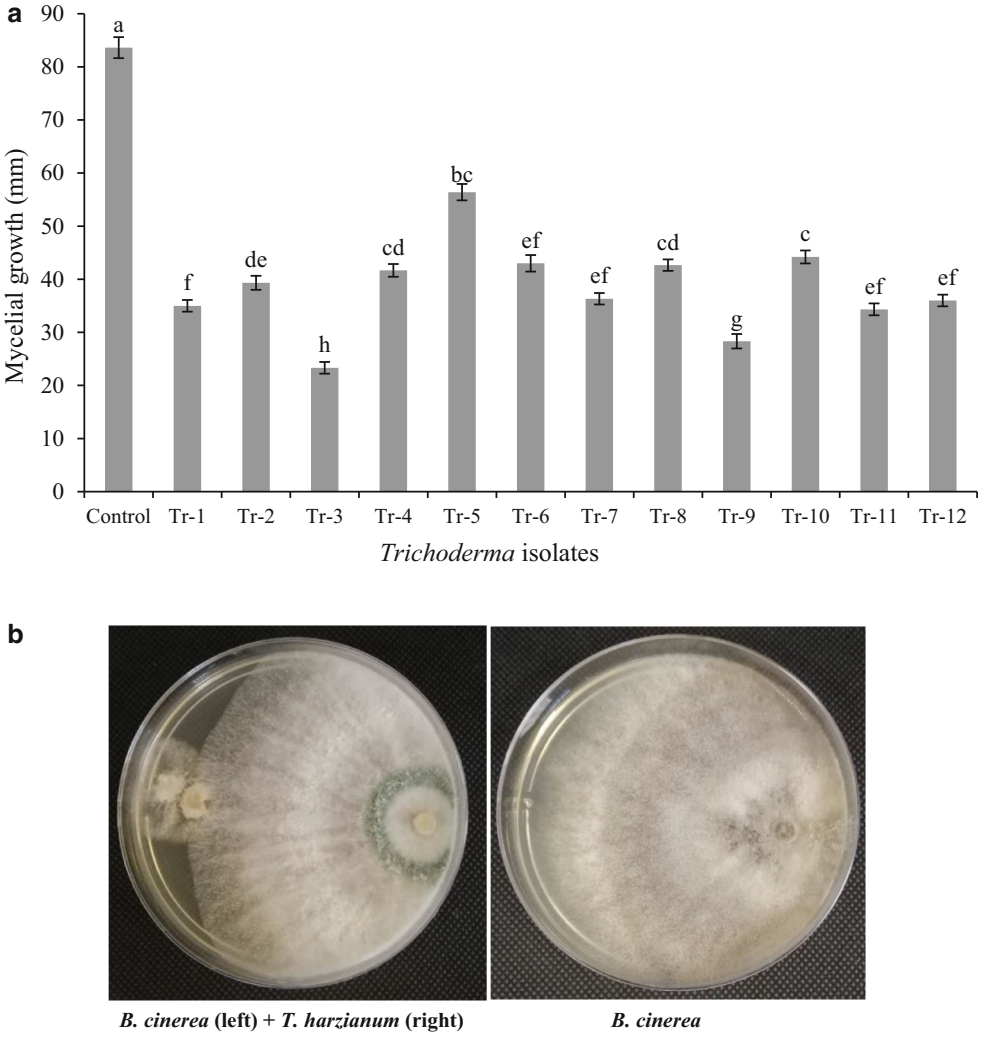

Fig. 1 **a** In vitro screening of native *Trichoderma* isolates for antagonistic potential against *Botrytis cinerea* strain BC-1101. Values followed by different *letters* indicate that means are significantly different from each other according to Fisher's least significant difference test at $p = 0.05$. *Error bars* represent the mean ± standard error. **b** In vitro antagonistic activity of indigenous *Trichoderma harzianum* against *Botrytis cinerea* strain BC-1101

hooked, flask shaped) were measured using a binocular microscope (Shenzhen Boshida Instrument Co., Ltd, China) and based on morphological characteristics. All isolates demonstrated homogeneity to *Trichoderma* spp. (Fig. 2a), which was further confirmed using the reported *Trichoderma* spp. characteristics (Myung-Soo et al. 2005; Kumar et al. 2016). All *Trichoderma* isolates were further subjected to evaluation of their antagonistic potential against *B. cinerea* strain BC-1101 and 12 isolates showed promising antagonistic inhibitory potential against *B. cinerea* mycelial growth in vitro. Among the 12 isolates, Tr-3 most strongly inhibited mycelial growth of *B. cinerea,* as the recorded growth (28.33 mm) was significantly lower (Fig. 1a) than mycelial growth in the control (83.62 mm) plate. Based on the antifungal activity of *Trichoderma*, the Tr-3 isolate (Fig. 1b) was selected for molecular identification by using specific primers for PCR analysis.

Molecular Characterization of Selected *Trichoderma* Species

Based on the antagonistic potential of *Trichoderma* isolates, the Tr-3 isolate significantly inhibited the in vitro mycelial growth of *B. cinerea* (Fig. 1a, b). Therefore, isolate Tr-3 was selected for molecular identification through PCR amplification of the ITS1 region. The length of the obtained product was about 620 bp and the Local BLAST analysis of the obtained sequence showed high similarity (80–90%) to the available sequences on NCBI that were identical to *Trichoderma harzianum* (KR868283.1), and there, isolate Tr-3 was identified as *Trichoderma harzianum*. The sequence of the identified *T. harzianum* Tr-3 strain was submitted to NCBI under accession number MW590687. The phylogenetic tree constructed by the sequence of *T. harzianum* showed high similarity to the other *T. harzianum* isolates available in NCBI (Fig. 2b).

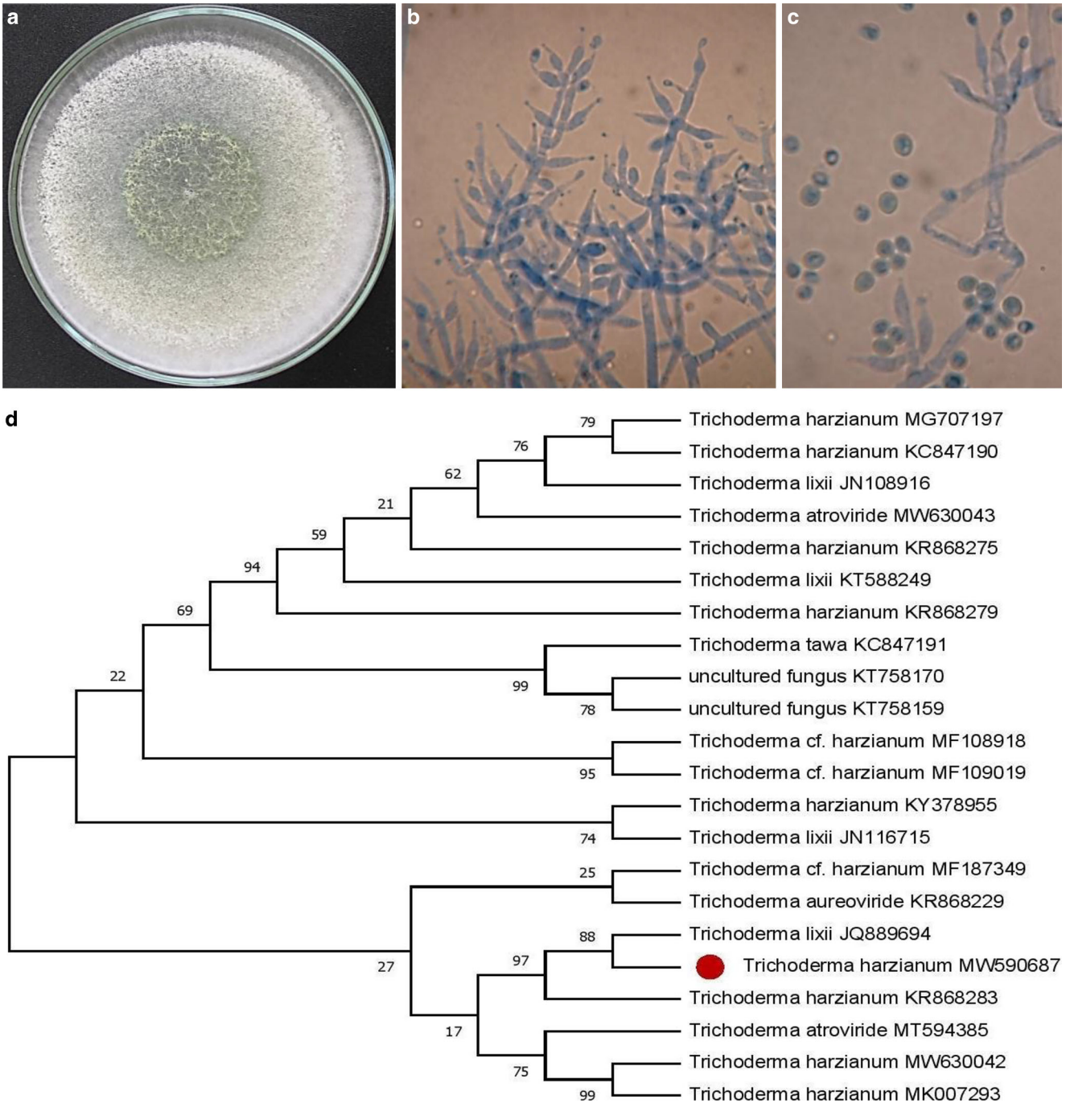

Fig. 2 **a** *Trichoderma harzianum* strain Tr-3 on potato dextrose agar (PDA). **a** Colony culture on PDA medium; **b** conidiophores and flask-shaped phialides; **c** smooth and subglobose conidia. **d** Phylogenetic tree of *Trichoderma harzianum* Tr-3 strain, constructed via analysis of internal transcribed spacer 1 sequences with the neighbor-joining algorithm in the MEGA 6X package (Pennsylvania State University, State College, PA, USA). The *numbers* over branches represent the coefficient of bootstrap, tested by 100 replications

In Vitro Application of Nanoparticles

In vitro application of ZnO-NPs at different concentrations showed a significant reduction in the mycelial growth of *B. cinerea* (Fig. 3b). At lower concentrations (10 ppm) of nanoparticles, higher (35.5 mm) mycelial growth was recorded, which significantly reduced with an increase in the concentration of nanoparticles. A remarkable reduction in mycelial growth was observed at 100 ppm (Fig. 3a). Mycelial growth at all concentrations, i.e., 10, 25, 50, and 100 ppm, was significantly lower, at 35.5, 23.68, 14.5, and 7.72 mm, respectively, compared to mycelial growth in con-

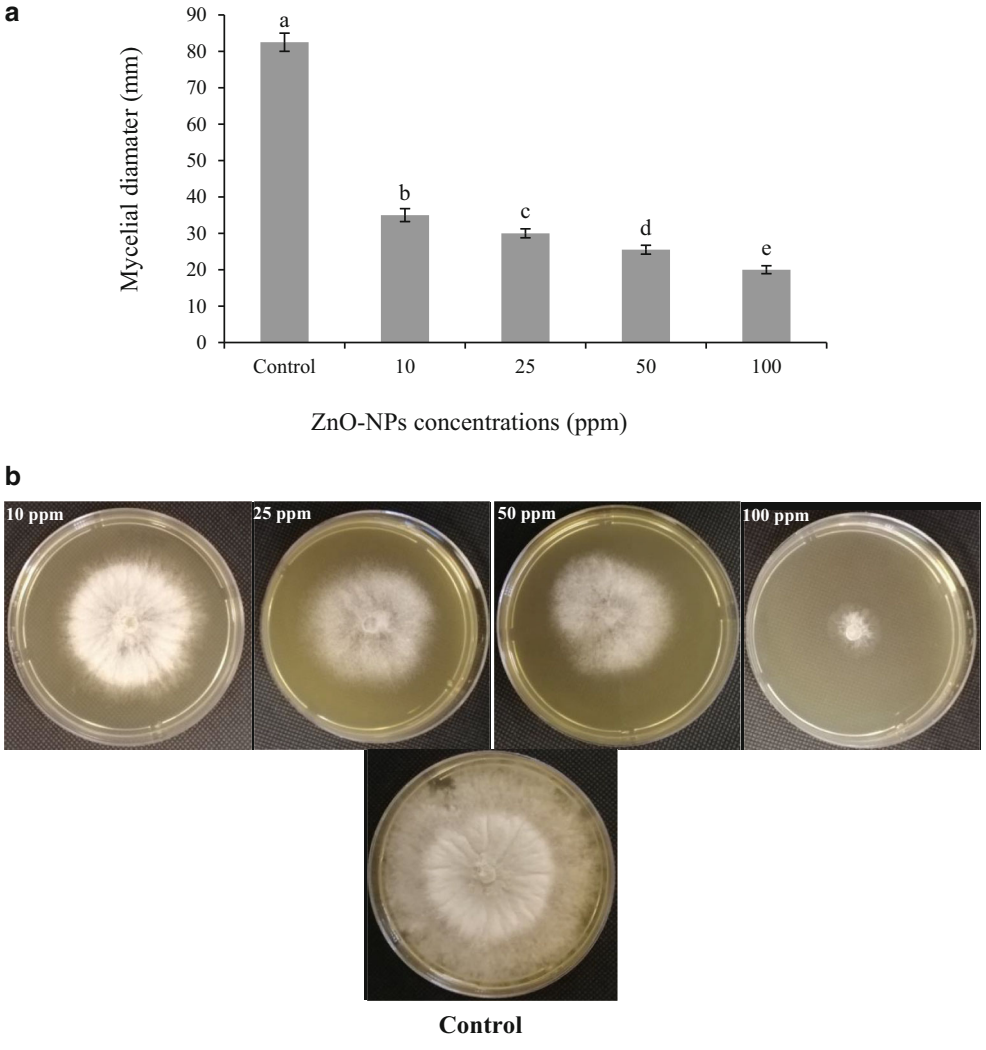

Fig. 3 **a** In vitro effect of ZnO nanoparticle (ZnO-NP) concentrations on mycelial growth inhibition of *B. cinerea*. Values followed by different *letters* indicate that means are significantly different from each other according to Fisher's least significant difference test at $p = 0.05$. *Error bars* represent the mean ± standard. **b** In vitro effect of ZnO-NP concentrations on mycelial growth inhibition of *B. cinerea*

trol (82.5 mm) plates. However, the in vitro effect of ZnO-NPs on the mycelial growth of *B. cinerea* pathogen was prodigious. The results presented herein show that the increased concentration of nanoparticles greatly influenced the growth of *B. cinerea*, which confers the strong antifungal activity of ZnO-NPs.

Application of Nanoparticles and *Trichoderma harzianum* in the Greenhouse

Effect on Disease Severity

Foliar application of ZnO-NPs (10 mg/L) and bioagent on tomato plants after artificial inoculation of *B. cinerea* pathogen significantly alleviated disease severity (%) in the greenhouse. The foliar application of both treatments demonstrated an auspicious effect for the reduction of disease, as disease severity on plants treated with *T. harzianum* was 27.25% and considerably lower than on *B. cinerea*-treated plants (86.43%). However, disease severity after ZnO-NPs foliar application was significantly lower (14.37%), followed by *T. harzianum*-treated plants. Foliar application of *T. harzianum* reduced disease intensity by up to 74.17% compared to infected controls, whereas the application of ZnO-NPs significantly reduced disease (by 84.41%) under greenhouse conditions. The results presented here demonstrate that application of ZnO-NPs

Table 1 Greenhouse effects of ZnO nanoparticles (ZnO-NPs) and *T. harzianum* on disease severity (%) after artificial inoculation of *B. cinerea* on tomato plants

Treatment	Disease severity (%)	Disease reduction (%)
Infected control	82.18 ± 0.58 a	–
T. harzianum	21.25 ± 1.64 b	74.14
ZnO-NPs (10 mg/L)	12.81 ± 1.11 c	84.41
Healthy control	0.00 ± 0.00 d	0

Values followed by different letters indicate that means are significantly different from each other while same letters indicate that means are not significantly different from one another according to Fisher's least significant difference test at $p = 0.05$

positively affected the initiation of *B. cinerea* infection on tomato plants in the greenhouse and causes an astonishing reduction in disease severity as compared to *T. harzianum* (Table 1).

Effects on Plant Physiological Parameters

Effects of *T. harzianum* and ZnO-NPs on plant physiological parameters, i.e., plant height and fresh and dry weight of root and shoots, were measured and the results show that foliar application of ZnO-NPs and *T. harzianum* treatments not only attenuates disease severity but also boosts plant height as compared to healthy and infected controls (Fig. 4a). Moreover, as a result of NP treatments, plant height was significantly higher (113.59 cm) than in *T. harzianum*-treated plants (104.92 cm) as well as infected (52.58 cm) and healthy controls 74.91 cm. While the application of nanoparticles and bioagent also enhanced the fresh and dry weight of roots, which ultimately increases (Fig. 4b, c) plant growth, the fresh and dry weight of plants was also increased (Fig. 4d, e). These results demonstrate that nanoparticles and indigenous *Trichoderma* can endorse plant growth, which may also protect the tomato plant from *B. cinerea* infection. These findings showed that application of bioagents and nanoparticles considerably enhanced the plant biomarkers (Fig. 4).

Effect on Enzyme Activity

The catalase (CAT) activity of the plants treated with ZnO-NPs was significantly higher than in control plants. The results of this study showed that CAT activity increased in ZnO-NP-treated plants, at 495.5 µmol/min mg^{-1} protein as compared to *T. harzianum*-treated plants with 138 µmol/min mg^{-1} protein. CAT activity of the control was 323.17 µmol/min mg^{-1} protein, followed by *T. harzianum*-treated plants (Fig. 5a).

The PO activity of ZnO-NP-treated plants was significantly higher compared to the control. Maximum PO activity was recorded in ZnO-NP-treated plants (9.88 µmol/min/

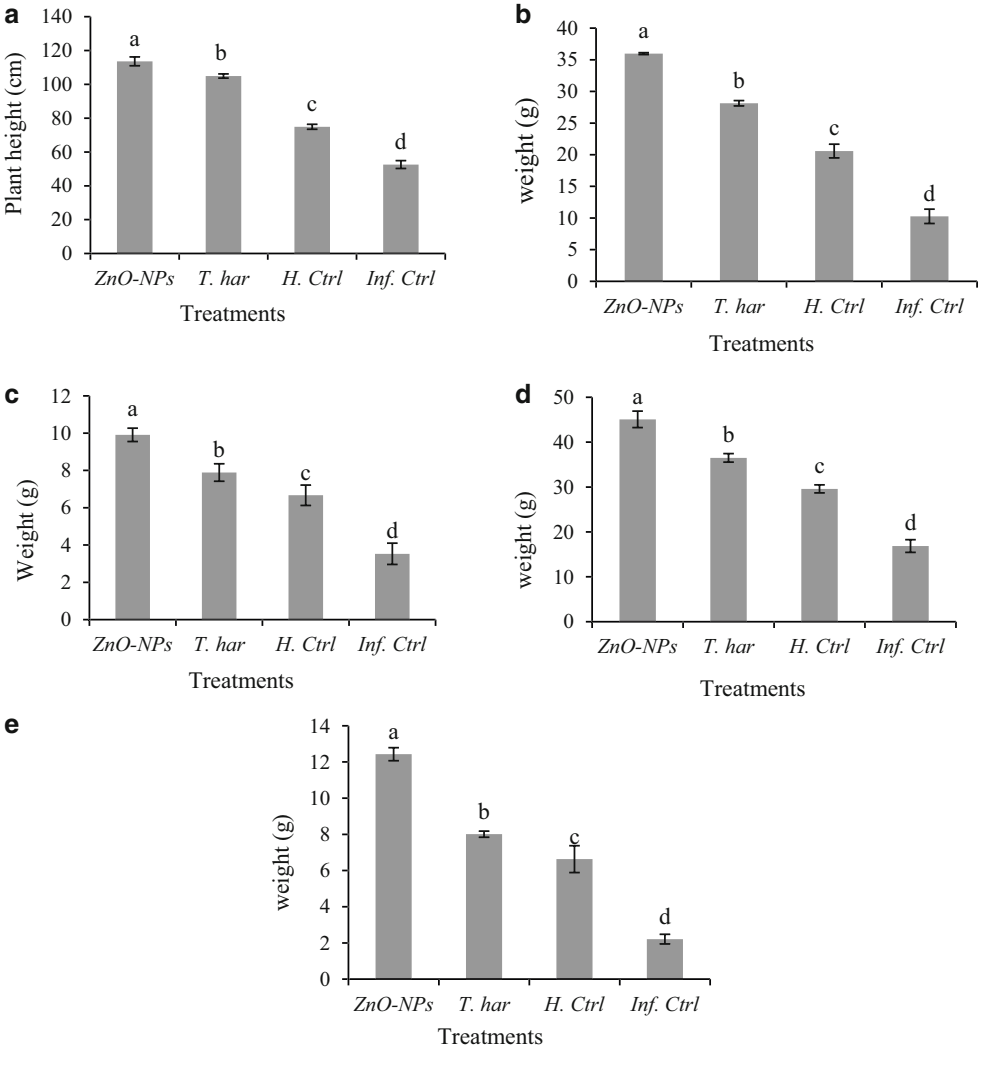

Fig. 4 Effect of *T. harzianum* and ZnO nanoparticles (*ZnO-NPs*) on plant physiological parameters. **a** Plant height (cm); **b** root fresh weight (g); **c** root dry weight (g); **d** shoot fresh weight (g); **e** shoot dry weight (g). Values followed by different *letters* indicate that means are significantly different from each other according to Fisher's least significant difference test at $p = 0.05$. *Error bars* represent the mean ± standard error; *T. har Trichoderma harzianum*, *H. Ctrl* healthy control; *Inf. Ctrl* infected control

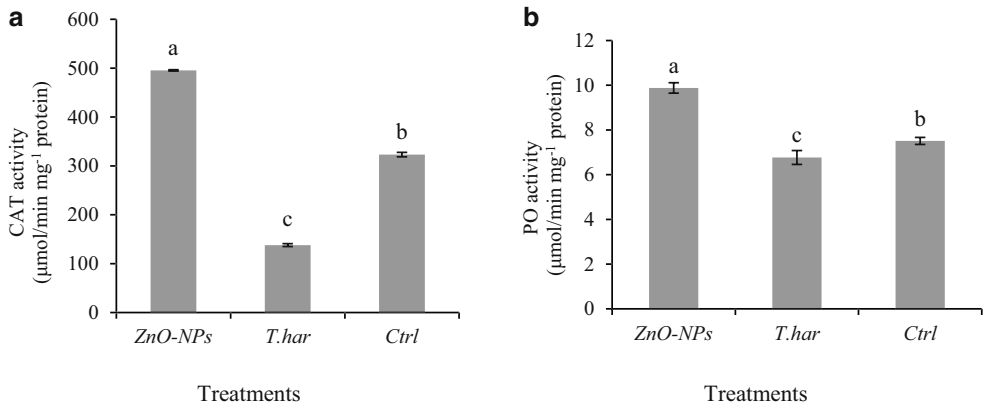

Fig. 5 Effect of ZnO nanoparticles (*ZnO-NPs*) and *T. harzianum* foliar applications on the antioxidant enzyme activity: catalase (*CAT*) activity (**a**) and peroxidase (*PO*) activity (**b**) in tomato plants. All data represent the means of replicates and *error bars* represent the mean ± standard error. Values followed by different *letters* indicate that means are significantly different from each other according to Fisher's least significant difference test at $p = 0.05$. *T. har Trichoderma harzianum*, *Ctrl* infected control

mg protein), and was lower in control (7.51 µmol/min mg^{-1} protein) and *T. harzianum*-treated plants (6.77 µmol/min/mg protein; Fig. 5b).

Discussion

Biological control of plant diseases caused by various fungal pathogens has been recommended as a substitute for chemical fungicides (Drobya et al. 2016). Therefore, the present study focused on fungicide alternatives and an ecofriendly approach for the control of gray mold disease of tomato using ZnO-NPs and indigenous *Trichoderma* spp. Various *Trichoderma* spp. as biocontrol agents have been widely reported on and used to control various phytopathogenic diseases including *B. cinerea* that causes gray mold disease (Ghazanfar et al. 2018; Silva et al. 2019; Fenta et al. 2019), because *Trichoderma* spp. produce various types of antibiotics, certain enzymes, volatile/non-volatile compounds, and secondary metabolites which hinder the growth of *B. cinerea* pathogen (Dukare et al. 2018; Carolina da Costa et al. 2021). Studies have reported the antagonistic potential of *T. harzianum* against mycelial growth of different pathogens such as in in vitro dual-culture assays of *T. harzianum* against *Rhizoctonia solani* (Srivastava 2021). Furthermore, the current results are confirmed by another study by Purwantisari et al. (2021), which reported biocontrol of *Phytophthora infestans* by *T. harzianum* and a significant growth reduction of *P. infestans*. Moreover, some volatile and non-volatile metabolites produced by *Trichoderma* spp. have the capability of restraining the fungal mycelium as shown by a study in which *T. piluliferum* as a biocontrol agent against *Colletotrichum musae* showed 62% mycelial growth inhibition (Carolina da Costa et al. 2021). Additionally, up to 42% mycelial growth suppression of *Colletotrichum gleosporioides* was recorded by *T. harzianum* (Rahman et al. 2013).

In this study, in vitro application of ZnO-NPs at different concentrations showed significant growth reduction of *B. cinerea*. An increase in the concentration of ZnO-NPs drastically decreased the in vitro mycelial growth, and lower mycelial growth was recorded at a higher concentration of ZnO-NPs (100 ppm) as compared to other concentrations. Former studies have reported that the concentration 5×10^{-3} M showed astonishing mycelial growth inhibition of *B. cinerea,* but the exposure of *B. cinerea* plates to homemade LED light inhibited 80% of mycelial growth (Luksiene et al. 2020). In another study, the ZnO-NP concentrations 0, 3, 6, and 12 mmol/L also inhibited growth of *B. cinerea*, and at a higher concentration (12 mmol/L), significant mycelial growth inhibition (78.8%) was recorded (He et al. 2011; Arciniegas-Grijalba et al. 2017). The results of these reported investigations firmly support the current results. In the present study, a significant reduction in disease severity (84.41%) with foliar application of ZnO-NPs was recorded and numerous studies have also reported a reduction in disease severity (%) with foliar application of ZnO-NPs (Rossi et al. 2019; Faizan et al. 2021), thus strongly supporting the present results. Foliar application of *T. harzianum* also suppresses *B. cinerea* initiating infection on tomato plants in the greenhouse and ultimately activates the defense mechanisms in plants that cause a reduction in disease severity. Previously, foliar application of *T. harzianum* in the greenhouse significantly reduced (54–65%) the pathogenicity of *B. cinerea* on strawberries (Barakat and Al-Masri 2017). Furthermore, a reduction in disease severity (%) by foliar application of *T. harzianum* against various fungal phytopathogens has also been documented (Menzel et al. 2016; Barakat and Al-Masri 2017; Srivastava 2021) and supports the current results. In the

present study, foliar application of ZnO-NPs reduced disease severity (84.41%), followed by *T. harzianum* (74.14%). Most recent studies have also reported a significant reduction in disease severity with foliar application of ZnO-NPs and *T. harzianum* (Faizan et al. 2021; Srivastava 2021), which supports the current findings. Plant growth, fresh, and dry weight was also studied to observe the influence of ZnO-NPs and *T. harzianum*, and their stimulation of the growth rate of plants promotes their potential as pathogen biological control agents.

In the presence of nanoparticles and *T. harzianum*, the enzyme content in plant leaves was positively influenced. In the present study, the PO and CAT activities in ZnO-NP-treated plants were higher than in *T. harzianum*-treated plants, as conferred by Mohammad Faizan et al. (2021). An increase in PO-like activity may be attributable to an increase enzyme activity (Gao et al. 2007). However, the synergetic effect of nanoparticles and *T. harzianum* against *Sclerotinia sclerotiorum* has also been documented (Bilesky-José et al. 2021), which significantly suppressed the pathogen and increased the levels of protein and carbohydrate and chlorophyll content, because the addition of nanoparticles in *Trichoderma* caused slackening of electron transport enzyme activity which ultimately diminished ATP and NADH formation.

Conclusion

The results presented herein conclude that indigenous *Trichoderma harzianum* and zinc-based nanoparticles have great potential for suppressing the gray mold fungal pathogen. Application of *T. harzianum* not only suppressed pathogen initiation but also elevated plant biomass by acting as plant growth promoter. Application of fungicide to manage this disease has become a great challenge due to resistance development in pathogens against fungicides with diverse modes of action. Furthermore, zinc oxide nanoparticles might have lower toxicity toward humans and animals than synthetic fungicides. Therefore, *Trichoderma* species and nanoparticles may be considered as an important and effective tool for the control of gray mold disease and studies should address the impact of NPs on plant health, specifically focusing on antioxidant pathways. Future studies should be focused on the development of zinc- and *Trichoderma*-based compositions that hinder fungal pathogens. Moreover, application of zinc nanoparticles in the field is important to monitor their influence on human health and impact on environment. These studies will prove to be representative when considering the effect of zinc nanoparticles and *Trichoderma* on destructive disease-causing fungal pathogens and will ultimately provide cost-effective and integrated disease management strategies for sustainable tomato production.

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Author Contribution All authors contributed equally in the manuscript, Imran, Abdel-Rahim, and Abo-Elyousr suggested the idea of the work and contributed to data curation and their validation as well as writing original draft. Bagy and Abdel-Rahim contributed to the formal analysis of the data, El-Sharnouby, Sallam, and Ali contributed to the reviewing and editing the manuscript. All authors reviewed and approved the final version of the manuscript.

Declarations

Conflict of interest M. Imran, K.A. Abo-Elyousr, M. El-Sharnouby, E.F. Ali, N.M. Sallam, H.M.M.K Bagy, and I.R. Abdel-Rahim declare that they have no competing interests.

Ethical standards This manuscript represents original research, and has not been submitted in full or in part to any other journals for publication.

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