

# The Root Endophytic Fungus *Serendipita indica* Decreased the Phytotoxicity of Zinc Oxide Nanoparticles to Alfalfa (*Medicago sativa L.*)

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## Research Article

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## Abstract

Zinc oxide nanoparticles (ZnO-NPs) are among the most commonly used nano-fertilizers (NF). However, elevated levels of ZnO-NPs in soil may affect plant growth and development due to its potential toxicity when accumulated in large amounts in plant tissues. This research was conducted using an *in situ* rhizobox system with the aims of evaluating Zinc uptake from nano-zinc oxide amended rhizosphere soil by alfalfa plant and the effect of plant growth promoting microorganisms on alleviating the phytotoxicity of ZnO-NPs. Treatments included microbial inoculations (*Sinorhizobium meliloti*, *Serendipita indica*) and different ZnO-NPs concentrations (0, 400 and 800 mg Kg<sup>-1</sup>) with three replications. The results indicated that *S. indica* minimized the phytotoxicity of ZnO-NPs to alfalfa by enhancing growth rate and decreasing Zinc (Zn) translocation from root to shoot. Compared with plants inoculated with *S. meliloti*, co-inoculation with *S. indica* increased the shoot dry weight by 18.33% and 8.05% at 400 and 800 mg Kg<sup>-1</sup> ZnO-NPs. However, at the highest level of ZnO-NPs (800 mg kg<sup>-1</sup>), root inoculation of *S. indica* and *S. indica* + *S. meliloti* decreased Zn transfer factor by 60.2% and 44.3% compared to *S. meliloti*, respectively. Furthermore, a distinct relation between tolerance of *S. indica*-colonized plant to ZnO-NPs and the ability of *S. indica* in inhibiting or retarding degradation of polyunsaturated lipids through prevention of excess reactive oxygen species formation was observed. Malondialdehyde content of inoculated plants with *S. indica* either alone or in combination with *S. meliloti* was significantly lower than non-inoculated plants ( $p < 0.01$ ). Zn-induced oxidative stress was mitigated by *S. indica* through enhanced activities of catalase and peroxidase enzymes. The findings of the present study indicate the potential use of endophytes fungus *S. indica* for ensuring food safety and security, and human health in heavy metal-polluted soil by reducing the phytoavailability of heavy metals in the aerial parts of the host plants.

## Introduction

Agriculture sector is facing an intense pressure for achieving considerable efficiency in food production to feed the consistently growing human population (Fróna et al. 2019). With the limited arable lands and scarce agricultural resources, further growth, or even maintenance of current yield levels will depend crucially on increased consumption of organic and mineral fertilizers (Hafeez et al. 2013). However, this approach has resulted in serious deterioration of ecosystems and environment especially in the developing countries with heavy dependence on agriculture for employment, income and food supplies.

Adverse environmental impacts of high rates of mineral fertilizers used in low-input agricultural systems has been triggered a renewed interest in enhancing fertilizer use efficiency (Rahman and Zhang 2018). In this sense, nanotechnology through Nano-fertilizers (NFs) has provided a new frontier in sustainable agriculture systems that promises to enhance the nutrient use efficiency of inputs and minimize relevant problems (He et al. 2019). Certain specific properties of NFs which facilitate nutrient use efficiency are enhanced mobility of NFs due to very small size (less than 100 nm) and high solubility in water that increase availability of encapsulated nutrients inside the NFs in rhizosphere (Jampilek and Králová 2015).

Plant growth and several physiological processes are highly dependent on zinc (Zn) because of its fundamental role in biochemical and metabolic processes such as photosynthesis, respiration, nucleic acid synthesis and protein, carbohydrate and lipid metabolism (Auld 2001). Moreover, Zn plays a central role in

detoxification mechanisms in plants such as the reactive oxygen species (ROS) related pathways (Broadley et al. 2007). Fertilizers with oxides (ZnO) and sulfates ( $\text{ZnSO}_4 \cdot \text{H}_2\text{O}$  &  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ ) forms improves soil productivity, however, high utilization of zinc fertilizers in soils with low plant available Zn, would result in over supplying Zn and causes toxicity to plants, and humans, as well as soil microbes (Kabata-Pendias 2010).

Zinc oxide nano-fertilizer is among the most commonly used metal-based nano materials in agriculture to alleviate Zn deficiency in soils (Rajput et al. 2018). New reports show that ZnO-NPs have the potential for delivering Zn via soil or leaves with increased and stimulated crop yield and Zn use efficiency; e.g., rice, maize, wheat, sugarcane, sunflower and cowpea (Bandyopadhyay et al. 2014; Li et al. 2012; Moghaddasi et al. 2017; Monreal et al. 2016; Wang et al. 2013). However, despite the exciting outcomes of nano-fertilizers, increased application of ZnO-NPs may result in appearance of toxicity in plants, animals, human and malfunctioning of soil microbial communities (Chai et al. 2015; Lin and Xing 2007, 2008). For plants this happens when Zn concentrates at the root surface and subsequently internalizes and accumulates in large amounts in plant tissues (Ma et al. 2010). The interaction of plant cell with nanoparticles leads to changes in transcription profile of genes and associated biological pathways which eventually affect plant growth and development (García-Sánchez et al. 2015; Moreno-Olivas et al. 2014). The phytotoxicity of ZnO-NPs on plant physiological traits like seed germination, biomass, leaf number, and root elongation have been reported in many agricultural crops (Monreal et al. 2016). Also, the negative impacts of ZnO-NPs may include oxidative damages to biological membranes (Kim et al. 2012; Mukherjee et al. 2014; Noori et al. 2020), decreased photosynthetic rate (Barhoumi et al. 2015; Mukherjee et al. 2014; Wang et al. 2016) and decrease in plant growth hormones synthesis (Castiglione et al. 2011; Vankova et al. 2017).

Although the quantity of nanomaterials currently reaching the soil is relatively small, however, increase of metal-based nanomaterials ( $\text{TiO}_2$ ,  $\text{Fe}_2\text{O}_3$ , ZnO) due to application of pesticides, insecticides and fungicides in soil is expected which may be taken up by roots and ultimately entered into food chain (Rico et al. 2011).

Heavy metal tolerant-plant growth promoting (HMT-PGP) microbes ensure plant survival and growth in contaminated soils by modulating plant growth, altering physico-chemical properties of soil to enhance metal bioavailability and binding metal ions present in the external environment on the cell surface or transport them into the cell (Mishra et al. 2017). Selected strains of rhizosphere Plant Growth Promoting Bacteria (PGPB) and Plant Growth Promoting Fungi (PGPF) contribute to plant protection from toxicity of metal-based NP as they perform multiple functions such as improvement of soil quality, enhancement of plant growth, detoxification, and removal of HM from soil (Mishra et al. 2017; Sepehri and Khatabi 2020). Rhizobia are among the most important PGPB found in the rhizosphere with ability to fix nitrogen in agricultural systems through the symbiotic association with legumes (Hao et al. 2014). In case of PGPF, root entophytic fungus, *Serendipita indica* formerly known as *Piriformospora indica* (Singhal et al. 2017) contributes to growth enhancement of host plants growing in harsh environmental conditions (Liu et al. 2020).

Although studies have investigated the ability of HMT-PGP microbes to alleviate toxicity of metal oxide particles such as ZnO on the host plants, however, the potential effects of PGP microorganisms in improving plant growth and development in soils contaminated with metal-based NPs have not yet been completely elucidated for most NPs. Also, it is well documented that PGPB and PGPF can greatly affect the immobilization of heavy metals by secreting organic acids, siderophores, 1-aminocyclopropane-1-carboxylic (ACC)-

deaminase, phytohormones and enzymatic transformation (Rizwan et al. 2016; Sharma and Archana 2016; Vimal et al. 2017).

Therefore, the main objective of the current study is to evaluate phototoxicity of nanoparticulate ZnO (nano-ZnO) to alfalfa and the synergistic action of PGPB (*Sinorhizobium meliloti*) and PGPF (*Serendipita indica*) to boost plant performance in Zn-contaminated rhizospheric soils.

## Materials And Methods

### Characteristics of ZnO-NPs

Zinc oxide nanoparticles (ZnO-NPs) of 10 nm mean diameter were obtained from the Iranian Nanomaterials Pioneers Company, NANOSANY (Mashhad, Iran). The sample of nano-zinc oxide was characterized under a transmission electron microscope (TEM) (EM208S, Philips) coupled with X-ray powder diffraction (XRD) (EQUINOX 3000, France) (Graphical abstract). ZnO-NP suspensions with concentration of 400 and 800 mg l<sup>-1</sup> were prepared, and carefully sonicated before mixed with the soil to minimize the aggregation effect.

### Soil Sampling for Greenhouse Experiment

Soil samples were collected from the 0–30 cm top layer of the agricultural fields located in Agricultural Research Station at Shiraz University, Shiraz-Iran. The soils were homogenized, sieved (< 2 mm) and analyzed for physio-chemical properties. The soil was sterilized by placing it in an autoclave (25 min at 121 °C) to eradicate any initial microbial community. Before seeds were transplanted into the soil, a dispersion of deionized water and ZnO-NPs were added to the soil with 0 (control), 400 and 800 mg Zn kg<sup>-1</sup> concentrations (dry based).

### Bacterial and Fungal Inoculums

*S. indica* was cultured on CM (complex medium) and the spore suspension was collected after 4 weeks of fungal incubation at 24°C±1°C in dark. Fungal inoculum was prepared by scratching the surface of CM medium with a mixture of sterile distilled water and Tween-20 (20 ml water and 10 µl Tween-20). The suspension of spores was filtered through Miracloth (CalBiochem, Bad Soden, Germany) to remove excess medium, and then washed three times with distilled H<sub>2</sub>O containing Tween-20. After each washing step, the spores were collected by centrifugation at 4000 rpm for 7 min. The spore pellet was finally suspended again in distilled H<sub>2</sub>O and adjusted to ~ 5 × 10<sup>5</sup> spores ml<sup>-1</sup> using a hemocytometer (Ghabooli et al. 2013).

The bacterial isolate was selected among the superior *Sinorhizobium* strains previously screened based on their ability to produce 1-aminocyclopropane-1-carboxylate (ACC) deaminase, indole-3-acetic acid (IAA), N<sub>2</sub> fixation and siderophore production. Our studies revealed that the selected strain had nfe, putA, acdS, genes involved in nodulation and symbiotic nitrogen fixation (Talebi et al. 2008). Also, we confirmed that *S. indica* has also ability to produce siderophore and regarded as a siderophore-producing microbe (Sepehri and Khatabi 2020).

To prepare the bacterial suspension, the bacterium was cultured on Nutrient Broth (NB) medium and incubated for 48 h and 120 rpm continuous shaking at 28 °C. The bacterial cells were centrifuged at 5000 rpm for 5 min and the pellets were washed twice with 10 mM MgSO<sub>4</sub>. The bacterial pellets diluted with 10 mM MgSO<sub>4</sub> to make a final concentration of 108 CFU ml<sup>-1</sup> (Saleem et al. 2017).

### Greenhouse Experiment

The greenhouse experiment was conducted as a completely randomized design with three replicates per treatment at Shiraz University, Shiraz, Iran. Experimental treatments included: ZnO-NPs at three levels mentioned above, and microbial inoculation factor at four levels (control, *Serendipita indica*, *S. meliloti*, and *S. indica+S. meliloti*).

The experiment was carried out in 36 rhizobox (200 mm × 130 mm × 200 mm) based on the design by Wang et al. (2002) with some minor modifications. To separate rhizosphere soil from bulk soil, each rhizobox was subdivided into 3 adjacent compartments by using nylon mesh of 25 µm pore size: A central compartment for planting (rhizosphere compartment) and two lateral compartments on both sides (bulk soil). All compartments were filled with soil amended with desired amounts of ZnO-NPs layer by layer using the same filling procedure in all cases. Controls were also set up in three replicates.

Alfalfa (*M. saliva* L.) seeds of cultivar Hamedani were sterilized for 5 min in NaClO solution (0.75% Cl) followed by 70% alcohol for 1 min and rinsed with sterilized distilled water at least ten times. The seeds were germinated in 45-mm plastic petri plates containing aqueous agar (0.8% [w/v] agar) at 25 °C for 2 days. The seedlings were inoculated with *S. indica* by immersion in spore suspension (~ 5 × 10<sup>5</sup> spores ml<sup>-1</sup>), and subsequently by gentle shaking (75 rpm) for 3 h. Bacterial inoculation was done by adding 1 ml of 48 h-old bacterial culture (1 × 10<sup>8</sup> bacterial cells ml<sup>-1</sup> of broth culture) to the soil surrounding each root seedling using a pipette. In co-inoculation treatment, *S. meliloti* was inoculated in the same manner in rhizoboxes assigned for the study of individual bacterial inoculation 2 days after *S. indica* inoculation. An equal quantity of sterile bacterial and fungal media was added to the control treatment (non-inoculated) to make the treatment conditions uniform.

The inoculated and non-inoculated alfalfa plantlets with bacterial and/or fungal isolates were kept under control growth conditions (26 ± 1°C with 16/8 h light/dark photoperiod and relative humidity of 80–85%). Soil moisture was kept approximately constant (near 70% field capacity) by periodical watering in order to replace consumed water. No pesticides were applied and weeds were removed manually.

In order to visualize root colonization by *S. indica* microscopically, *S. indica*-inoculated alfalfa roots were harvested 14 days after inoculation. Roots were softened by 10 % KOH solution for 15 min, acidified with 1 M HCl for 10 min, and stained with 0.02 % Trypan blue overnight, and then were distained with 50% lacto-phenol for 1 h prior to microscopic observation under a light microscope (Dickson and Smith 1998; Phillips and Hayman 1970).

After 9 weeks of ZnO-NPs exposure, the shoots were cut at the crown and leaves washed thoroughly with distilled water before analysis. Subsequently, the collected samples from the last well-developed leaves were frozen at -80 °C prior to the evaluation of antioxidant enzymes activities, photosynthetic pigments and MDA

content. At week 2 of the flowering stage, the plants were harvested and dry weight of shoot and root parts were measured after oven drying samples at 70 °C for 48 h. The shoot and root concentrations of Zn were determined with Atomic Absorption Spectrophotometer (Perkin-Elmer 3030) according to standard method (Horwitz et al. 1975).

### **Carotenoids and Chlorophyll Contents**

Carotenoids, chlorophyll a (Chl a) and chlorophyll b (Chl b) of fresh fully expanded leaves were determined based on the Arnon'smethod (Arnon 1967). 0.1 g of fresh leaf tissues were homogenized in 80% acetone, centrifuged at 4000 rpm for 20 min and by using spectrophotometer, the optical density of the supernatant was read at 470, 663 and 645 nm wave lengths for Carotenoids, Chl a and Chl b, respectively.

### **Hydrogen Peroxide ( $H_2O_2$ ) and Lipid Peroxidation**

Hydrogen peroxide ( $H_2O_2$ ) content in control and treated plants was measured according to the method of Nukuntornprakit et al. (2015). About 0.2 g of fresh leaf tissues from each replicate was homogenized in 2 mL of 1%(w/v) trichloroacetic acid (TCA) and centrifuged at 10,000× g for 10 min at 4°C. The supernatant (250 µl) was mixed with 250 µl of phosphate buffer (PBS, 100 mM, pH=7) and 500 µl of 1M potassium iodide (KI). The assaymixture was vortexed and its absorbance was measured at 390 nm using a microplate reader spectrophotometer (Epoch Biotech, USA). The  $H_2O_2$  content was determined by using a standard curve of  $H_2O_2$  represented as  $\mu\text{mol g}^{-1}$  fresh weight.

Lipid peroxidation in terms of malondialdehyde (MDA) formation was determined according to the method of Heath and Packer (1968). 0.1 g of fresh leaf tissue was homogenized in 0.5 % thiobarbituric acid (TBA) in 20% trichloroacetic acid (TCA). The assay mixture was heated at 95 °C in the water bath for 30min, cooled immediately and centrifuged at 10,000× g for 10 min. The absorbance was read at 532nm.The value of non-specific absorption at 600 nm was subtracted. MDA concentration was calculated using an extinction coefficient of 155 mM<sup>-1</sup> cm<sup>-1</sup> and expressed as nmol g<sup>-1</sup> fresh weight.

### **Antioxidant EnzymesActivities**

Enzyme extraction was performed by grounding 0.5 g of fresh leaf tissues with 2 mL of chilled PBS (50 mM, pH=7) containing 2 mM ethylenediaminetetraacetic acid (EDTA) and 1 % (w/v) polyvinylpyrrolidone (PVP). The homogenate was centrifuged at 13,000 ×g for 10 min at 4°C, the supernatant was taken for detection of Catalase (CAT), Peroxidase (POD), and Superoxide dismutase (SOD) activities.

The CAT and POD activities were measured using the method described by Chance and Maehly (1955). CAT activity was determined by adding 50 µL of leaf enzyme extract to the reaction medium containing 50mM PBS (pH=7) and 15mM  $H_2O_2$  as substrate. Decomposition of  $H_2O_2$  was detected by the reduction of absorbance at 240nm after 1 min of reaction. One unit of enzyme activity was defined as a 0.1 change of absorbance per min and it was defined as U g<sup>-1</sup> fresh weight.

POD activity was determined using guaiacol as substrate. The reaction mixture (3 mL) contained 50 µL of crude enzyme extract, 50mM PBS (pH=7), 13mM guaiacol, and 20mM  $H_2O_2$ . Peroxidase activity was detected

by measuring the absorbance at 470 nm after 2 min of reaction. One unit of POD activity was defined as the amount of enzyme that caused 0.1 change in absorbance at 470 nm per min under the specified conditions. The specific POD activity was defined as U g<sup>-1</sup> fresh weight.

SOD activity was measured following the method described by Beachamp and Fridovich (1971). The reaction mixture (2 mL) contained 100 µL of crude enzyme extract in 50 mM PBS (pH=7.8), including 13Mm methionine, 75 µM nitroblue tetrazolium (NBT), 0.1 mM EDTA, and 2 µM riboflavin and was illuminated using a fluorescent lamp for 15 min at ambient temperature. The absorbance was measured at 560 nm. Two mixtures without enzyme extract were maintained under the dark and light, and used as blank and control, respectively. One unit of SOD activity was detected as the amount of enzyme causing 50% reduction of NBT and the specific SOD activity was defined as U mg<sup>-1</sup> fresh weight.

## Statistical Analysis

Analysis of variance (ANOVA) of data was performed using SAS 9.1 software. Treatment means were compared by determining the least significant difference (LSD) at 5% ( $p \leq 0.05$ ) probability level.

# Results

## Soil Characteristics before the Onset of the Experiment

Geographic locations and some physical and chemical characteristics of the applied soil for the greenhouse experiment are presented in Table 1. The results showed that unlike low organic soil carbon content, the amount of zinc usable was sufficient for the plant and due to low electrical conductivity in the studied soil, there was no salinity problem. Among the chemical characteristics, soil pH is considered as the major variable due to its profound effect on numerous chemical reactions, including essential plant nutrients, phytotoxic elements and pollutants. A high percentage of Iranian arable land as well as the soil used in this experiment, due to high pH and calcium carbonate equivalent, are calcareous which could have a significant impact on the availability of elements.

## Effect of Microbial Inoculation on pH Values in Rhizosphere and Bulk Soil

The pH in the rhizosphere soils amended with ZnO-NPs was lower than bulk soil pH (Table 2). The rhizosphere soil pH was significantly lower in microbial treatments over the control (non-inoculated). Among the microbial treatments, the highest pH value under ZnO-NPs stress recorded in *S.meliloti*. The root inoculation of *S. indica* either alone or in combination with *S. meliloti* caused a decrease of the rhizosphere soil pH of about 0.5 units compared to the control suggesting that this fungus can help to reduce soil acidity.

## Effects of Microbial Inoculation on Plant Biomass

ZnO-NPs resulted in phenotypic changes as visualized by reduction of shoot and root biomass (Fig. 1&2). Relative to plants colonized with *S. indica* either alone or in combination with *S. meliloti*, symptoms of Zn toxicity were noticed on the aboveground and belowground parts of non-inoculated plants and *S. meliloti* inoculated ones.

The shoot dry weights of the non-inoculated plants for 400 and 800 mg Kg<sup>-1</sup> ZnO-NPs were significantly reduced (by 31.5 and 32.8 %, respectively,  $p<0.001$ ) compared to non-exposed plants which illustrates Zinc toxicity. This was also observed in tendency for root biomass. At high concentration of ZnO-NPs (800 mg Kg<sup>-1</sup> Zn), root dry weight of the non-inoculated plants decreased by 45.4 % relative to the control.

*S.indica* significantly mitigated the adverse effects of Zn toxicity by displaying significant higher plant growth observed in inoculated plants when compared with non-inoculated and/or inoculated plants with *S. meliloti*. Zinc toxicity on plants inoculated with *S. meliloti* was clearly visible at all of the applied treatments of ZnO-NPs. The shoot and root dry weights of *S. meliloti*-inoculated plants decreased 26.1% and 19.4% when exposed to 400 mg Kg<sup>-1</sup> ZnO-NPs, respectively. A significant reduction in shoot and root weights of plants inoculated with *S. meliloti* were also observed.

Despite the impact of ZnO-NPs on *S.meliloti*-inoculated plants, *S. indica* played a major role in decreasing Zn toxicity when inoculated in combination with *S. meliloti*. Compared with the single-inoculation of *S. meliloti*, co-inoculated plants exposed to 400 mg Kg<sup>-1</sup> ZnO-NPs showed an increase of 18.3% of shoot dry weight.

### Photosynthetic Pigments

The content of Photosynthetic Pigments (carotenoids, chlorophyll a and chlorophyll b) of alfalfa plants were reduced with increasing concentrations of Zn in both inoculated and non-inoculated plants (Fig. 3).

Under normal conditions, individual inoculation of microbial treatments resulted insignificant increase in chlorophyll a content over the control ( $p<0.01$ ). *S. indica*-inoculated plants exposed to ZnO-NPs showed the highest content of chlorophyll a, chlorophyll b and carotenoids. At the highest level of ZnO-NPs (800mg Kg<sup>-1</sup>), *S. indica* increased the amount of chlorophyll a, chlorophyll b and carotenoids by 17.2 %, 12.6 % and 11 % compared to non-inoculated plants, respectively. *S. meliloti* either alone or in combination with *S. indica* produced similar effects on chlorophyll a and carotenoids, so that no significant difference was detected for different concentrations of ZnO-NPs. In the case of chlorophyll b, double inoculation of *S. meliloti* and *S. indica* had significant effect for 400 mg kg<sup>-1</sup> ZnO-NPs, however, the effect of *S. meliloti* was the same as the controls. The content of chlorophyll b in plants inoculated with *S. meliloti* + *S. indica* at 400 mg kg<sup>-1</sup> ZnO-NPs treatment was increased by 12.4% when compare to *S. meliloti*-colonized plant. The effect of co-inoculation of *S. meliloti* with *S. indica* on chlorophyll b at the highest concentration of ZnO-NPs showed no significant differences to non-inoculated plants.

### Zinc Accumulation in Plant Tissues

Zinc concentration in the shoot and root parts of both inoculated and non-inoculated alfalfa plant was significantly increased by increasing Zn concentration. *S. indica*-inoculated plants exposed to 400 and 800 mg Kg<sup>-1</sup> ZnO-NPs had the lowest amount of Zn in the shoot tissue (Fig. 4a). Co-inoculation of *S. indica* and *S. meliloti* at the level of 400 ZnO-NPs showed no significant difference compared to the individual inoculation of *S. meliloti*. However, at level of 800 mg kg<sup>-1</sup> ZnO-NPs, the concentration of Zn in the shoot of plants co-inoculated with *S. meliloti* and *S. indica* was significantly lower than the plants inoculated with *S. meliloti* ( $p<0.001$ ).

Upon exposure to 400 mg Kg<sup>-1</sup> ZnO-NPs, Zn content in the roots colonized with the individual inoculations of microbial treatments was significantly higher than the other treatments. At the highest level of ZnO-NPs (800 mg kg<sup>-1</sup>), co-inoculation of *S. indica* and *S. meliloti* led to an increase in root Zn concentration, while *S. meliloti* inoculation had no effect on Zn accumulation when compared to the control plants (Fig. 4b).

### Zinc Transfer Factor (TF)

The influence of the microbial inoculation on plant Zn uptake and translocation was further scrutinized by calculating the translocation factor (TF) values (Fig. 5). Increasing concentrations of ZnO-NPs had no significant effect on the TF in *S. indica* treatment, while the TF of plants inoculated with *S. meliloti* increased with the increasing level of ZnO-NPs.

There was a significant difference in TF values between the non-inoculated and inoculated plants under different ZnO-NPs treatments. When plants exposed to 400 mg kg<sup>-1</sup> ZnO-NPs, all microbial inoculations significantly decreased TF compared to the non-inoculated plants. Under such conditions, co-inoculation of *S. indica* and *S. meliloti* led to a relatively high TF, while *S. indica* inoculation showed the lowest TF value. The value of TF in *S. indica*+ *S. meliloti* treatment was 152 and 73.9% higher than the values obtained for individual inoculation of *S. indica* and *S. meliloti*.

At the highest Zn level (800 mg kg<sup>-1</sup> ZnO-NPs), *S. meliloti* inoculation significantly increased the TF of Zn by 60.2% and 44.3% compared to *S. indica* either alone or in combination with *S. meliloti*. However, there was no significant difference between plants inoculated with either *S. indica* or *S. indica*+ *S. meliloti*.

### Malondialdehyde (MDA) and Hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) Production

The variation in Malondialdehyde (MDA) content as an index of lipid peroxidation in leaves of alfalfa plants is shown in (Fig. 6a). Comparison between inoculated plants illustrated that ZnO-NPs application caused a significant increase in MDA content of plants inoculated with *S. meliloti*. Upon exposure to 400 mg Kg<sup>-1</sup> ZnO-NPs, MDA content of *S. meliloti*-inoculated plants increased by 39.9% and 33.5% compared to plants inoculated with *S. indica* and *S. meliloti*+*S. indica*, respectively. However, no significant difference was detected between *S. meliloti*-inoculated and control plants exposed to 400 and 800 mg Kg<sup>-1</sup> ZnO-NPs.

MDA content of non-inoculated plants was significantly higher than inoculated plants with *S. indica* either alone or in combination with *S. meliloti*. Although there was no significant difference between plants inoculated with either *S. indica* or *S. meliloti*+*S. indica*, the rate of decline in MDA content was high for *S. indica*.

As shown in the Fig. 6b, there was no significant change in the H<sub>2</sub>O<sub>2</sub> formation in shoots of inoculated plants exposed to 400 mg Kg<sup>-1</sup> ZnO-NPs when compared to non-inoculated plants. However, a significant decrease in H<sub>2</sub>O<sub>2</sub> formation was observed in plants inoculated with *S. indica* alone or in combination with *S. meliloti* compared to *S. meliloti*-inoculated plants upon exposure to 800 mg Kg<sup>-1</sup> ZnO-NPs. The amount of H<sub>2</sub>O<sub>2</sub> production in plants inoculated with *S. meliloti*+*S. indica* decreased by 41.2% compared to inoculated plants with *S. meliloti*.

## Antioxidant Enzymes

For the two applied levels of ZnO-NPs, all microbial treatments either alone or in combination exhibited no significant difference on activity of SOD. It's worth to mention that under the highest concentration of ZnO-NPs ( $800 \text{ mg Kg}^{-1}$ ), the activity of SOD in the leaves of inoculated plants did not significantly differ from the controls (Table 3). The highest activity of CAT and POD antioxidant enzymes under 400 and  $800 \text{ mg Kg}^{-1}$  ZnO-NPs treatments was observed in plants inoculated with *S. indica*. The effect of co-inoculation of *S. indica* with *S. meliloti* CAT activity was the same as *S. meliloti* if compared with the controls (non-inoculated) at  $400 \text{ mg Kg}^{-1}$  ZnO-NPs. However, at  $800 \text{ mg Kg}^{-1}$  ZnO-NPs, the lowest CAT activity was found for *S. meliloti*-inoculated plants. The activity of CAT in inoculated plants with *S. meliloti* decreased by 38.8% and 23.4% compared with *S. indica* and *S. meliloti+S. indica* treatments, respectively. The highest POD activity was found for *S. indica* colonized plants at the level of  $400 \text{ mg Kg}^{-1}$  ZnO-NPs, which was about 87.6% and 39.4% higher than *S. meliloti* and *S. indica+S. meliloti*, respectively. The POD activity was preferentially stimulated by *S. indica*, while *S. meliloti* and co-inoculation of the microbes produced similar effects on the POD activity at the highest level of Zn ( $800 \text{ mg Kg}^{-1}$  ZnO-NPs). However, no significant difference was detected between individual inoculation of *S. indica* and inoculation of this fungus with *S. meliloti* under such conditions.

## Discussion

Rhizosphere soil possess different chemical and biological characteristics compared to bulk soil which can alter heavy metals (HM) bioavailability and plant HM uptake (Motaghian and Hosseinpur 2013). The changes in chemical properties of rhizosphere compared to bulk soil, might be due to the changes in pH induced by plant exudates, microbial activity or a combination of these (Seshadri et al. 2015).

The results showed that the value of pH in post-harvest rhizosphere soils of both non-inoculated and inoculated plants with the microorganisms was slightly lower than the initial pH and the value in the bulk soil (Table 2).The lower pH of the rhizosphere compared to the bulk soil explains possible functions of the large proportion of root exudates (including sugars, amino acids, and organic acids) in attracting microbes to the root that facilitates plant-microbe interactions in the rhizosphere (Sasse et al. 2018). Under metal-stressed conditions, secretion of root exudates containing certain secondary metabolites into the rhizosphere is a detoxification strategy of the plants to tolerate and encounter HM toxicity (Bais et al. 2006). We observed a relationship between rhizosphere soil pH and Zn uptake by plant roots indicating that pH is a principal factor influencing the mobility and bioavailability of heavy metals (Pikuła and Stępień 2007). By comparing pH values in the rhizosphere soils of the inoculated plants, we demonstrated that *S. indica* inoculation alone or in combination with *S. meliloti* had a substantial influence on decreasing pH under normal and Zn stress conditions. One potential explanation is that *S. indica* inoculation greatly affected the organic acid exudation rate and the composition of root exudates. It has been reported that *S. indica* directly increases plant root biomass by producing indole-3-acetic acid (IAA), which increases the surface area for secretion of root exudates including organic acids in rhizosphere (Strehmel et al. 2016). Increased production of ethylene during root colonization by *S. indica* is another factor which contributes to localized soil acidification (Khatabi et al. 2012). The excessive uptake of cations ( $\text{NH}_4^+$ ) relative to anions ( $\text{NO}_3^-$ ) by the plants inoculated with *S. indica+S. meliloti* due to the increased levels of biologically fixed  $\text{N}_2$  might be another reason for rhizosphere

acidification (Hinsinger et al. 2003). Our results indicate the beneficial effects of synergistic interaction between *S. indica* and *S. meliloti* on alfalfa growth and nodulation. Compared with plants inoculated with *S. meliloti*, co-inoculation of this bacterium with *S. indica* increased the shoot dry weight by 18.33% and 8.05% at 400 and 800 mg Kg<sup>-1</sup>ZnO-NPs. Our results were consistent with the previous studies which confirmed the beneficial effect of simultaneous inoculation of endophytic fungi and *Rhizobium* on the nodulation, nitrogen fixation and growth of legumes (Hazarika et al. 2000; Li et al. 2020; Sampathkumar and Ganeshkumar 2003).

The amount of Zn accumulation in the root clearly implies that *S. indica* could effectively increase Zn bioavailability to the plant root. To prove the influence of *S. indica* on localized soil acidification and subsequently Zn bioavailability to the plant root, Zn concentration in plant roots was measured. The results indicated that at the highest level of ZnO-NPs level (800 mg kg<sup>-1</sup>), individual and co-inoculation of the endophytic fungus *S. indica* with *S. meliloti* significantly increased Zn concentration in roots when compared with plants colonized by *S. meliloti* alone. One explanation could be that tolerance mechanisms adopted by *S. indica*-inoculated plants to counteract excess Zn toxicity primarily include exclusion mechanisms that impede the entry of Zn ions to the root cells by secreting root exudates in the rhizosphere (Strehmel et al. 2016). In addition, the induction of siderophores synthesis by *S. meliloti* and *S. indica* in the presence of ZnO-NPs can increase the bioavailability of Zn (Sepehri and Khatabi 2020). Here, we showed that the ZnO-NPs toxicity in alfalfa can be modulated by endophytic fungus *S. indica*. The increased growth of plants inoculated with *S. indica* at 800 mg kg<sup>-1</sup>ZnO-NPs could be related to the ability of the fungus to produce siderophore which promotes plant growth and nutritional status particularly iron and at the same time enhanced Zn uptake through plant roots. The results also confirm our previous findings about *S. indica* role in successful survival and growth of alfalfa in cadmium contaminated soils (Sepehri and Khatabi 2020). Also, the high potential of *S. indica* to decrease Zn translocation from the root to shoot might also contribute to better performance of alfalfa under ZnO-NPs stress. Further studies are necessary to identify the precise mechanism by which *S. indica* takes up high levels of Zn into the root of the host plant. In addition to the effect of *S. indica* in decreasing pH of the studied rhizosphere, the density of fungal mycelium in rhizosphere zone can also play directly to the uptake and translocation of Zn to the host roots.

On exposure to ZnO-NPs, plants inoculated with *S. indica* displayed the highest content of chlorophyll a, chlorophyll b and carotenoids. The effect of *S. indica* to enhance plant tolerance to environmental stresses through improved photosynthesis has been well documented (Franken 2012). Several proteomics studies have indicated the significant up-regulation of the key enzymes of the Calvin cycle and the photosynthetic electron-transport chain in the leaves of *S. indica*-inoculated plants under different abiotic stresses (Alikhani et al. 2013; Ghabooli et al. 2013). This study showed the beneficial effect of *S. indica* on chlorophyll a, chlorophyll b and carotenoid contents of alfalfa grown in contaminated soils with ZnO-NPs. Improved nutritional status in plants inoculated with *S. indica* can lead to increase in biosynthesis of photosynthesis pigments to absorb light energy for photosynthesis (Gao et al. 2008; Gómez-Sagasti 2015; Sepehri and Khatabi 2020). Accumulation of heavy metals in plants will result in induced H<sub>2</sub>O<sub>2</sub> accumulation and lipid peroxidation which eventually cause oxidative stress (Tripathi, et al. 2012).

This study also revealed that Zn uptake in alfalfa induced oxidative stress as indicated by the H<sub>2</sub>O<sub>2</sub> and MDA accumulation data. The enhanced lipid peroxidation in the shoots of the control and inoculated plants with *S.*

*meliloti* exposed to 800 mg Kg<sup>-1</sup>ZnO-NPs may be linked to increased Zn contentin the shoots (Kumari et al. 2011). Low accumulation of MDA in inoculatedplants with individual or combination of *S. indica* and *S. meliloti* treatments clearly demonstrated that the studied microbial treatments could partially counteract ZnO-NPs stress. The MDA is mainly formed by the ROS-induced degradation of polyunsaturated lipids (Del Rio et al. 2005; Pryor and Stanley 1975). Therefore, a clear relation is provided between the observed tolerance of *S. indica*-colonized plant to ZnO-NPs and the ability of *S. indica* in inhibiting orretarding degradation of polyunsaturated lipids through preventing excess ROS formation (Sun et al. 2010).

Most abiotic stresses, such as heavy metals pollution can activate a common mechanism involving the production of ROS in plants (Dimkpa et al. 2012; Kim et al. 2009; Ma et al. 2015). The inhibition effects of HM can arise as secondary effects by regulating specific pathways such as antioxidant enzyme systems. The activity of Superoxide Dismutase (SOD) showed similar pattern in *S. indica*-colonized plants and plants inoculated with other microbial treatments under ZnO-NPs treatments (400 and 800 mg Kg<sup>-1</sup>). In other words, SOD activity showed no significant changes resulted from *S. indica* inoculation. This implies a decreased need in this antioxidant enzyme scavenging reactive oxygen species (ROS) in *S. indica*-colonized plants. So, we propose that probably the other detoxification related enzymes; CAT and POD are playing greater contribution to counteract oxidative stress in inoculated plants with *S. indica*. These results robustly demonstrated the importance of synergistic impacts of microbial inoculations in protecting alfalfa as the host plant against the negative effects of ZnO-NPs by increasing the activities of POD and CAT as a protective mechanism in scavenging ROS.

## Conclusions

The results indicated that ZnO-NPs affected photosynthesis and plant growth which were accompanied by a significant increase in Zn, H<sub>2</sub>O<sub>2</sub> and MDA accumulation. The selected strains of rhizosphere microorganisms especially the endophytic fungus *S. indica* contributed to plant protection from toxicity of ZnO-NPs. The greater root biomass of *S. indica*-colonized plants may be considered as an avoidance mechanism of plants grown in the soils polluted by ZnO-NPs, since it favors the retention of grater Zn ions in the root interface and thereby restricts the amount of Zn reaching the leaves. We observed a significant decrease of the rhizosphere soil pH upon root inoculation of alfalfa with *S. indica* either alone or in combination with *S. meliloti*. Therefore, soil acidification during root colonization by *S. indica* is another factor influences Zn uptake by plant roots. Furthermore, with regards to the Zn concentrations found in root tissue of *S. indica*-inoculated plants, simultaneously having high biomass production and tolerance against ZnO-NPs toxicity, we confirmed that *S. indica* serve as a highly effective approach in reducing the phyto availability of ZnO-NPs in the aerial parts of the host plants.

## Declarations

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## Compliance with Ethical Standards

**Conflict of Interest:** The authors declare that they have no conflict of interest.

**Ethics Approval:** This article does not contain any studies with human or animal subjects.

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**Authors' contribution:** LT performed the experiments, analyzed data and wrote the first draft of the manuscript. MS supervised the research, participated in its design and gave final approval of the version to be submitted. JY provided technical help in conducting the experiments as a scientific advisor. MZ and RGH-F served as scientific advisors and made a substantial contribution in interpretation of data. BK participated in setting-up the experiments and critically revised the manuscript. All authors read and approved the final version of the manuscript.

**Data Availability:** The datasets generated during the current study are available from the corresponding author on reasonable request.

**Consent to Participate:** Not applicable.

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## Tables

**Table 1** Some physical and chemical properties of the soil.

Soil texture	Sample location		PH	ECe	CEC	Zn	CCE	OC	Clay	Silt	Sand
	Longitude	Latitude	ds m <sup>-1</sup>	cmol <sup>+1</sup> kg <sup>-1</sup>	mg Kg <sup>-1</sup>	%					
Clay Loam	52° 46' E	29° 50' N	8.2	0.35	15	0.94	42.5	0.41	35.6	39	25.4

pH and EC<sub>e</sub> (electrical conductivity) in 1:2.5 soil/water (W/V) suspension, CEC (cation exchange capacity), CCE (calcium carbonate equivalent), OC (organic carbon), Zn (DTPA extractable).

**Table 2** Effect of microbial inoculation on pH values in rhizosphere and bulk Soil.

	ZnO NPs (mg kg <sup>-1</sup> )			ZnO NPs (mg kg <sup>-1</sup> )		
	0	400	800	0	400	800
	Bulk soil			Rhizosphere soil		
Control	8.22±0.01 ab	8.25±0.02 <sup>a</sup>	8.17±0.01 <sup>b,c</sup>	8.12±0.01 cd	8.09±0.01 de	8.07±0.01 def
<i>S.indica</i>	8.16±0.04 bc	8.21±0.02 <sup>ab</sup>	8.04±0.03 <sup>ef</sup>	7.66±0.04 g	7.64±0.01 g	7.61±0.01 gh
<i>S.meliloti</i>	8.19±0.02 ab	8.22±0.01 <sup>ab</sup>	8.15±0.01 <sup>b,c</sup>	8.06±0.02 def	8.03±0.03 ef	8.01±0.03 f
<i>S.indica + S.meliloti</i>	8.16±0.02 bc	8.19±0.01 <sup>ab</sup>	8.01±0.01 <sup>f</sup>	7.64±0.02 g	7.66±0.02 g	7.56±0.01 h

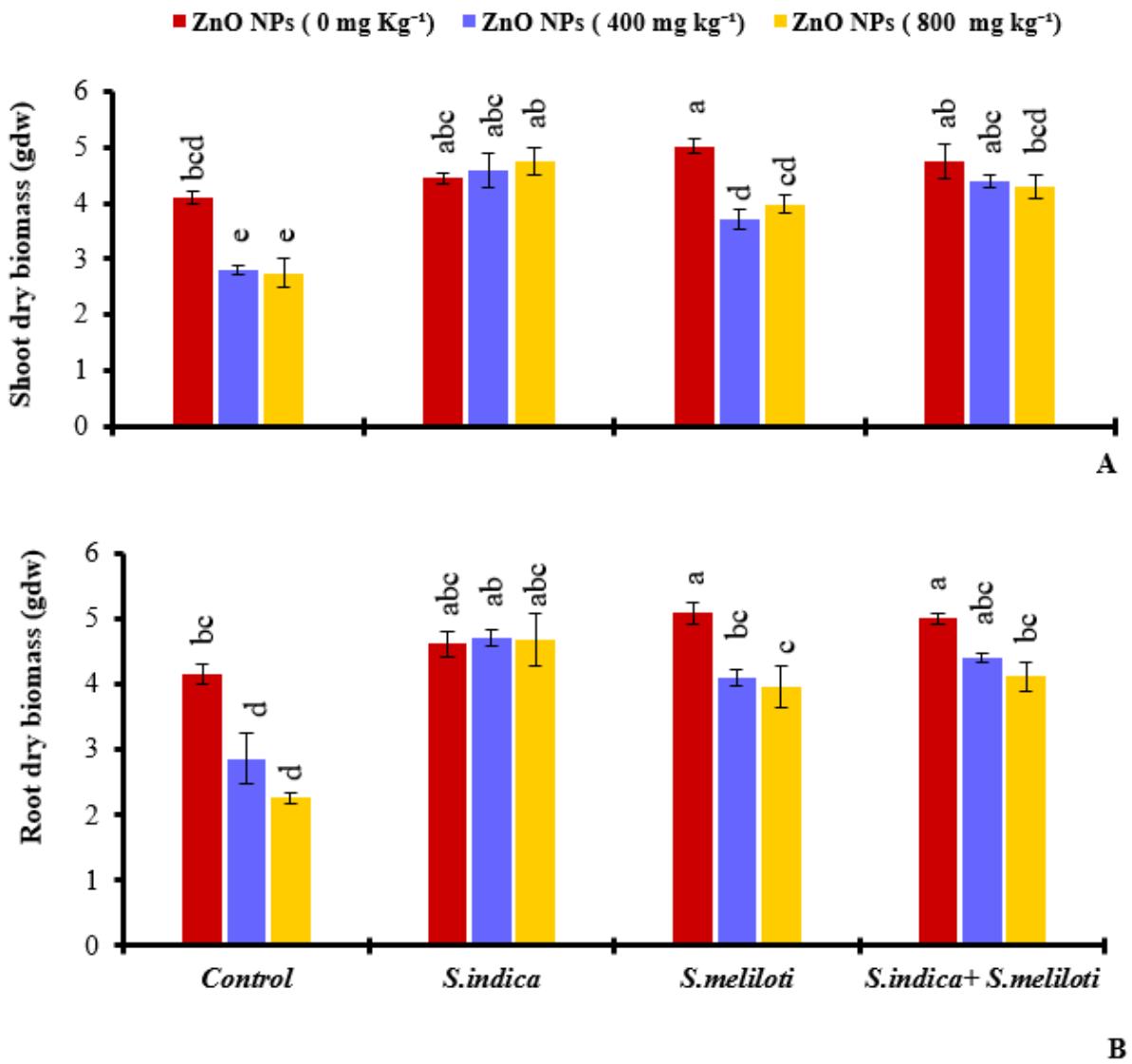
Values are expressed as means ± standard deviation (*n*=3), different *small letters* indicate significant difference at 0.05 level.

**Table 3** Effect of microbial inoculation on SOD, CAT and POD activities of alfalfa plant leaves grown under ZnO-NPs stress.

Microbe	ZnO-NPs (mg Kg⁻¹)		
	0	400	800
SOD activity (U mg⁻¹ FW)			
<i>control</i>	0.259±0.028 <sup>bcd</sup>	0.218±0.051 <sup>de</sup>	0.351±0.006 <sup>ab</sup>
<i>S.indica</i>	0.316±0.009 <sup>abc</sup>	0.310±0.024 <sup>abc</sup>	0.333±0.012 <sup>ab</sup>
<i>S.meliloti</i>	0.232±0.022 <sup>cde</sup>	0.282±0.057 <sup>abcd</sup>	0.358±0.011 <sup>a</sup>
<i>S.indica+ S.meliloti</i>	0.183±0.029 <sup>e</sup>	0.317±0.011 <sup>abc</sup>	0.3224±0.023 <sup>abc</sup>
CAT activity (U g⁻¹ FW)			
<i>control</i>	0.147±0.002 <sup>f</sup>	0.263±0.016 <sup>cde</sup>	0.226±0.034 <sup>e</sup>
<i>S.indica</i>	0.283±0.007 <sup>bcd</sup>	0.44 ±0.043 <sup>a</sup>	0.432±0.017 <sup>a</sup>
<i>S.meliloti</i>	0.245±0.018 <sup>de</sup>	0.326 ±0.028 <sup>bc</sup>	0.264±0.020 <sup>cde</sup>
<i>S.indica+ S.meliloti</i>	0.105±0.002 <sup>f</sup>	0.313±0.037 <sup>bcd</sup>	0.345 ±0.005 <sup>b</sup>
POD activity (U g⁻¹ FW)			
<i>control</i>	2.11±0.21 <sup>f</sup>	3.53±0.26 <sup>cd</sup>	3.53±0.35 <sup>cd</sup>
<i>S.indica</i>	4.36±0.61 <sup>bc</sup>	5.87±0.03 <sup>a</sup>	5.21±0.52 <sup>ab</sup>
<i>S.meliloti</i>	2.43±0.11 <sup>ef</sup>	3.13±0.11 <sup>de</sup>	3.81±0.35 <sup>cd</sup>
<i>S.indica+ S.meliloti</i>	3.66±0.13 <sup>cd</sup>	4.21±0.52 <sup>bc</sup>	4.31±0.07 <sup>bc</sup>

For each parameter, values are expressed as means ± standard deviation ( $n=3$ ), different *small letters* indicate significant difference at 0.05 level.

## Figures



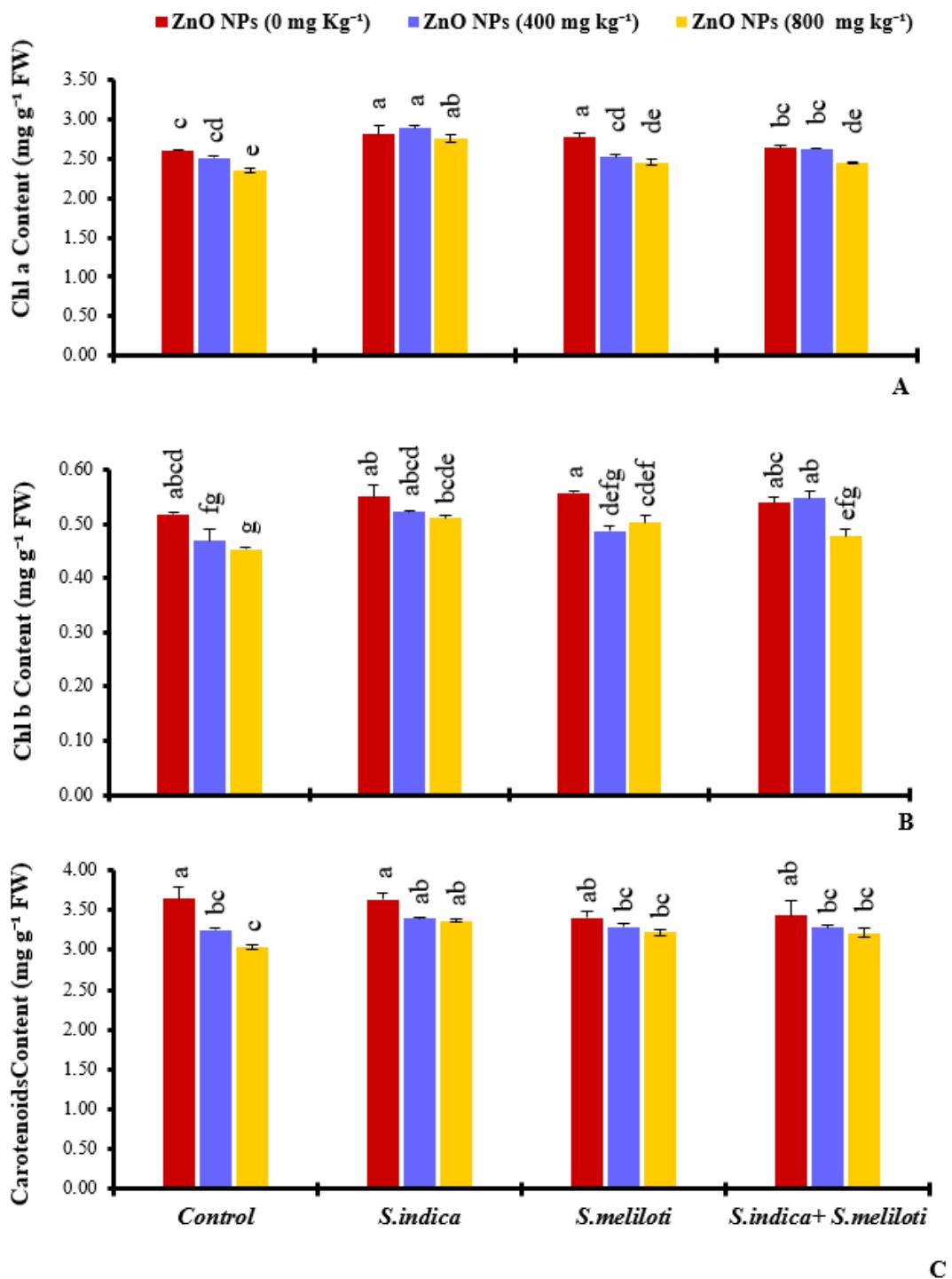
**Figure 1**

Effect of microbial inoculation on the shoot (A) and root(B) dry biomass of alfalfa plant grown under ZnO-NPs stress. Values are expressed as means standard deviation (n=3); different small letters indicate significant difference at 0.05 level.



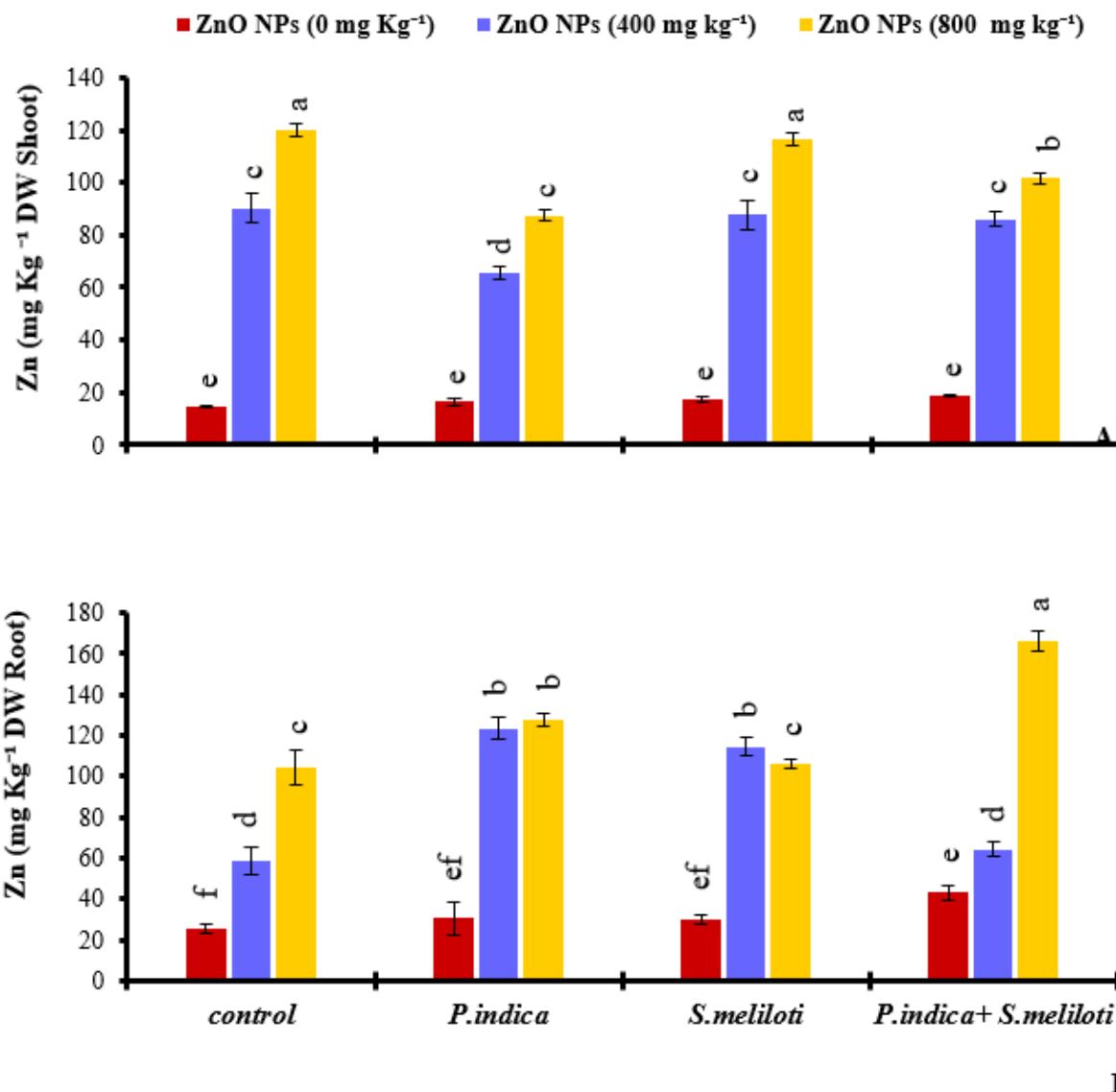
**Figure 2**

Effect of microbial inoculation on the shoot and root biomass of alfalfa plant exposed to 800 mg Kg-1 ZnO-NPs.



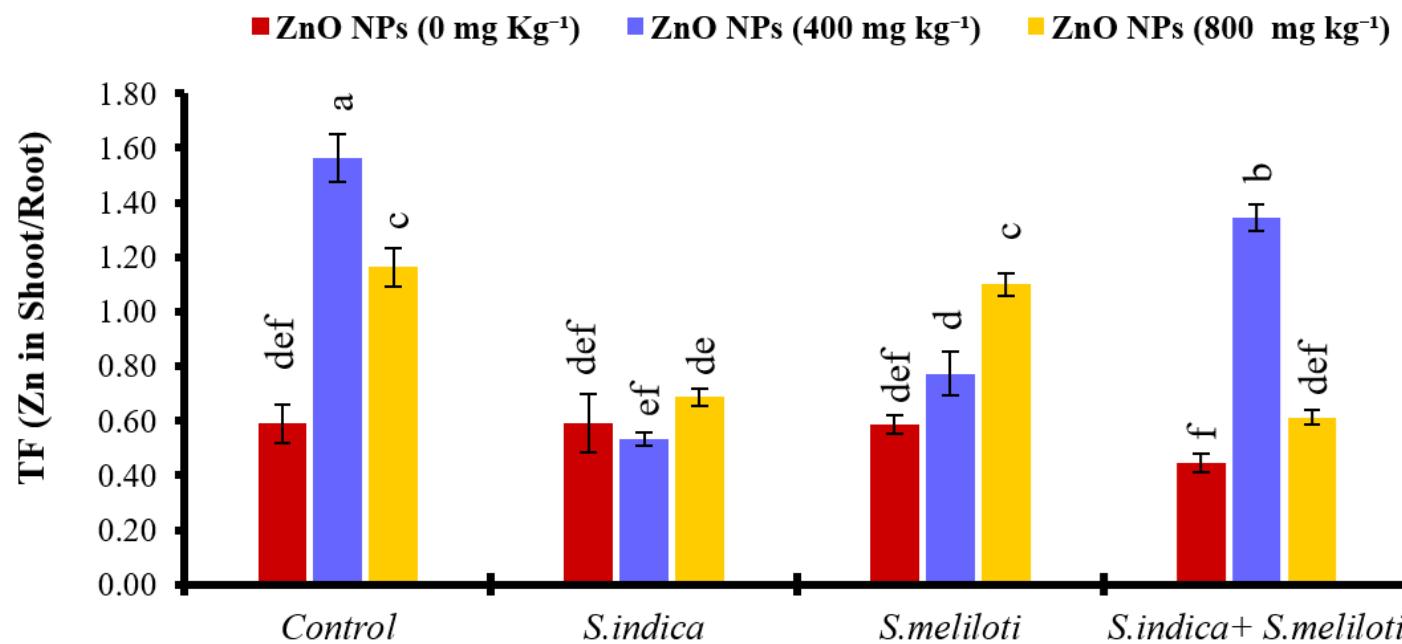
**Figure 3**

Effect of microbial inoculation on chlorophyll (chl) content a (A) and b (B) and carotenoids content (C) of alfalfa plant grown under ZnO-NPs stress. Values are expressed as means standard deviation (n=3); different small letters indicate significant difference at 0.05 level.



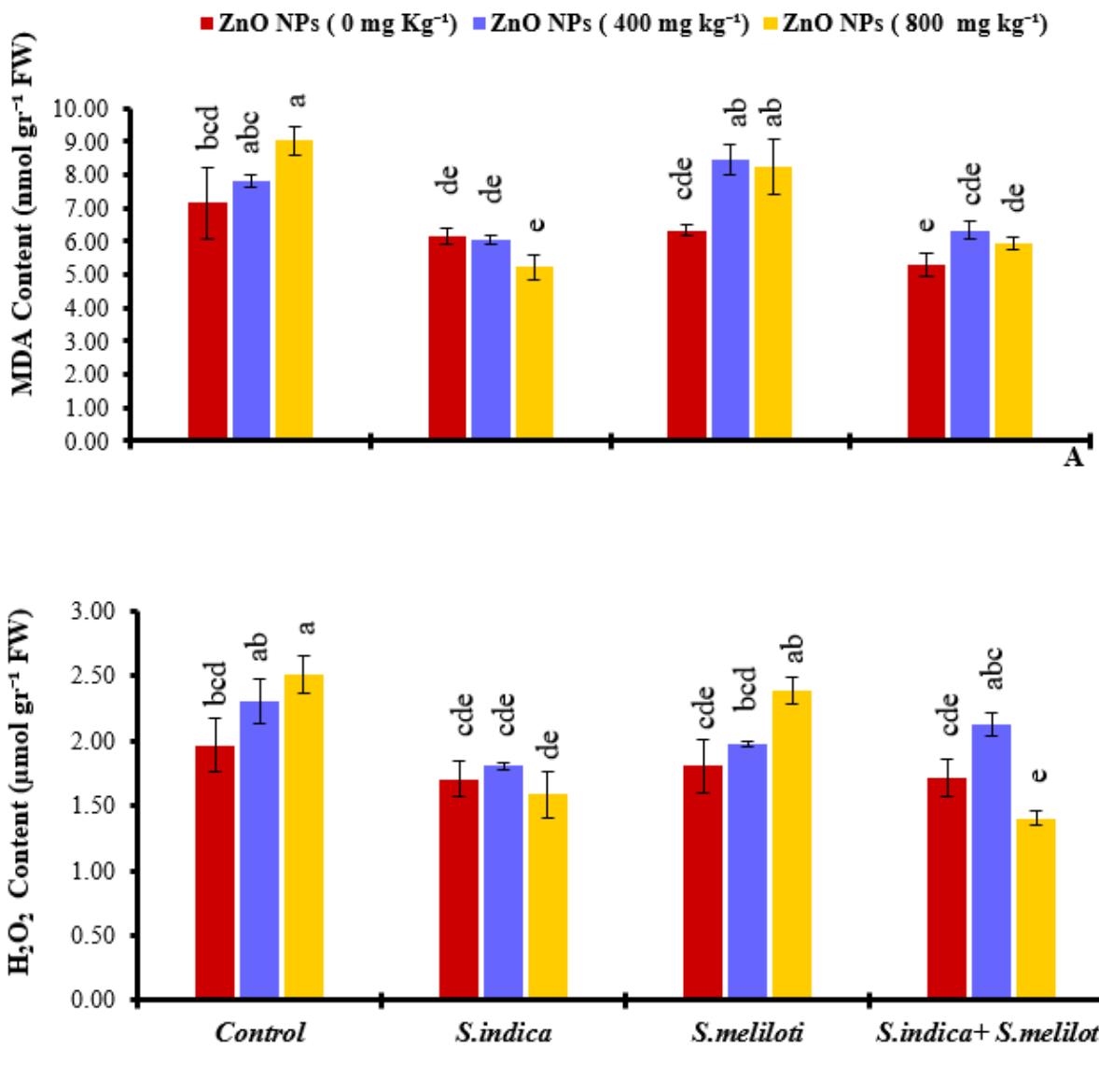
**Figure 4**

Effect of microbial inoculation on the concentration of Zn in the shoot (A) and root (B) of alfalfa plant grown under ZnO-NPs stress. Values are expressed as means standard deviation ( $n=3$ ), different small letters indicate significant difference at 0.05 level.



**Figure 5**

Effect of microbial inoculation on the translocation factor (TF ration Zn content in shoot to Zn content in root) of alfalfa plant grown under ZnO-NPs stress. Values are expressed as means standard deviation ( $n=3$ ); different small letters indicate significant difference at 0.05 level.



**Figure 6**

Effect of microbial inoculation on the Malondialdehyde (A) and hydrogen peroxide (B) contents of alfalfa plant leaves grown under ZnO-NPs stress. Values are expressed as means standard deviation (n=3); different small letters indicate significant difference at 0.05 level.

## Supplementary Files

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