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ORIGINAL PAPER

Positive effects of *Funneliformis mosseae* inoculation on reed seedlings under water and TiO_2 nanoparticles stresses

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Abstract

TiO_2 nanoparticles (TiO_2NPs) is one of the most widely used nanomaterials. Arbuscular mycorrhizal fungi (AMF) are an important and widely distributed group of soil microorganisms, which promote the absorption of nutrients by host plants and increase their tolerance to contaminants. However, the effects and mechanisms of AMF on plant TiO_2NPs tolerance in wetland habitats are not clear. In this experiment, under the conditions of three soil moisture contents (drought 50%, normal 70% and flooding 100%) and four TiO_2NPs concentrations (0, 100, 200 and 500 mg kg^{-1}), the effects of *Funneliformis mosseae* on the growth, antioxidant enzyme activities, osmotic substances and the absorption and accumulation of Ti in the *Phragmites australis* (reed) seedlings were studied. The results showed that the inoculation of *F. mosseae* under three moisture content conditions significantly increased the plant nutrition and root activities of reeds. Compared with the non-inoculated control, inoculation with *F. mosseae* increased the activities of antioxidant enzymes, the contents of chlorophyll, proline, soluble protein, and free amino acids, and significantly reduced the contents of malondialdehyde (MDA) and reactive oxygen species (ROS) of leaves. The accumulating ability of inoculated reeds to Ti was significantly higher than that of non-inoculated controls ($P < 0.05$), and inoculation of *F. mosseae* changed the distribution of Ti in reeds, increased the accumulation of Ti in roots. It's confirmed that inoculation of *F. mosseae* under three water conditions could improve the plant growth and nutrition, the activities of antioxidant enzymes, and enhance the reeds tolerance to TiO_2NPs in this study.

Keywords Arbuscular mycorrhizal fungi · TiO_2NPs · Reeds · Water stress · Wetland

Introduction

Since the 1970s, China has experienced dramatic environmental degradation, including water pollution, as a by-product of rapid economic development and industrialization (Ministry of Environmental Protection 2015). Pollutant discharge causes widespread organic pollution, toxic pollution, and eutrophication, along with severe ecological destruction. Metal nanoparticles have the dual effects of metal toxicity and nanotoxicity, and are more harmful to the environment, which have become one of the research hotspots of nanotoxicology (Zhang et al. 2013). TiO_2 nanoparticles (TiO_2NPs) have been widely used in many fields such as medical, food, cosmetics, wastewater treatment, etc. due to their dual affinity of oil and water, excellent UV absorption characteristics, efficient photocatalytic properties, and good resistance to photochemical corrosion and electrical conductivity (Wen and Wang 2017). In 2010, global TiO_2NPs production estimate has reached 88,000 tons per year (Keller and Lazareva 2014). In recent years, TiO_2NPs have been detected in urban surface waters with high concentrations. The concentration of Ti in the water of Quyang Wastewater Treatment Plant reached 768 μg L^{-1} (Liu et al. 2013, the threshold is 0.1 mg L^{-1} according to the GB 3838–2002), and the concentrations of TiO_2NPs in Arizona wastewater were 181 ~ 1233 μg L^{-1} (Westerhoff et al. 2011). Water pollution can cause damages to plants, such as negatively affect the nutrition absorption and growth, inhibited the photosynthesis and enzyme activities, etc., through the circulation of

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atmospheric airflow and agricultural irrigation water (Asli and Neumann 2009; Du et al. 2011; Nowack et al. 2012; Concha-Guerrero et al. 2014; Ruffini Castiglione et al. 2016; Zhang et al. 2018). In agricultural soils, the bioavailability of the different NPs often is not predictable, due to their tendency to aggregate, to adsorb/precipitate on solid phase, as well as to be coated by organic molecules (Pachapur et al. 2016). Many studies showed that high concentration of TiO_2NPs had negative effects on plant growth (Servin et al. 2012; Wen and Wang 2017). According to the results of Ghosh et al. (2010), 319 mg L^{-1} of TiO_2NPs increased the level of lipid peroxidation in onion (*Allium cepa* L.) roots, caused DNA damage and inhibited plant growth. Many plants have been found to have the ability to resist pollutions, and many wastewater treatment and water remediation technologies based on aquatic macrophyte have been developed, for example, constructed wetland. According to the reports, nanomaterials could adsorb on the surface and enter the interior of plants through the nano- or micro-scale openings on the surface of plants (Birbaum et al. 2010; Schreiber 2011). Wang et al. (2012) studied the transport process of nano-CuO in maize (*Zea mays* L.) seedlings, and revealed that the nanoparticles could transfer in xylem from root to stem utilizing transmission electron microscope (TEM) and energy dispersive spectroscopy (EDS), which lays a theoretical foundation for the solution of the wastewater polluted by nanomaterials. The remediation of nano-material contaminated water is an arduous project, there are relatively few data on the aquatic toxicological effects of nanomaterials, and there is no standard detection method to evaluate the potential effects of nanomaterials on their related ecological receptors.

The main mechanisms for the removal of pollutants in the aquatic plant system are assimilation and absorption of plants, the mineralization and oxidation of rhizosphere microorganisms and the purification of other mediators (such as adsorption and degradation, etc.). Moreover, there are synergism and interaction effects between these mechanisms (Yin et al. 2017). Arbuscular mycorrhizal fungi (AMF) are a class of ancient fungi that can form mycorrhiza with most of the plants on the earth. AMF have various positive effects on host plants, such as improving the host's N and P nutrition status (Yang et al. 2015), enhancing the host resilience, including heavy metal, salt, drought, disease, waterlogging and cold (Chen et al. 2015), and promoting the host's rhizosphere microbial activity and the stability of the host plant community structure (Zeng et al. 2017). However, current researches on mycorrhizal remediation of heavy metal mainly were focused on soil or sludge (Hu et al. 2013; Zu et al. 2015; Chen et al. 2017b), there are few reports on the application of AMF in purifying sewage in wetlands (Fester 2013; Twanabasu et al. 2013; Xu et al. 2018). The occurrence of this may be attributed to the limited oxygen in natural wetland soil, which will limit the survival of AMF in roots. However, more and more studies have shown that AMF is adaptable to aquatic environment (Kohout et al. 2012; Tisserant et al. 2013). Moreover, the previous study of our group showed that plants of 99 families living in 31 different wetland habitats had been found to be associated with AMF, even including submerged aquatic plants and several plant species that were thought to be non-mycorrhizal (Cyperaceae, Chenopodiaceae, and Plumbaginaceae) (Xu et al. 2016). Therefore, AMF have the potential to enhancing decontamination in wetland.

Phragmites australis, an emergent aquatic plant, have developed roots and large biomass, is one of the most common aquatic plant used in constructed wetland. *P. australis* can accumulate many kinds of heavy metals and is an excellent heavy metal tolerant plant (Vymazal and Březinová 2016; Bello et al. 2018). In addition, its well-developed aerenchyma can transport oxygen to the rhizosphere, thus creating favorable condition for the formation of AMF. Although the effects of TiO_2NPs on plants have been studied (Asli and Neumann 2009; Ghosh et al. 2010; Servin et al. 2012; Wen and Wang 2017), however, most of these reports are focused on plants, lacking the synergistic study of microorganisms, and the related mechanisms are less understood. In the present study, the effects of AMF on the removal of TiO_2NPs by *P. australis* were focused, and the effects of three factors (water conditions, *F. mosseae* and TiO_2NPs) and their interactions on plant growth were studied. The results will provide theoretical basis for the bioremediation of artificial nanoparticles, and also enrich the application field of AMF.

Materials and methods

Experimental materials

The reed rhizomes were collected from Hankou River Beach (Wuhan, China) (30°36′26.97″N, 114°18′38.00″E), and the seedlings were germinated in the laboratory. When the seedling height reached 5 cm, they were transplanted to the pots filled with *F. mosseae* inoculum, which consisting of roots, spores and hyphae, was obtained by trap culture with *Zea mays* L. *F. mosseae* (BGC XJ01A) spores were purchased from the Institute of Plant Nutrition and Resources, Beijing Academy of Agricultural and Forestry Sciences, China. TiO_2NPs (particle size of 60 nm, anatase, purity of 99.8%) was purchased from Shanghai Jianghu Co., Ltd. The substrate, mixture of vermiculite and standard sand (1:1 in volume), was passed through a 1.4 mm sieve, rinsed five times with tap water, and then sterilized at 180 °C for 2 h in an oven, and then taken out for 12 d before use. The plastic pots ($8.00\times6.00\times15.00$ cm) were soaked in 0.1% $KMnO_4$ solution, and washed by sterile water.

Experimental design

Treatments were factorial combinations of three factors: (1) inoculation with *F. mosseae* (FM) and autoclaved *F. mosseae* (NM), (2) three relative water content of substrates (100, 70 and 50%), and (3) four TiO_2NPs concentrations (0, 100, 200, and 500 mg kg^{-1}). The experiment had a randomized design with 24 treatments and three replicates. 400 g of the sterilized substrate was placed in each pot. Half of these pots (36) received 112 g *F. mosseae* inoculum, with the other half receiving 112 g autoclaved *F. mosseae* inoculum before planting. On August 1st, 2017, the seedlings with the same growth rate were selected and transplanted into the pots filled with different concentrations of TiO_2NPs, which was ultrasonicated for 30 min at 300 W using a cell pulverizer (JY92-II, Ningbo Xinzhi Biotechnology Co., Ltd. China) before application. The pots were placed in an artificial climate incubator (GHP-300, Shanghai Sanfa Technology Instrument Co., Ltd., China, Fig S1) for 60 days with the following condition parameters: 25 °C, 4450 lx light intensity, and 14 h light at day time; 20 °C, 0 lx light intensity, and 10 h dark time (Ge et al. 2008; Gu et al. 2010). The pots were weighed every day at 18:00 to keep water content constant, and modified Hoagland nutrient solution ($Ca(NO_3)_2$,945 mg L^{-1}, KNO_3 506 mg L^{-1}, NH_4NO_3 80 mg L^{-1}, $MgSO_4$ 493 mg L^{-1}, KH_2PO_4 136 mg L^{-1}, iron salt solution 2.5 ml L^{-1}, trace element solution 5 ml L^{-1}, pH 6.0) was added to all pots once every 5 days. At the end of the experiment, samples were collected to determine the relevant indicators.

Methods

After the end of the experiment, the reeds were harvested. The plant height, branch number and root length were measured firstly, then the aboveground parts and the roots were immediately washed by sterile water and weighed. Part of the roots were used to determine the root activity and mycorrhizal infection rate. Root activity was measured by the TTC (2,3,5-triphenyl tetrazolium chloride) method (Gao 2006). Root fragments were stained according to the modified method of Phillips and Hayman (1970), and the mycorrhizal infection rate was determined as the percentage of infected root segments in the total root segments. Part of the leaves were placed in an ice bath for the determination of relative water content, soluble protein, free amino acids, proline, ROS, and MDA, and the remaining leaves were transferred to a − 80 °C refrigerator for the determination of the activities of superoxide dismutase (SOD), catalase (CAT) and peroxidase (POD). The leaves were oven-dried at 105 °C for 30 min and 80 °C for 48 h, and then weighed. The relative water content of leaves was calculated as the ratio of dry weight to fresh weight. The concentrations of Chlorophyll and ROS were determined by spectrophotometry, and the concentrations of MDA were tested according to the thiobarbituric acid colouration method (Gao 2006). The activities of SOD, CAT and POD were determined using the nitrogen blue tetrazolium method (NBT), UV absorption method, and guaiacol method, respectively (Wang et al. 2003). The concentrations of proline and free amino acids were determined by ninhydrin method, and the soluble protein was measured with Coomassie Brilliant Blue G-250 (Li 2000). The total N and P concentrations of reeds were tested by Kjeldahl method and vanadium molybdenum yellow colorimetric method, respectively. The concentrations of Ti were determined by ICP-MS (Prodigy 7, Leemanlabsinc, USA).

Data analysis

All treatments were performed with three replicates, and the mean and standard deviation (Mean ± SD) were calculated from data of the three parallel groups. SigmaPlot V 10.0 (Systat Software, San Jose, CA) was used for mapping, SPSS 20.0 (SPSS Inc., Chicago, USA) and SAS version 6.1 (SAS Institute Inc., Cary, NC) were used to performed the differential significance analysis. A three-way analysis of variance (ANOVA) was carried out to test effects of main factors (inoculation of *F. mosseae*, soil moisture contents, and TiO_2NPs concentrations) and their interactions on the growth, antioxidant enzyme activities, osmotic substances and Ti accumulation of reed seedlings. Duncan's multiple range test at the 5% level of probability was used for post hoc comparison to separate treatment differences.

Results

The growth and nutritional status of reed seedlings under different treatment

The infection rates of reeds by *F. mosseae*

As shown in Fig. 1, the inoculated roots of reeds showed high infection rates. Without the stress of TiO_2NPs, the infection rates under drought and normal water conditions exceeded 50%, and reached 62.50% under drought stress. Under the same water condition, the *F. mosseae* infection rate was the highest with 0 mg kg^{-1} application of TiO_2NPs, and decreased with the increase of TiO_2NPs concentration.

Under the same TiO_2NPs concentration, the infection rates of *F. mosseae* under drought stress were significantly higher than those under normal and flooding state ($P < 0.05$), and the infection rates of *F. mosseae* were the lowest under flooding stress. The mycorrhizal infection rate of about 10%

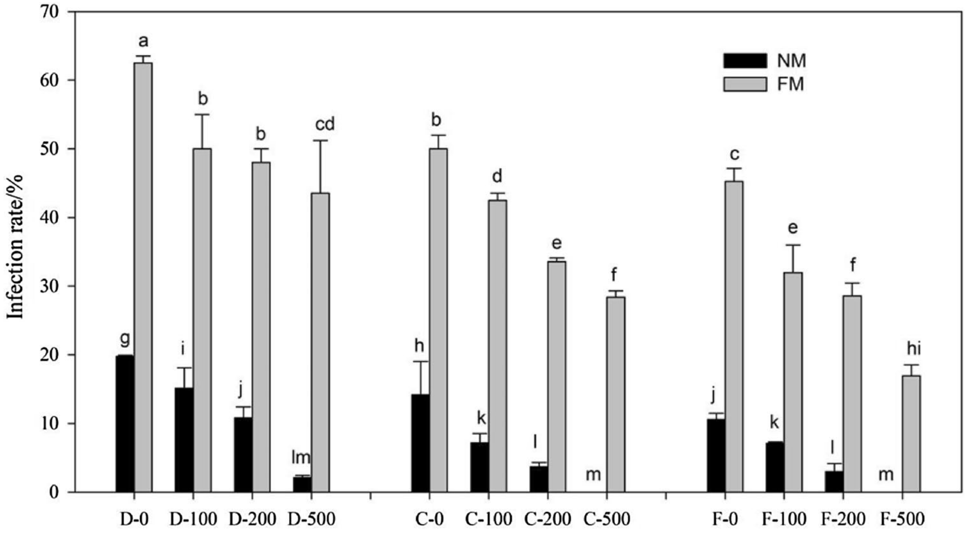


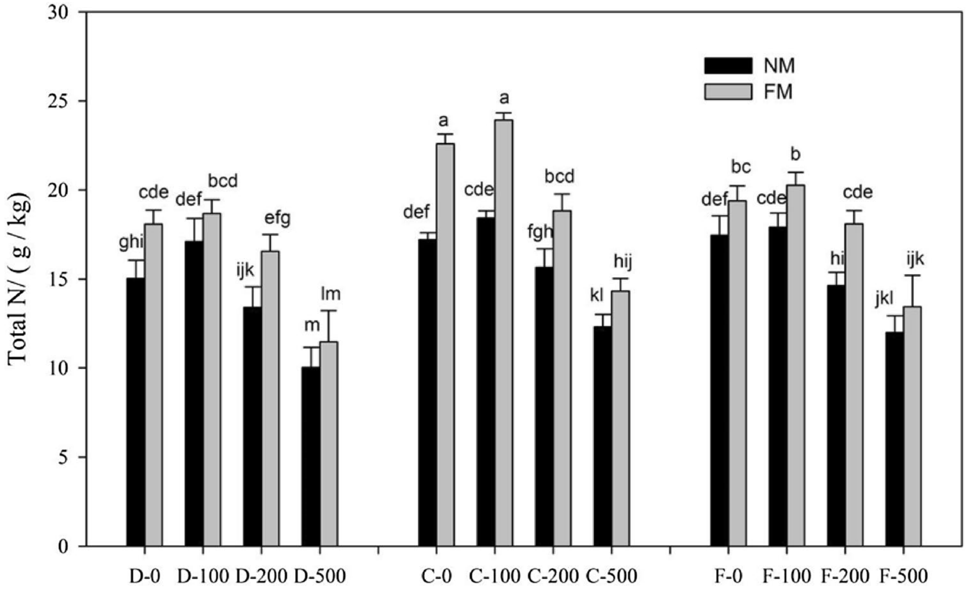
Fig. 1 Root infection rate of *F. mosseae* under different water conditions and TiO_2NPs concentration stress. D, C, and F represent drought stress, normal water, and flooding stress, respectively; 0, 100, 200, and 500 represent the concentration of applied TiO_2NPs; FM represents the treatments which inoculated with *F. mosseae*, and NM represents the treatments which inoculated with autoclaved *F. mosseae*. The data shown are the means and standard deviation from three replicate samples, letters show significant differences between treatments according to Duncan's multiple range test ($P < 0.05$)

of the non-inoculated seedlings should be caused by contamination of the spores in air or water.

The growth and nutrition of reed seedlings

As shown in Figs. 2 and 3, inoculation of *F. mosseae* promoted the reeds absorption of N and P. Under normal water and 0 mg kg^{-1} TiO_2NPs conditions, the total N concentration of the inoculated reeds was 1.31 times of the non-inoculated control. Under normal water and 200 mg kg^{-1} TiO_2NPs conditions, the total P concentration of the inoculated reeds was 1.10 times of the non-inoculated control. The N and P concentrations increased at low concentration (100 mg kg^{-1}) of applied TiO_2NPs and decreased at higher concentrations (200 and 500 mg kg^{-1}). Under the same concentration of TiO_2NPs, the N and P concentrations of the inoculated seedlings were significantly higher than the non-inoculated seedlings ($P < 0.05$), and the N and P concentrations under normal water condition were significantly higher than the drought and flooding stress conditions ($P < 0.05$).

Fig. 2 Effects of inoculation of *F. mosseae* on total N concentration under different water and TiO_2NPs concentration stress. D, C, and F represent drought stress, normal water, and flooding stress, respectively; 0, 100, 200, and 500 represent the concentration of applied TiO_2NPs; FM represents the treatments which inoculated with *F. mosseae*, and NM represents the treatments which inoculated with autoclaved *F. mosseae*. The data shown are the means and standard deviation from three replicate samples, letters show significant differences between treatments according to Duncan's multiple range test ($P < 0.05$)



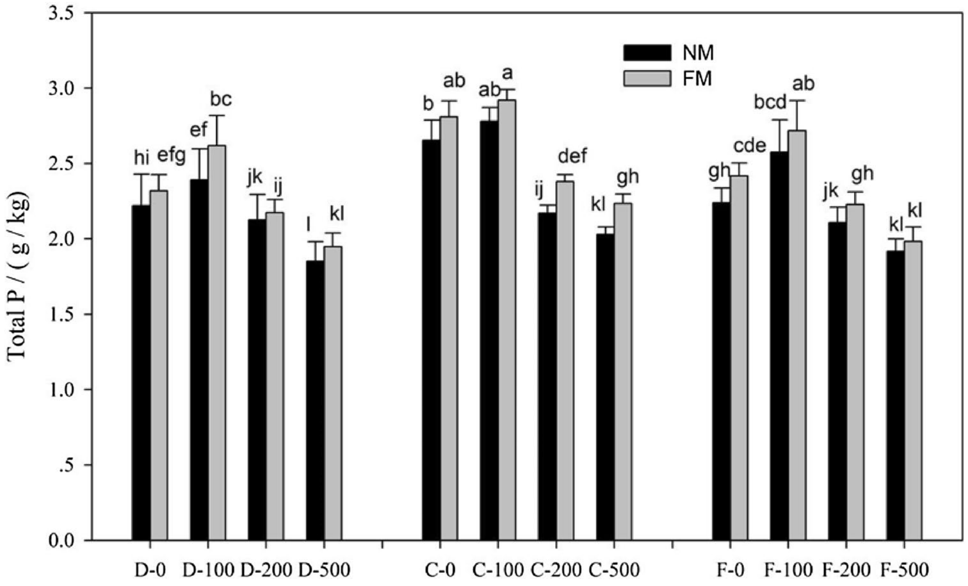


Fig. 3 Effects of inoculation of *F. mosseae* on total P concentration under different water and TiO_2NPs concentration stress. D, C, and F represent drought stress, normal water, and flooding stress, respectively; 0, 100, 200, and 500 represent the concentration of applied TiO_2NPs; FM represents the treatments which inoculated with *F. mosseae*, and NM represents the treatments which inoculated with autoclaved *F. mosseae*. The data shown are the means and standard deviation from three replicate samples, letters show significant differences between treatments according to Duncan's multiple range test ($P < 0.05$)

Table 1 Effects of *F. mosseae* on the growth of reeds under water stress and TiO_2NPs stress

Soil relative water content/%	TiO_2NPs Con./ mg kg^{-1}	Inoculation	Height/cm	Branch number	Root length/cm	Shoot fresh weight/g	Root fresh weight/g	Relative water content/%
30	0	NM	13.85 ± 0.42hi	5.67 ± 0.58hi	8.25 ± 0.21hi	0.62 ± 0.02ghi	0.25 ± 0.01hij	65.91 ± 2.27ij
		FM	21.17 ± 2.71cd	12.33 ± 0.58c	10.43 ± 0.29efg	0.75 ± 0.02def	0.36 ± 0.03ef	70.00 ± 1.25c
	100	NM	16.47 ± 0.65fgh	6.00 ± 0.00ghi	9.20 ± 0.10gh	0.68 ± 0.04 fg	0.31 ± 0.05fgh	67.33 ± 0.99hij
		FM	22.40 ± 2.13c	14.67 ± 0.58ab	12.00 ± 1.18cde	0.84 ± 0.02 cde	0.45 ± 0.02cd	71.00 ± 1.00ab
	200	NM	12.17 ± 0.96ijk	4.00 ± 0.00kl	7.43 ± 0.15i	0.55 ± 0.03ijk	0.16 ± 0.03 k	63.64 ± 3.03kl
		FM	14.93 ± 0.29ghi	6.00 ± 1.00ghi	9.23 ± 0.47gh	0.60 ± 0.02hij	0.34 ± 0.01 fg	69.00 ± 1.00hij
	500	NM	9.73 ± 1.37 k	3.33 ± 0.58 l	6.73 ± 0.61i	0.43 ± 0.06 l	0.15 ± 0.05 k	59.70 ± 2.99 l
		FM	12.35 ± 0.95ijk	4.67 ± 0.58jkl	7.33 ± 0.90i	0.49 ± 0.03jkl	0.19 ± 0.04jk	67.74 ± 2.15jkl
50	0	NM	20.63 ± 2.86 cd	6.00 ± 1.00ghi	9.43 ± 0.21gh	0.74 ± 0.01ef	0.35 ± 0.05ef	72.90 ± 0.93hij
		FM	27.58 ± 0.08b	15.00 ± 1.00ab	13.67 ± 1.29a	1.05 ± 0.06b	0.49 ± 0.03bc	76.67 ± 1.11ab
	100	NM	19.43 ± 0.74de	8.00 ± 1.00efg	11.30 ± 1.22def	0.89 ± 0.05c	0.44 ± 0.05 cd	74.84 ± 1.26efg
		FM	31.75 ± 0.78a	15.33 ± 1.53a	15.63 ± 1.71ab	1.26 ± 0.09a	0.58 ± 0.06a	78.00 ± 1.00a
	200	NM	14.20 ± 0.62ghi	5.67 ± 0.58hi	8.25 ± 0.32hi	0.52 ± 0.01jkl	0.31 ± 0.02fgh	71.08 ± 1.20ij
		FM	16.77 ± 0.67 fg	10.67 ± 1.15d	12.20 ± 1.30 cd	0.86 ± 0.02 cd	0.43 ± 0.01 cd	73.55 ± 1.24d
	500	NM	10.80 ± 1.84jk	5.33 ± 0.58ij	7.30 ± 0.10i	0.48 ± 0.02jkl	0.20 ± 0.03ijk	69.14 ± 1.23jk
		FM	12.57 ± 0.93ij	7.00 ± 1.00fgh	10.60 ± 0.89efg	0.75 ± 0.06def	0.33 ± 0.04 fg	72.44 ± 1.57ghi
70	0	NM	17.70 ± 3.96ef	7.33 ± 0.58fgh	8.33 ± 0.12hi	0.65 ± 0.04fgh	0.28 ± 0.01gh	73.47 ± 2.04fgh
		FM	25.75 ± 2.15b	13.67 ± 0.58bc	9.93 ± 0.67fgh	0.88 ± 0.20c	0.48 ± 0.09bc	76.62 ± 1.30bc
	100	NM	20.93 ± 1.17 cd	8.67 ± 1.53ef	9.43 ± 0.06gh	0.74 ± 0.01ef	0.32 ± 0.02 fg	75.89 ± 1.79ef
		FM	26.57 ± 2.75b	14.33 ± 0.58ab	13.17 ± 1.67bc	1.06 ± 0.15b	0.58 ± 0.06b	79.19 ± 0.98ab
	200	NM	14.97 ± 0.68ghi	5.33 ± 0.58ij	7.53 ± 0.31i	0.53 ± 0.03jkl	0.19 ± 0.01jk	70.00 ± 1.43jk
		FM	16.37 ± 1.00fgh	9.00 ± 1.00e	9.33 ± 0.32gh	0.68 ± 0.01 fg	0.40 ± 0.07de	72.08 ± 1.02e
	500	NM	10.17 ± 0.58jk	5.00 ± 1.00ij	6.83 ± 1.13i	0.45 ± 0.05 kl	0.18 ± 0.05k	68.24 ± 2.35jk
		FM	12.25 ± 0.21ijk	5.67 ± 0.58 hi	7.53 ± 0.32i	0.56 ± 0.07ijk	0.26 ± 0.05hi	70.23 ± 1.53ij

Different letters in the same column indicate significant differences ($P < 0.05$) assessed by Duncan's multiple range test, mean ± standard deviation

FM inoculated with *F. mosseae*, *NM* non-inoculated controls

As shown in Table 1, under the stresses of water and TiO_2NPs, the relative water content, plant height, root length, number of branches, and fresh weight of inoculated reeds were significantly higher than those of non-inoculated controls. Comparing with the non-inoculated plants, the inoculation of *F. mosseae* increased the relative water content by 1.99% ~ 8.04%, especially under drought and 500 mg kg^{-1} TiO_2NPs conditions with a promotion of 8.04%. The plant height of inoculated seedlings were 1.11 ~ 1.63 times of the non-inoculated seedlings. The root length of the inoculated seedlings were 1.09 ~ 1.48 times of the non-inoculated seedlings, the interaction between normal water and 100 mg kg^{-1} TiO_2NPs was the most significant (1.48, $P < 0.05$). The aboveground fresh weight of the inoculated reeds were 1.08 ~ 1.65 times of the non-inoculated controls, and the promotion effect was the most significant ($P < 0.05$) under normal water and 200 mg kg^{-1} TiO_2NPs conditions. The underground fresh weight of the inoculated seedlings were 1.25 ~ 2.09 times of the non-inoculated controls, and the interaction between the flooding and the 200 mg kg^{-1} TiO_2NPs was the most significant ($P < 0.05$).

Under the same concentration of TiO_2NPs, the plant height, root length, aboveground and underground fresh weight, relative water content and total N and P of the plants under flooding and drought conditions were lower than those under normal water condition. Under the same water condition, the plant height, root length, branch number, aboveground and underground fresh weight, total N and P concentrations significantly increased ($P < 0.05$) at low TiO_2NPs concentration (100 mg kg^{-1}), and then decreased under higher concentrations (200 and 500 mg kg^{-1}).

The root activity of reeds seedlings

Root activity is an important physiological indicator of roots. It was found that the root activities of the inoculated and non-inoculated reeds increased firstly and then decreased with the increase of TiO_2NPs concentration under different water conditions (Fig. S2). 100 mg kg^{-1} of TiO_2NPs caused a significant increase in root activity ($P < 0.05$), indicating that proper TiO_2NPs stress was beneficial to the growth and nutrient uptake of reed seedlings. Under the stress of higher concentration of TiO_2NPs (200 and 500 mg kg^{-1}), the root activities of the inoculated and non-inoculated reeds decreased significantly. The root activities of inoculated seedlings were higher than the non-inoculated seedling, which indicated that *F. mosseae* could increase the root activity of plants under the stress of TiO_2NPs.

The chlorophyll a and b contents of reed seedlings under different treatments

As shown in Table S1, no matter whether inoculated or not, the contents of chlorophyll a and chlorophyll b changed in the same trend, low concentration of TiO_2NPs (100 mg kg^{-1}) significantly increased the contents of chlorophyll a and chlorophyll b under three water conditions ($P < 0.05$), while high concentrations of TiO_2NPs (200 and 500 mg kg^{-1}) significantly decreased chlorophyll contents. Compared with the non-inoculated seedlings, the chlorophyll a and chlorophyll b contents of inoculated reed leaves were higher under different water conditions and TiO_2NPs stress, and the promotion effect of *F. mosseae* was more significant under high concentration of TiO_2NPs. Under the interaction of drought and 500 mg kg^{-1} of TiO_2NPs, the chlorophyll a and chlorophyll b contents of inoculated reed leaves were 2.59 and 2.56 times of the non-inoculated reeds, respectively, 2.31 and 3.04 times under the interaction of normal water and 500 mg kg^{-1} of TiO_2NPs, respectively. The increase of chlorophyll content is conducive to the absorption and transformation of photosynthesis and the enhancement of plant carbon oxidation. Therefore, *F. mosseae* inoculation can effectively improve their host' photosynthesis efficiency.

The antioxidant enzyme activities in leaves of reeds seedling under different treatment

Figure 4a–c showed that inoculation with *F. mosseae* increased SOD, CAT and POD activities in leaves under different water conditions and TiO_2NPs concentrations, compared with the non-inoculated controls. The differences of SOD, CAT and POD activities between inoculated and non-inoculated reeds were significant ($P < 0.05$). Under drought, normal and flooding conditions, the SOD and POD activities of inoculated reeds significantly increased under 100 mg kg^{-1} TiO_2NPs stress, the SOD activities of inoculated reeds were 1.72, 1.86 and 1.94 times of the non-inoculated controls, respectively, and the POD activities of inoculated reeds were 1.68, 1.25 and 1.49 times of the non-inoculated controls, respectively. Under the same concentration of TiO_2NPs, the SOD and POD activities of reed leaves under drought and flooding conditions were significantly higher than those under normal water condition ($P < 0.05$), indicated that reeds were stressed under drought and flooding conditions and produced a large number of MDA and ROS, which promoted the synthesis of SOD and POD to eliminate excessive oxygen free radicals. Under drought condition, the CAT activities of inoculated reed leaves was 1.45, 2.11, 4.03 and 12.91 times of the non-inoculated ones under the four levels of TiO_2NPs stress, respectively ($P < 0.05$). Under the interaction of drought and 500 mg kg^{-1} of TiO_2NPs stress, the CAT activity of inoculated reeds increased significantly,

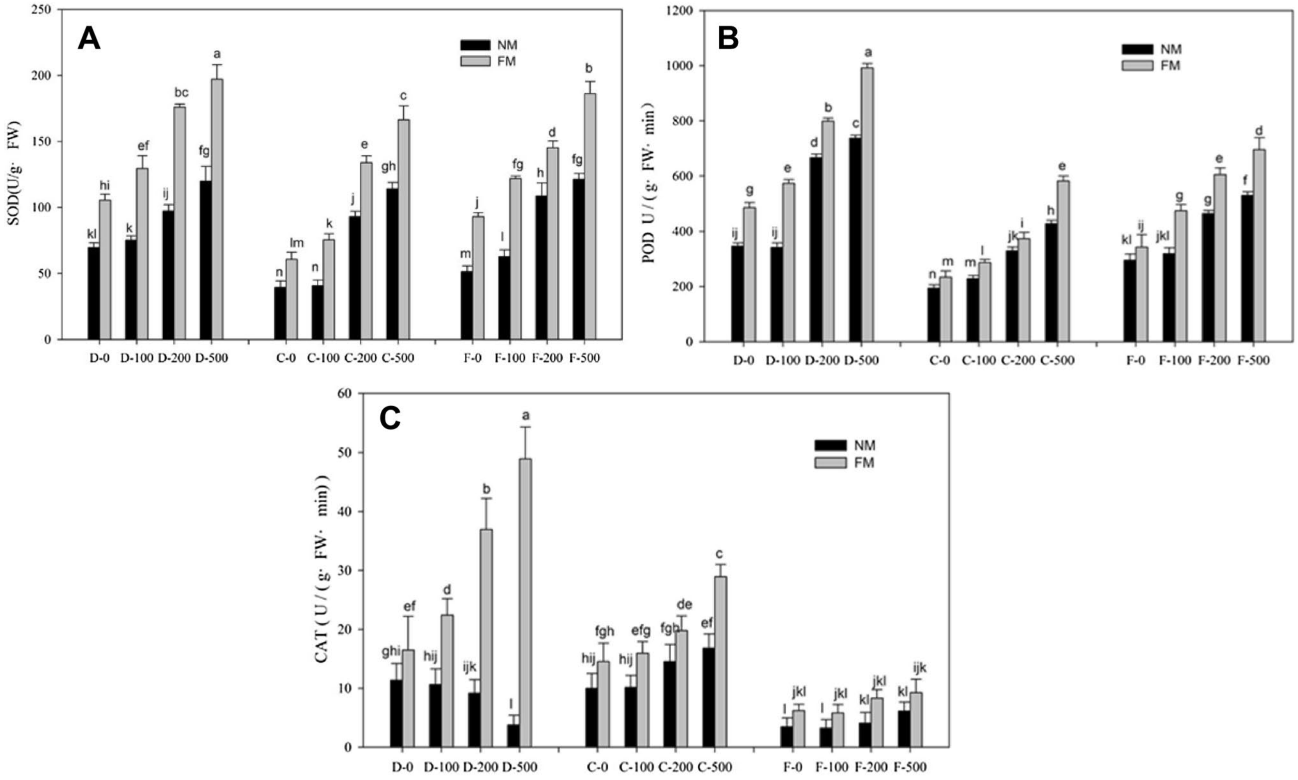


Fig. 4 SOD (**a**), POD (**b**), CAT (**c**) activities of inoculated and non-inoculated reeds under different water conditions and TiO_2NPs concentrations. D, C, and F represent drought stress, normal water, and flooding stress, respectively; 0, 100, 200, and 500 represent the concentration of applied TiO_2NPs; FM represents the treatments which inoculated with *F. mosseae*, and NM represents the treatments which inoculated with autoclaved *F. mosseae*. The data shown are the means and standard deviation from three replicate samples, letters show significant differences between treatments according to Duncan's multiple range test ($P < 0.05$)

which was 12.91 times of the non-inoculated control. As shown in Fig. 4c, no matter inoculated with *F. mosseae* or not, CAT activities were significantly lower under flooding condition than drought and normal water conditions ($P < 0.05$).

The contents of osmotic substances, ROS and MDA in reed leaves under different treatment

As shown in Table 2, the MDA and ROS contents of inoculated and non-inoculated reeds under flooding and drought stresses were significantly higher than those under normal water condition ($P < 0.05$), and the MDA and ROS contents were the highest under drought stress. Under the same water condition, the contents of MDA and ROS increased with the concentration of TiO_2NPs applied.

In the present study, *F. mosseae* inoculation significantly decreased the contents of MDA and ROS ($P < 0.05$). The ROS contents of inoculated reeds decreased by 2.04% (under drought and 500 mg kg^{-1} TiO_2NPs conditions) ~ 48.86% (under normal and 100 mg kg^{-1} TiO_2NPs conditions) compared with those of non-inoculated reeds. The MDA contents of inoculated reeds decreased by 37.63% ~ 67.01% compared with those of non-inoculated controls, and the MDA contents decreased as much as > 60% under water flooding stress.

According to the results of Table 2, under different water conditions, the contents of soluble protein, proline and free amino acid in leaves were significantly ($P < 0.05$) increased by inoculation of *F. mosseae* and as much as 1.37~3.85, 1.02~1.63 and 1.24~3.79 times of the non-inoculated controls, respectively. The contents of three osmotic regulators significantly decreased with the increase of substrate water content ($P < 0.05$). Under the same water condition, proline and free amino acid contents increased significantly with the increase TiO_2NPs concentration ($P < 0.05$). The soluble protein content decreased with the increase of TiO_2NPs concentration.

The distribution of Ti in the reed seedlings under different treatment

As shown in Fig. 5, with the increase of TiO_2NPs concentration in the substrate, the Ti accumulation in the roots and shoots increased gradually regardless of inoculation or not. *F. mosseae* inoculation increased the uptake of

Table 2 Contents of osmotic substances, ROS and MDA in leaves of different treated reeds

Soil relative water content/%	TiO_2NPs Con./mg kg^{-1}	Inoculation	ROS/(μg g^{-1} FW)	MDA/(μmol g^{-1} FW)	Proline/(μg g^{-1} FW)	Soluble protein/(mg g^{-1} FW)	Free amino acid/(mg g^{-1}· FW)
30	0	NM	30.19 ± 1.09fgh	16.44 ± 0.59fg	106.13 ± 3.67lm	34.50 ± 5.00hi	6.80 ± 0.33 m
		FM	25.38 ± 2.70hij	9.48 ± 0.30jk	209.66 ± 7.05h	98.09 ± 1.74a	17.82 ± 0.34ef
	100	NM	39.92 ± 2.13d	18.74 ± 0.54e	149.46 ± 8.40j	41.60 ± 2.35gh	10.26 ± 0.20i
		FM	30.16 ± 2.01fgh	11.69 ± 0.29i	233.64 ± 9.37fg	98.74 ± 2.30a	23.53 ± 0.53c
	200	NM	57.04 ± 5.92b	27.53 ± 1.26c	190.66 ± 8.07i	16.86 ± 5.81jk	12.42 ± 0.16 h
		FM	53.04 ± 2.41bc	14.92 ± 0.32gh	336.75 ± 17.53cd	85.31 ± 7.56bcd	41.42 ± 0.67a
	500	NM	69.97 ± 3.64a	38.13 ± 1.39a	264.97 ± 14.09e	15.55 ± 2.78kl	17.76 ± 0.69ef
		FM	68.55 ± 4.23a	16.47 ± 0.32fg	492.27 ± 16.37a	75.34 ± 4.77def	42.03 ± 1.08a
50	0	NM	20.91 ± 3.48j	10.60 ± 1.47ij	94.60 ± 3.18m	27.84 ± 5.68ij	3.70 ± 0.29p
		FM	11.57 ± 3.29k	6.12 ± 0.47lmn	96.37 ± 8.58m	90.38 ± 11.43abc	14.71 ± 0.72 g
	100	NM	26.86 ± 2.05hij	15.41 ± 1.18gh	123.28 ± 5.35kl	29.61 ± 3.07ij	5.32 ± 0.26n
		FM	13.74 ± 3.48k	7.03 ± 0.37lm	170.87 ± 6.72m	95.19 ± 9.62ab	17.25 ± 0.45f
	200	NM	31.43 ± 2.40efg	19.49 ± 1.42e	153.10 ± 8.96j	24.01 ± 2.648jk	8.72 ± 0.24jk
		FM	27.84 ± 3.62ghi	9.63 ± 0.58jk	323.77 ± 9.90d	83.39 ± 10.56cde	22.60 ± 0.53d
	500	NM	50.01 ± 2.65c	26.26 ± 2.15c	240.39 ± 13.01f	20.27 ± 3.28jkl	9.29 ± 0.39j
		FM	31.86 ± 4.45ef	9.96 ± 0.65ij	401.49 ± 15.48b	75.00 ± 10.87def	31.57 ± 0.74b
70	0	NM	26.44 ± 4.48hij	14.05 ± 2.07h	67.09 ± 3.52n	23.79 ± 3.01jk	2.29 ± 0.12q
		FM	22.82 ± 2.69ij	5.13 ± 0.24n	69.01 ± 2.16n	74.25 ± 3.37ef	10.97 ± 0.23i
	100	NM	33.79 ± 1.22e	17.82 ± 0.75ef	95.62 ± 3.74m	20.75 ± 2.83jkl	4.49 ± 0.11o
		FM	24.93 ± 3.05hij	5.88 ± 0.38mn	103.28 ± 13.19m	89.50 ± 5.00abc	15.05 ± 0.41 g
	200	NM	42.14 ± 2.11d	21.25 ± 0.48d	110.76 ± 6.95lm	15.84 ± 3.56kl	7.48 ± 0.19 lm
		FM	29.33 ± 2.79fgh	7.89 ± 0.15kl	218.11 ± 13.44gh	67.83 ± 2.89f	17.93 ± 0.40ef
	500	NM	55.09 ± 2.19bc	29.54 ± 2.16b	130.70 ± 6.60k	11.75 ± 3.04l	8.10 ± 0.37kl
		FM	40.37 ± 5.47d	11.75 ± 0.24i	343.31 ± 10.96c	46.83 ± 7.94g	18.18 ± 0.27e

Different letters in the same column indicate significant differences ($P < 0.05$) assessed by Duncan's multiple range test, mean ± standard deviation

FM inoculated with *F. mosseae*, *NM* non-inoculated controls

Ti by reeds. Under the stress of 100 mg kg^{-1} TiO_2NPs, the amounts of Ti uptake were not significantly different between the inoculation and the non-inoculation reeds. However, under the stress of 200 and 500 mg kg^{-1} TiO_2NPs, the inoculation of *F. mosseae* significantly improved the absorption of Ti by reeds. Under the interaction of flooding and 500 mg kg^{-1} TiO_2NPs, the Ti accumulation in the inoculated reeds was 1.67 times of the non-inoculated control. Meanwhile, it was found that *F. mosseae* inoculation significantly increased Ti accumulation in the root of hosts under all the three water conditions. *F. mosseae* inoculation significantly reduced Ti transport coefficient (Ti content ratio between shoot and root) in reeds ($P < 0.05$), promoted more Ti accumulate in the reed roots and inhibited the Ti transferred to shoot. Therefore, inoculation with *F. mosseae* not only increased the total uptake of Ti, but also changed the distribution of Ti in reeds, increased the accumulation of Ti in roots and weakened the toxicity of Ti to host.

The interaction effects of water stress, TiO_2NPs stress and *F. mosseae* on reed seedlings

According to the results of Table 3, that water stress, TiO_2NPs stress and *F. mosseae* inoculation have significant effects on reed growth (reed height, branch number, fresh shoot weight, fresh root weight, chlorophyll), antioxidant enzyme activities (SOD, POD and CAT), osmotic adjustment substances (proline, soluble protein and free amino acid), root characteristics (root length, infection rate and root activity) and reed nutrient (above- and below-ground total N and total P).

There was no significant interaction between water stress and *F. mosseae* on reed height, but significant effects on other indices ($P < 0.001$). There was no significant interaction between *F. mosseae* and TiO_2NPs stress on root length and leaf relative water content, but significant effects on shoot fresh weight and ROS content ($P < 0.05$), and other indices ($P < 0.001$). Water stress and TiO_2NPs stress had no

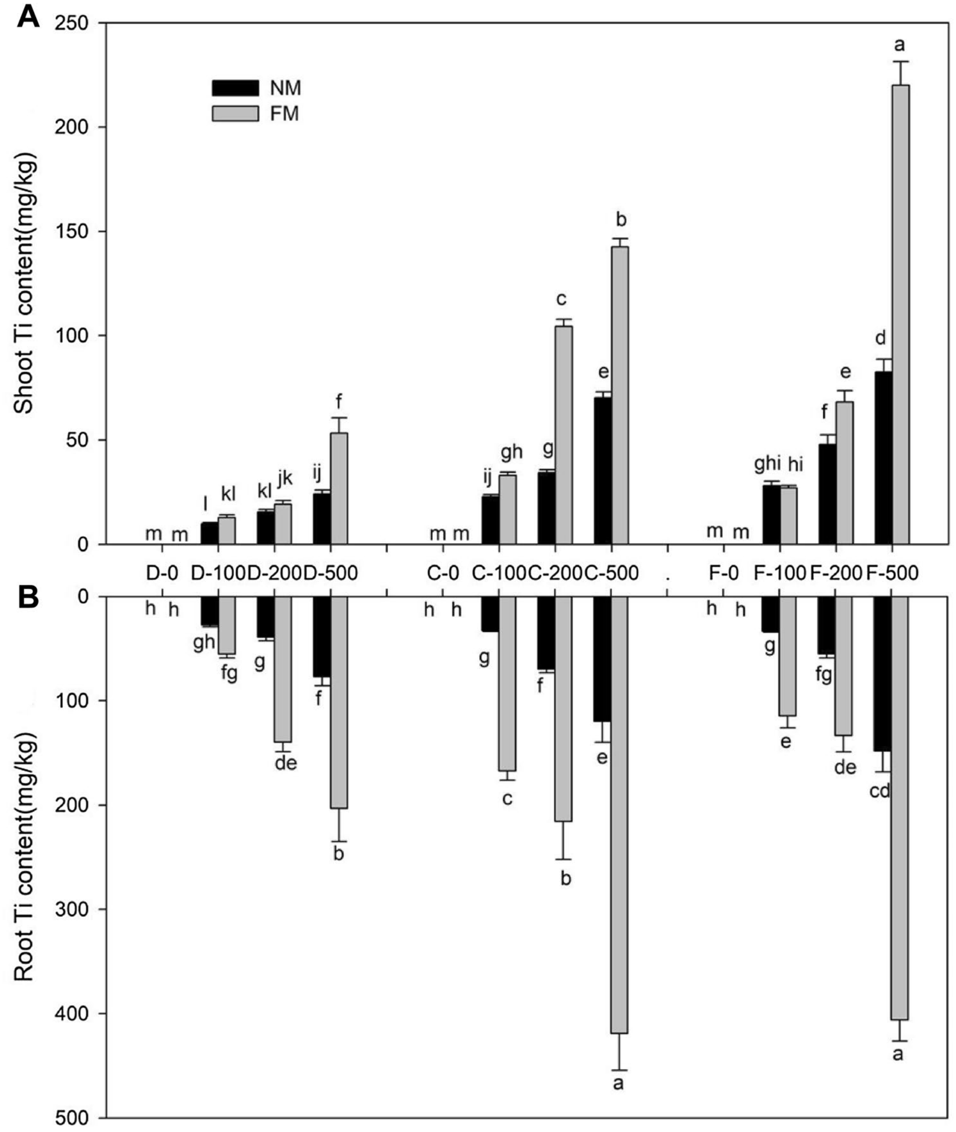


Fig. 5 Ti accumulation in above- (**a**) and below- (**b**) ground of inoculated and non-inoculated reeds under water stress and TiO_2NPs stress. D, C, and F represent drought stress, normal water, and flooding stress, respectively; 0, 100, 200, and 500 represent the concentration of applied TiO_2NPs; FM represents the treatments which inoculated with *F. mosseae*, and NM represents the treatments which inoculated with autoclaved *F. mosseae*. The data shown are the means and standard deviation from three replicate samples, letters show significant differences between treatments according to Duncan's multiple range test ($P < 0.05$)

significant interaction on branch, root length, underground fresh weight, leaf relative water content and soluble protein, but had significant influences on shoot fresh weight and other indices. *F. mosseae*, water stress and TiO_2NPs stress had no significant interaction on root length, above- and below-ground fresh weight and relative water content of leaves, but had significant effects on reed height, branch, soluble protein ($P < 0.05$), and other indices ($P < 0.001$).

Discussion

Studies have shown that *F. mosseae* can form a good symbiotic relationship with reed roots. The infection rate of *F. mosseae* in the present study decreased significantly with the increase of soil water content, which was consistent with the result of the negative correlation between *F. mosseae* infection rate and water depth in the study of Miller (2000), while Wirsel (2004) indicated that permanent flooding could even eliminate the association. This might be due to an increase in the moisture content of the matrix, resulted in a decrease in the oxygen content of the matrix, which limited the symbiosis of *F. mosseae* with reeds (Cooke et al. 1993; Peat and Fitter 1993). However, it's not always the case. Ipsilantis and Sylvia (2007) found flooding had no adverse effect on mycorrhizal root colonization. The degree of AMF colonization of plant living in wetland habitats varied with plant species. Root colonization of AMF in different mangrove species (*Avicennia marina*, *Aegiceras corniculatum*, *Sonneratia caseolaris*, and *S. apetala*) at Futian Mangrove area ranged from 10 to 20%, however, in other mangrove species (*Bruguiera gymnorhiza*, *Acanthus ilicifolius*, and *Kandelia candel*) ranged from 50 to 80% (Wang et al. 2010). As concluded in our previous study, mycorrhizal status in

Table 3 Three-way analysis of variance of water stress, TiO$_2$NPs stress and inoculation with *F. mosseae* on the growth of reed seedlings

Indicators	AMF (F value)	W (F value)	C (F value)	AMF × W (F value)	AMF × C (F value)	W × C (F value)	AMF × W × C (F value)
Height	195.54***	40.84***	231.77***	1.79^{NS}	19.84***	6.25***	2.73*
Branch number	593.65***	38.37***	196.86***	6.06**	56.39***	1.59^{NS}	2.84*
Root length	127.19***	39.32***	48.59***	8.73**	2.37^{NS}	1.30^{NS}	1.64^{NS}
Shoot fresh weight	186.06***	58.73***	125.83***	18.53***	3.59*	2.78*	0.53^{NS}
Root fresh weight	283.52***	63.19***	124.45***	3.50*	5.45**	0.80^{NS}	1.69^{NS}
Relative water content of leaves	90.94***	128.81***	54.07***	4.40*	0.44^{NS}	1.68^{NS}	1.04^{NS}
Total Chl content	6581.12***	756.00***	3921.36***	132.05***	130.47***	55.68***	65.44***
SOD activity	1103.95***	146.46***	616.22***	21.80***	19.12***	6.57***	4.69**
POD activity	724.59***	1165.26***	1064.31***	47.80***	27.36***	44.56***	3.78**
CAT activity	214.10***	162.30***	54.45***	102.18***	44.75***	7.36***	15.58***
Proline content	1811.60***	670.39***	1546.76***	71.76***	324.70***	22.56***	18.88***
Soluble protein	1746.97***	44.62***	48.53***	4.85*	4.39**	1.51^{NS}	2.61*
Free amino acid	17,841.97***	3425.16***	2367.35***	614.30***	375.30***	224.53***	162.33***
MDA content	2254.17***	236.70***	438.51***	15.61***	108.99***	9.58***	5.22***
ROS content	125.82***	226.96***	292.84***	5.82**	3.10*	14.38***	3.59**
Shoot Ti content	1103.49***	896.54***	1515.19***	131.39***	366.09***	226.82***	109.73***
Root Ti content	813.87***	95.94***	381.69***	42.09***	84.91***	23.03***	8.09***
Total N	132.39***	46.17***	159.19***	6.07**	3.74*	1.08^{NS}	1.60^{NS}
Total P	21.99***	34.33***	95.23***	0.40^{NS}	0.13^{NS}	2.44*	0.36^{NS}

NS not significant, *AMF* represents inoculation of *F. mosseae*, *W* represents water stress, *C* represents TiO$_2$NPs stress

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$

wetland habitats depends not only on the plants (mainly the degree of aerenchyma and the number of lateral roots), the AMF (different tolerance to oxygen-deficient environment by concentrating oxygen in the root or rhizosphere), and the influences of flooding on root morphology, anatomy and physiology (e.g., continuous flooding might lead to a progressive decrease of the roots available for AMF colonization by both diminishing lateral roots formation and widening the area occupied by the aerenchyma), but also on the environmental and edaphic factors, such as oxygen concentration, pH, temperature and so on, that affect the AMF growth (Ban et al. 2017).

In the present study, drought and flooding stress significantly inhibited the growth of reeds ($P < 0.05$). At the same time, under three water stress conditions without TiO$_2$NPs application, compared with non-inoculated reeds, *F. mosseae* inoculation significantly increased root activity, total N and P contents in the aboveground and underground of the plant ($P < 0.05$), chlorophyll content and photosynthetic efficiency of the leaves ($P < 0.05$), thus promoted the growth of *P. australis*, which were consistent with the inoculation effects of *F. mosseae* on other plants (Smith and Read 2010; Xie et al. 2014; Cao et al. 2017).

The effects of TiO$_2$NPs on plant growth, nutrient uptake, antioxidant enzymes, chlorophyll and osmotic regulators in leaves are very significant ($P < 0.001$). Under the same water condition, TiO$_2$NPs had a promotion effect at low concentration (100 mg kg^{-1}) and an inhibition effect at higher concentrations (200 and 500 mg kg^{-1}) on the growth of reeds. It was speculated that this was due to its unique photocatalytic effect, low concentration of TiO$_2$NPs promoted the synthesis of chlorophyll and enhanced photosynthesis, which resulted in the promotion effect on reed growth. Studies have also shown that TiO$_2$NPs can promote the synthesis of chlorophyll in *Solanum lycopersicum* and *Arabidopsis thaliana* leaves (Tiwari et al. 2017; Ze et al. 2011). However, in this study, high concentration of TiO$_2$NPs inhibited the plant growth, which is consistent with the results of Song et al. (2012). Lyu et al. (2017) also reviewed that Ti could improve plant activity at low concentrations, and posed toxicity to plants at high concentrations. < 100 mg L^{-1} Ti did not have significant toxicity to willows (Seeger et al. 2009). Wen and Wang (2017) reported that reeds showed slower or even stagnant growth, leaf chlorosis, plant wilting or death under high concentrations of TiO$_2$NPs stress (200 and 500 mg L^{-1}). High concentration of TiO$_2$NPs can induce the production of ROS in cells, leading to lipid peroxidation damage, hindering photosynthesis and ultimately affecting leaf growth (Lan et al. 2018). Wen and Wang (2017) also reported that TiO$_2$NPs significantly caused lipid peroxidation in the reeds to produce a large amount of MDA, which had toxic effects on reeds. Therefore, when ROS exceeds a certain amount, enzymes such as SOD cannot eliminate a large amount of active oxygen and oxygen radicals, leading cell membrane peroxidation and producing MDA, which may cause damage to the membrane system and organelles.

In this study, inoculation of *F. mosseae* significantly promoted the N, P contents, increased SOD, CAT and POD activities and reduced the accumulation of ROS and MDA production in the cells of reed ($P < 0.001$), comparing with the non-inoculated reed seedlings, indicating that inoculation of *F. mosseae* alleviated the damage of reed caused by TiO_2NPs stress and improved reed resistance. Wang et al. (2016) found AMF inoculation alleviated ZnONPs induced phytotoxicity to maize (*Zea mays* L.) by increasing mineral nutrients and antioxidant capacity at high doses (> 800 mg kg^{-1}). Wang et al. (2018) found AMF inoculation had positive effects on the growth, and P nutrition of *Sorghum bicolor* under ZnONPs stress. When exposed 500 μg/g ZnONPs, AMF inoculation increased shoot and root biomass of *Trigonella foenum-gracum* (Siani et al. 2017). According to the above reports, AMF can promote host growth and nutrition not only under high concentration of TiO_2NPs stress, but also ZnONPs stress.

At the same TiO_2NPs concentration, ROS and MDA contents under drought and flooding stress were significantly higher than those under normal water stress, and the activities of POD and SOD under drought and flooding conditions were higher than normal water condition. However, under the flooding condition, the activity of CAT enzyme was significantly lower than drought and normal water conditions. It was inferred that excessive ROS in reeds exceeded the scavenging capacity of enzymes and destroyed the enzyme synthesis system under flooding and TiO_2NPs conditions, resulting in a significant decrease in CAT activities (Wang et al. 2011).

The effects of inoculation of *F. mosseae* on leaf proline content were different. Zhang et al. (2014) found the inoculated plants maintained lower proline content than the non-inoculated plants throughout the growth period. However, Pollastri et al. (2018) found that, after inoculation with *F. mosseae*, some changes in plant metabolism occurred, including a significant increase in proline accumulation. In the present study, the contents of free amino acids and proline in reed leaves were significantly increased by inoculating *F. mosseae* under different conditions, indicating the accumulation of these osmotic regulators in leaves decreased the osmotic potential of cells, assisted the cells or tissues to hold water and prevent dehydration, maintain the normal metabolic activity of the cells, facilitated the absorption of water from the substrate to the cells, and thus improved the stress resistance of plants (Zhao et al. 2017). The soluble protein content decreased with the increase of TiO_2NPs concentration, which might attribute to inhibition of N absorption by root or inactivation of related enzyme under the stress of high concentration of TiO_2NPs.

In this study, the Ti contents in the aboveground and underground parts of the reeds inoculated with *F. mosseae* were significantly higher than those of the non-inoculated reeds ($P < 0.05$), this might be due to the inoculation of *F. mosseae* promoted the growth of reeds by increasing the absorption of nutrients such as N and P, and expanding the root range of plants and the absorption area of roots (Miransari 2013; Chen et al. 2017a). *F. mosseae* inoculation significantly reduced Ti transport coefficient in reeds ($P < 0.05$), accumulated more Ti in the roots and inhibited Ti transformation to the above-ground parts. In accordance with our results, Wang et al. (2016) also reported AMF inoculation (*Glomus versiforme and Glomus caledonium*) decreased Zn partitioning to shoots of maize, at high ZnONPs doses (800 mg kg^{-1}). However, AMF inoculation have variable effects on metal nanoparticles uptake and translocation. Noori et al. (2017) reported mycorrhizal (*Rhizophagus intraradices*) and non-mycorrhizal *S. lycopersicum* exposed to 36 mg kg^{-1} of 2-nm Ag-NPs accumulated 1300 and 1600 μg/g Ag in their tissues, respectively, mycorrhizal plants accumulated 14% less Ag compared to non-mycorrhizal plants. AMF inoculation (*R. intraradices*) ameliorated inhibitory effects of ZnONPs to *T. foenum-gracum* by promoting secretion of glycoprotein called glomalin (a potent metal chelator) within the rhizosphere, which significantly reduced (by almost half) Zn uptake by root and subsequent translocation to the shoot (Siani et al. 2017). In addition, Wang et al. (2018) found AMF inoculation (*F. caledonium*) significantly decreased Zn concentrations in *S. bicolor* shoots and did not significantly affect root Zn concentrations independently of ZnONPs addition levels, compared with non-inoculated plants. Therefore, the effects of AMF on the uptake and translocation of metal nanoparticles depended on AMF species, plant species, and soil properties.

Whether inoculated or not, only a small amount of underground Ti could be transferred to the shoots in this study. When the heavy metals in the substrate reached the toxic level, AMF could reduce the transfer of heavy metals to the ground by secreting certain substances and binding excess heavy metals to the mycorrhiza, or directly interact with heavy metals to change their bioavailability and toxic effects on plant growth (Vodnik et al. 2008; Yuan et al. 2013; Zhang et al. 2017; Liu et al. 2018). On the other hand, Larue et al. (2012) reported that the maximum diameter of TiO_2NPs that could accumulate in wheat roots was less than 140 nm, and if the initial diameter of TiO_2NPs was less than 36 nm, they would be transferred to the leaves. The anatase type TiO_2NPs used in the present study has a particle diameter of 60 nm, therefore, it was inferred that the TiO_2NPs used was difficult to transfer to the aboveground part of reeds.

The accumulated TiO_2NPs in the plant roots will be removed from the local environment with the harvest of the plant.

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Compliance with ethical standards

Conflict of interest The authors declare that there is no conflict of interest.

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