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Effects of zinc oxide nanoparticles on the growth, photosynthetic traits, and antioxidative enzymes in tomato plants

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Abstract

With the dramatic increase in nanotechnologies, it has become probable that biological systems will be exposed to excess of nanoparticles (NPs). However, the impact of NPs on plants remains to be explored. The aim of this research was to determine the effects of ZnO NPs on tomato (*Solanum lycopersicum* L.) plants. Plant growth, photosynthetic characteristics, chlorophyll fluorescence parameters, and activities of antioxidative enzymes were measured in 35-d-old plants. The ZnO NP treatments significantly inhibited tomato root and shoot growth, decreased the content of chlorophylls *a* and *b*, and reduced photosynthetic efficiency and some other chlorophyll fluorescence parameters in a concentration-dependent manner. However, the supernatant of ZnO NP suspensions did not affect growth of tomato, despite the presence of small amounts of Zn^{2+}. Taken together, these results suggest that toxic effects on tomato plants were from ZnO NPs, not from Zn^{2+} released into the solution; toxicity was likely caused by reduced chlorophyll content and damaged photochemical system, which in turn limited photosynthesis and led to the reduction in biomass accumulation. Also, ZnO NPs enhanced the transcription of genes related to antioxidant capacity, suggesting that ZnO NPs could enhance the defence response by increasing activities of antioxidant enzymes.

Additional key words: carotenoids, catalase, chlorophyll content, chlorophyll fluorescence, net photosynthetic rate, stomatal conductance, *Solanum lycopersicum*, transpiration rate.

Introduction

Nanoparticles (NPs) are unique materials with a characteristic dimension from 1 to 100 nm and remarkable structural and physicochemical characterristics. Due to their physical, chemical, optical, and biomedical properties (Nel *et al*. 2006), they found applications in medicine, biology, electronics, and engineering. Nanoscale materials can be divided into a number of different compound classes: metal oxides such as Fe_3O_4, Fe_2O_3, and TiO_2; clays, usually including montmorillonite, hydroxyapatite, kaolin; zero-valent metals such as iron, silver, and gold; carbonaceous nanomaterials; and semiconductor materials, including quantum dots, *etc*. (Klaine *et al*. 2008).

Zinc oxide NPs are one of the most commonly used metal oxide NPs with a range of applications in sunscreens and other personal care products, electrodes, biosensors, photocatalysts, and solar cells (Szabó *et al*. 2003). However, inappropriate handling, incidental

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Abbreviations: APX - ascorbate peroxidase; Car - carotenoids; CAT - catalase; Chl - chlorophyll; c_i - intercellular CO_2 concentration; E - transpiration rate; ETR - apparent electron transport rate; F_m - maximum fluorescence; F_v - variable fluorescence; F_v/F_m - maximum efficiency of PS II photochemistry; g_s - leaf stomatal conductance; NPs - nanoparticles; P_N - net photosynthetic rate; PS - photosystem; qP - photochemical quenching; qPCR - quantitative PCR; ROS - reactive oxygen species; SOD - superoxide dismutase; Φ_{PSII} - quantum yield of PS II photochemistry.
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and/or accidental release of NPs could result in environmental contamination (Dimkpa *et al*. 2012). This raises the immediate need to assess potential toxicological impacts of NPs on human health and the ecosystem (Nel *et al*. 2006, Bandyopadhyay *et al*. 2012a,b). Plants are the essential components of all ecosystems and they closely interact with their surrounding environment containing NPs. However, it is largely unknown whether any toxicity results from exposure to NPs or from released ions (Parsons *et al*. 2010).

Lin and Xing (2008) and Lin *et al*. (2009) demonstrated that NPs can enter into plant roots *via* the apoplastic pathway and they are transported to shoots through the vascular system. Thus, their uptake depends on the plant anatomy and composition, shape, and size of NPs. The uptake of ZnO NPs has been reported in *Lolium perenne* (Lin and Xing 2008), copper NPs in *Phaseolus radiatus* and *Triticum aestivum* (Lee *et al*. 2008), multiwalled carbon nanotubes in *Oryza sativa* (Lin *et al*. 2009) and *T. aestivum* (Wild and Jones 2009), and CdSe/ZnS quantum dots in *Poa annua* (Whiteside *et al*. 2009). Plants can accumulate metals in different cellular compartments such as vacuoles, nuclei, and cell wall (Schwab *et al*. 2016), and they can affect various physiological and biochemical processes (Lin and Xing 2007, 2008, Zhao *et al*. 2013a,b, Mukherjee *et al*. 2014a,b).

The ZnO NPs adversely affect plant growth of ryegrass (Lin and Xing 2007, 2008), maize (Lin and Xing 2007, Zhao *et al*. 2012), zucchini (Stampoulis *et al*. 2009), *Arabidopsis* (Lee *et al*. 2010), wheat (Dimkpa *et al*. 2012), rice (Boonyanitipong *et al*. 2011), buckwheat (Lee *et al*. 2013), cowpea (Wang *et al*. 2013), cucumber (Zhao *et al*. 2013b), duckweed (Thwala *et al*. 2013), soybean (Yoon *et al*. 2014), green pea (Mukherjee *et al*. 2014a,b), rape (Mousavi Kouhi *et al*. 2014), and alfalfa (Bandyopadhyay *et al*. 2015) in a dose dependent manner. For example, Lin and Xing (2007, 2008) reported that ZnO NPs affected root elongation in ryegrass, radish, and rape. Transcriptomics in *Arabidopsis* has shown that most of the genes induced by ZnO NPs occur in ontology groups annotated as stress response, including both abiotic and biotic stimuli (Landa *et al*. 2012).

The effect of NPs accumulation on plant metabolism and development depends on their size, concentration, and surface chemistry, as well as the chemical milieu of the subcellular sites to which the NPs are deposited (Dietz and Herth 2011). However, the mechanisms of possible phytotoxicity remain unknown. Metal oxide NPs promote the generation of reactive oxygen species (ROS), which are indicators of NP phytotoxicity (Choi and Hu 2008). ROS are key factors inducing DNA damage (Mehrabi and Wilson 2007), can be signaling molecules of abiotic and biotic stresses (Takahashi *et al*. 2011), and can also control programmed cell death (Gechev and Hille 2005). More severe consequences, such as genotoxicity, may occur when NPs enter cells (Karlsson 2010). Oxidative stress results when the balance between ROS production and antioxidant defence is disturbed (Gajewska and Skłodowska 2007). Plants have special mechanisms to remove or inactivate ROS, and plant cells possess an antioxidant system consisting of both nonenzymatic and enzymatic antioxidants, which include catalase (CAT), superoxide dismutase (SOD), and ascorbate peroxidase (APX).

Tomato (*Solanum lycopersicum* L.) was selected as the experimental species because it is very popular vegetable in the world, and it is commonly used in studies on the phytotoxicity of NPs (Ma *et al*. 2010). The aim of this study was to investigate the effects of ZnO NPs on tomato growth, photosynthetic traits, and antioxidative enzymes.

Materials and methods

Plants and treatments: Tomato (*Solanum lycopersicum* L.) cv. Moneymaker was used for the experiments. Seeds were sown in 17-cm diameter plastic pots containing 2.5 kg of washed sand. After germination, extra seedlings were removed to ensure that every pot contained four plants. The seedlings were sufficiently watered with Hoagland nutrient solution and evaporation was compensated by distilled water every two days. All pots were placed outdoor and sheltered from rain. Temperatures during the experiment were 24 - 28 °C in the daytime and 17 - 20 °C at night. The relative humidity was 50 - 60 % and plants were subjected to natural irradiance.

The pots with three-week-old seedlings were moistened thoroughly with suspensions containing 0, 200, 400, or 800 mg dm^{-3} ZnO NPs (*Sigma-Aldrich*, Saint Louis, MO, USA). The supernatant, obtained by centrifuging 800 mg dm^{-3} ZnO NP suspensions at 3 700 *g* for 10 min and filtered through 0.22 μm filters, was used to investigate the contribution of Zn^{2+} released from ZnO NPs to the observed phytotoxicity. The content of Zn in plant tissues was analyzed by inductively coupled plasma optical emission spectrometer (*ICP-OES*, *Prodigy*, *Leeman*, Hudson, NH, USA) at the wavelength of 213.9 nm.

Five-week-old plants were harvested and washed thoroughly with tap water and then with distilled water. After removing excess water with paper towels, the roots and shoots were separated, and fresh mass recorded. The samples were then oven-dried at 80 °C for 15 min, followed by vacuum-drying at 40 °C to a constant mass before dry mass was recorded.

Measurement of photosynthetic parameters: Net photosynthetic rate (P_N), leaf stomatal conductance (g_s), intercellular CO_2 concentration (c_i), and transpiration rate (E) of the fully expanded fifth rosette leaves of 5-week-old plants were measured using a portable gas-exchange system *LI-6400* (*LI-COR*, Lincoln, NE, USA) in the morning before the plants were harvested. An irradiance was 1 000 μmol m^{-2} s^{-1}, an ambient CO_2 concentration was 360 ± 10 μmol mol^{-1}, an air temperature was 22 °C, and a relative humidity about 50 %. A total of four leaves per pot were measured, and measurements were repeated four times for each leaf.

Chlorophyll fluorescence was measured using a *LI-6400* system with the *6400-40* leaf chamber fluorometer. Seedlings were kept in darkness for 30 min before recording the fluorescence of the same blades as used for measuring photosynthetic parameters. The minimal fluorescence (F_0) was determined under sufficiently low irradiance (< 1 μmol m^{-2} s^1). The maximal fluorescence (F_m) was determined after a 0.8-s saturation pulse at 4 200 μmol m^{-2} s^{-1} on the dark-adapted leaves (30 min). In the light-adapted leaves, the radiation of the saturation pulses to determine the maximal fluorescence (Fm′) was 6 000 μmol m^{-2} s^{-1} for 0.8 s, whereas the actinic light was 200 μmol m^{-2} s^{-1}. Measurements of the quantum yield of photosystem (PS) II photochemistry (Φ_{PSII}) were obtained by application of a saturation light pulse (6 000 μmol m^{-2} s^{-1} for 0.8 s) under ambient irradiance. The fluorescence parameters, such as variable fluorescence (F_v), maximum efficiency of PS II photochemistry (F_v/F_m), photochemical quenching (qP), and apparent electron transport rate (ETR), were calculated according to Schreiber *et al.* (1986).

Chlorophyll (Chl) and carotenoid (Car) content was measured as described by Lichtenthaler and Wellburn (1983). Briefly, pigments were extracted from leaves with 100 % ethanol. Absorption of the extracts was measured using a spectrophotometer (*721 TYPE*, *Shanghai Analysis Instrument*, Shanghai, China) and the content of Chl *a*, Chl *b*, and Car was calculated using the formulae of Lichtenthaler and Wellburn (1983).

Enzyme extraction and assay: Fresh leaves (500 mg) were frozen in liquid nitrogen and then ground in 4 cm^3 of solution containing 50 mM phosphate buffer (pH 7.0), 1 % (m/v) polyvinylpolypyrrolidone, and 0.2 mM ascorbic acid. The homogenate was centrifuged at 15 000 *g* for 30 min, and the supernatant collected for enzyme assays.

The SOD (EC 1.15.1.1) activity was assayed by measuring inhibition of the photochemical reduction of nitroblue tetrazolium (NBT) (Stewart and Bewley 1980). One unit of SOD activity was defined as the amount of enzyme that caused 50 % inhibition of the initial rate of the reaction in the absence of enzyme.

The CAT (EC 1.11.1.6) activity was determined as a decrease in absorbance at 240 nm for 1 min following the decomposition of hydrogen peroxide (H_2O_2) (Chance and Meahly 1955). The reaction mixture contained 50 mM phosphate buffer (pH 7.0) and 15 mM H_2O_2. One unit of CAT activity corresponded to 1 μmol of H_2O_2 consumed per minute per gram of protein using a coefficient of absorbance of 40 M^{-1} cm^{-1}.

The APX (EC 1.11.1.11) activity was measured as a decrease in absorbance at 290 nm for 1 min (Nakano and Asada 1981). The assay mixture consisted of 0.5 mM ascorbic acid, 0.1 mM H_2O_2, 0.1 mM EDTA, 50 mM sodium phosphate buffer (pH 7.0), and 0.15 cm^3 of enzyme extract. One unit of APX was defined as 1 nmol of ascorbate oxidized per minute per gram of protein.

RNA isolation and real time quantitative PCR: Total RNA were isolated from leaves of 5-week-old tomato plants by using *EasyPure*™ plant RNA kit (*TransGen Biotech*, Beijing, China) and following the manufacturer's protocols. The first-strand cDNA was synthesized using 2 μg of total RNA by oligo(dT)-primed reverse transcription using the *EazyScript* first-strand DNA synthesis super mix (*TransGen Biotech*) according to manufacturer's instructions. Real time qPCR was used to examine the expressions of chlorophyll synthesis genes, Car synthesis genes, and photosystem structure genes. The tomato gene *ACTIN2* (*ACT2*) was used as an internal control. The list of genes and primers is shown in Table 1 Suppl.

Statistical analysis was carried out according to Wang *et al*. (2015). Briefly, data were analyzed by one-way *ANOVA* using the statistical software *SPSS v. 22.0* (*IBM*, New York, NY, USA), and the means were compared by Student-Newman-Keuls test (*q*-test).

Results

The 200 mg dm^{-3} ZnO NPs had little if any effect on plant dry mass (Fig. 1*A*-*C*). However, a decrease of about 10 % of shoot and root dry masses was observed in 400 mg dm^{-3} ZnO NPs treated plants, and about 50 % inhibition in 800 mg dm^{-3} treated plants (Fig. 1*A*,*B*). High concentration of ZnO NPs had more severe inhibitory effects on root growth compared to shoot growth (Fig. 1*A*-*C*). This finding was also supported by the change in root/shoot ratio (Fig. 1*D*). The supernatant of 800 mg dm^{-3} suspensions had no significant inhibitory effects on tomato growth (Fig. 1), even though the content of released Zn^{2+} was 0.51 mg dm^{-3}.

The yellow leaf colour observed in ZnO NP treated plants (Fig. 2*A*) indicated a possible effect on chlorophyll

content. Our measurements showed that lower concentrations of ZnO NPs had little or no effect on Chl *a* and *b* content; however, there was about 60 and 70 % reductions in Chl *a* and *b* content in plants exposed to 800 mg dm^{-3} ZnO NPs, respectively (Fig. 2*B*,*C*). Consistent with this, the Chl *a*/*b* ratio increased (Fig. 2*D*) and Chl *a*+*b* content was reduced in plants exposed to ZnO NPs (Fig. 2*F*). The ZnO NP treatments increased Car content (Fig. 2*E*). The Car/Chl *a*+*b* ratio also increased in plants exposed to ZnO NPs, and an about eight-fold increase occurred in plants exposed to 800 mg dm^{-3} ZnO NPs (Fig. 2*G*). Consistent with these results, the expressions of chlorophyll synthesis genes examined, including *CAO*, *CHLG*, *CRD1*, *CHLI*, *HEMG*, *HEMB,* and *HEMC* (for full names see Table 1 Suppl.), were reduced in plants exposed to high concentrations of ZnO NPs (Fig. 1 Suppl.). It should be noted that a slight increase in expressions of *CAO* and *HEMG* was observed in plants treated with supernatant of 800 mg dm^{-3} ZnO NPs (Fig. 1 Suppl.). Also, expressions of Car synthesis genes, including *PSY* and *LYCB*, increased in response to ZnO NP treatments, but to different degrees (Fig. 1 Suppl.).

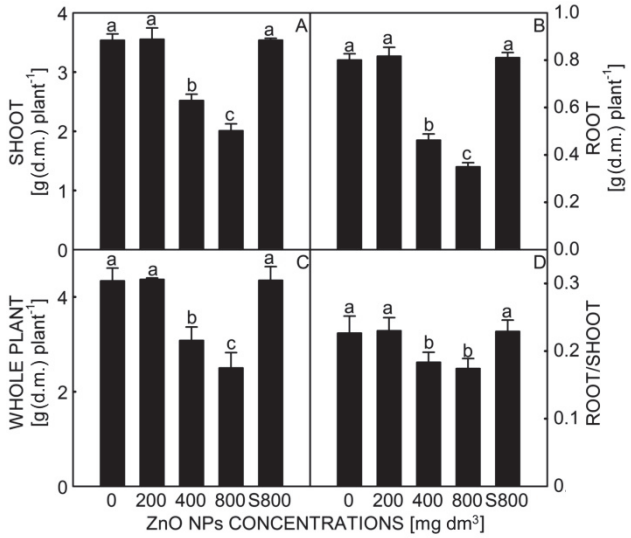

Fig. 1. Effects of ZnO NPs on growth of 5-week-old tomato plants treated with 0, 200, 400, and 800 mg dm^{-3} ZnO NPs or supernatant from 800 mg dm^{-3} ZnO NPs suspensions for two weeks. Shoot dry mass (DM) (*A*), root DM (*B*), whole plant DM (*C*), and root/shoot DM ratio (*D*). Means ± SDs, $n = 4$; means with different letters are significantly different at 5 % level.

As ZnO NP treatments decreased the content of chlorophylls (Fig. 2), we supposed that photosynthesis might be affected. To test this, we examined P_N, g_s, c_i, and E of fully expanded fifth leaves. High concentrations of ZnO NP treatments decreased the P_N (Fig. 3*A*). Even higher decrease was observed for g_s; in plants exposed to 800 mg dm^{-3} ZnO NPs, the g_s was only about 50 % of that in the control plants treated with water only or with supernatants from 800 mg dm^{-3} ZnO NP suspensions (Fig. 3*B*). The c_i was also greatly reduced in plants exposed to ZnO NPs, with a more than 30 % decrease in those exposed to 800 mg dm^{-3} ZnO NPs (Fig. 3*C*). A similar decrease in E was also observed in plants watered with ZnO NPs (Fig. 3*D*). Consistent with these results, expressions of photosynthesis genes including *SBPASE* and *FBPASE* were also reduced in high concentrations of ZnO NP treated plants (Fig. 2 Suppl.).

The chlorophyll fluorescence parameters F_v/F_m (Fig. 3*E*), Φ_{PSII} (Fig. 3*F*), qP (Fig. 3*G*), and ETR (Fig. 3*H*) decreased with increasing ZnO NP concentrations compared to controls. Notably, when tomato plants were exposed to 800 mg dm^{-3} ZnO NPs, the fluorescence parameters significantly decreased by about 24, 22, 26, and 25 % for F_v/F_m, qP, Φ_{PSII}, and ETR, respectively. Consistent with these results, the expressions of photosystem structure genes including *PSAA*, *PSBD*, *PSBF*, and *PSBH* were also reduced in plants treated with high concentrations of ZnO NP (Fig. 2 Suppl.).

Generally, to cope with ROS, plant cells have an antioxidant defense capacity including antioxidant enzymes such as SOD, CAT, and APX. Thus, we investigated whether their activities were affected. The SOD, CAT, and APX activities in tomato plants increased in a concentration-dependent manner with ZnO NP treatment; even though 200 mg dm^{-3} ZnO NPs had no effect on these activities. Consistent with these results, the expressions of respective genes *Cu/Zn2-SOD*, *Fe-SOD*, *APX2*, and *CAT1* increased in plants exposed to all high concentrations of ZnO NPs (Fig. 3 Suppl.).

Discussion

Accumulation of dry mass is an ideal indicator of tomato growth. In this study, although both shoot DM and root DM were strongly inhibited under high concentration of ZnO NPs, root DM was more inhibited, as supported by the change in root/shoot ratio (Fig. 1). These results are in accordance with those of Bandyopadhyay *et al*. (2015), who found that root biomass significantly decreased (80 %) in all ZnO NP treated alfalfa plants; and with López-Moreno *et al*. (2017), who reported ZnO NP treatments imposed a more severe inhibition of root growth than the same ionic treatments in maize. However, low concentrations of ZnO NPs had no significant effect on growth of tomato plants (Fig. 1), partly because ZnO NPs had little toxicity at low

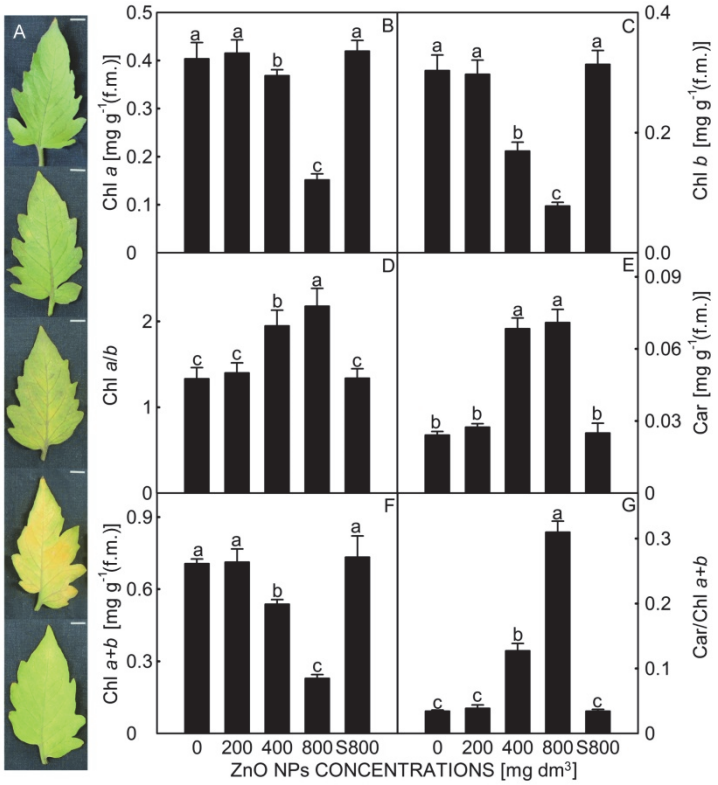

Fig. 2. Effects of ZnO NPs on content of chlorophylls (Chl) and carotenoids (Car) in 5-week-old tomato plants treated with 0, 200, 400, and 800 mg dm^{-3} ZnO NPs, or supernatant from 800 mg dm^{-3} ZnO NPs suspensions for 2 weeks. Photographs of tomato plants leaves (*A*; increased concentration of NPs from top to bottom; *bar* = 9 mm). Chl *a* (*B*), Chl *b* (*C*), Chl *a*/*b* ratio (*D*), Car (*E*), Chl *a*+*b* (*F*), and Car/Chl *a*+*b* ratio (*G*). Means ± SDs, *n* = 4; means with different letters are significantly different at 5 % level.

concentration, and also because tomato plants were somewhat tolerant to ZnO NP treatment. Therefore, high concentrations of ZnO NPs are considered to be phytotoxic to tomato plants in terms of reduction of growth. In addition, the supernatant of 800 mg dm^{-3} ZnO NP suspensions did not affect growth of tomato (Fig. 1), even though it contained a small amount of Zn^{2+}. In fact, the amount of Zn^{2+} was slightly higher than the actual content of released ions because some small ZnO NPs cannot be completely excluded through centrifugation and filtration (Lin and Xing 2007, Tso *et al.* 2010, Yang *et al*. 2015). Most researchers have reported no significant growth inhibition in the corresponding Zn^{2+} solutions. Lin and Xing (2007) reported that Zn^{2+} released from ZnO NP suspensions did not display any phytotoxicity on radish, rape, and ryegrass. Yang *et al*. (2015) reported that the phytotoxicity of ZnO NPs to maize and rice mainly depended on the NP itself, not the Zn^{2+} released from the ZnO NPs suspensions. Consistent with these observations (Lee *et al*. 2013, Yang *et al*. 2015), our study also indicated that the amount of Zn^{2+} released from ZnO NPs was negligible.

The phytotoxicity of NPs can be evaluated according to content of photosynthetic pigments which has been often considered as indicator in phytotoxicity assays (Miralles *et al*. 2012, Zhao *et al*. 2013a). In our data, content of Chl *a* and Chl *b* were reduced in rosette leaves of ZnO NP treated plants (Fig. 2), consistent with the correspondingly reduced expressions of chlorophyll synthesis genes (Fig. 1 Suppl.); whereas those in plants treated with supernatants of 800 mg dm^{-3} ZnO NP suspensions remained largely unaffected. However, Car

content showed slight increase in ZnO NP treated plants (Fig. 2), consistent with the observation that expressions of some Car synthesis genes, especially *LYCB*, dramatically increased (Fig. 1 Suppl.). Chlorophyll fluorescence can provide a large amount of information about photosynthetic mechanisms in plants (Hu *et al*. 2014a). In the present study, the chlorophyll fluorescence parameters F_v/F_m, Φ_{PSII}, qP, and ETR were reduced in ZnO NP treated plants (Fig. 3), implying some damage to the photochemical system. Consistent with reductions in chlorophyll content and parameters of chlorophyll fluorescence in plants treated with high concentrations of ZnO NPs, the P_N, g_s, c_i, and E also decreased (Fig. 3), suggesting that reduction in photosynthesis was caused by reduced chlorophyll content and damage to the photochemical system. SOD, CAT, and APX are indispensable enzymes required for ROS detoxification in plants. SOD is considered to be crucial in regulating the content of superoxide anion radical (Mittler *et al*. 2004). Our analysis showed that SOD activity was up-regulated by high concentrations of ZnO NPs, supported by enhancing transcription of *Cu/Zn2-SOD* and *Fe-SOD* genes (Fig. 3 Suppl.), which was in accordance with other studies (Hu *et al*., 2014b, Priyanka and Venkatachalam, 2016). CAT mainly prevents the accumulation of H_2O_2, generated as a byproduct of plant metabolic processes (Panda and Choudhury 2005). In our data, both the activity of CAT and the expression of *CAT1* gene increased at high ZnO NP concentrations (Fig. 3 Suppl.). Hernandez-Viezcas *et al*. (2011) also reported an increase in CAT activity with increasing ZnO NP concentrations in mesquite plants. APX, which is a central peroxidase in

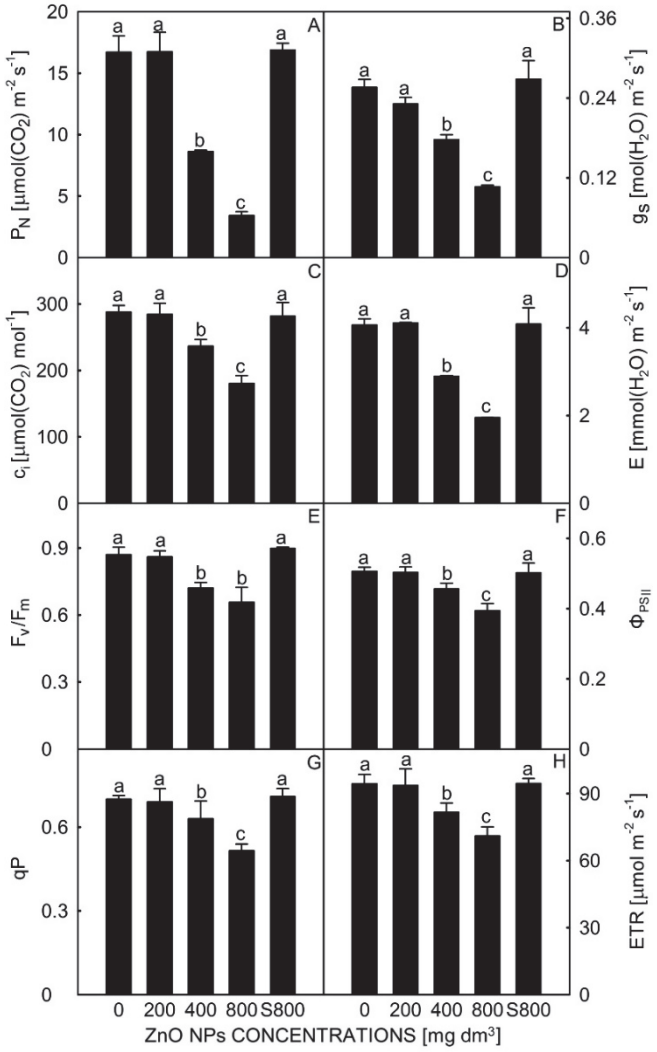

Fig. 3. Effects of ZnO NPs on gas exchange and Chl fluorescence of tomato plants. Net photosynthetic rate (P_N) (*A*), stomatal conductance (g_s) (*B*), intercellular CO_2 concentration (c_i) (*C*), transpiration rate (E) (*D*), maximum efficiency of PS II photochemistry (Fv/Fm) (*E*), quantum yield of PS II photochemistry (Φ_{PSII}) (*F*), photochemical quenching (qP) (*G*), and apparent electron transport rate (ETR) (*H*) in leaves of 5-week-old tomato plants treated with 0, 200, 400, and 800 mg dm^{-3} ZnO NPs, or with a supernatant from 800 mg dm^{-3} ZnO NP suspensions. The fifth leaves were used for the measurement by using a portable open-flow, gas-exchange system. Means ± SDs, $n = 4$; *bars* with different letters are significantly different at 5 % level.

H_2O_2 reduction, was activated in ZnO NP treated plants, supported by the data on *APX2* expression (Fig. 3 Suppl.). A similar response was reported by Cuypers *et al*. (2001) in common bean.

In conclusion, the ZnO NPs showed toxicity to tomato plants only at higher concentrations (400 and 800 mg dm^{-3}). They reduced plant growth and biomass production by affecting chlorophyll content and photosynthesis. The ZnO NPs enhanced the activities of antioxidant enzymes and the transcriptions of respective genes.

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