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ORIGINAL ARTICLE

Impact of zinc oxide and copper oxide nano-particles on physiological and molecular processes in *Brassica napus* L.

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Abstract In the present study, the effect of ZnO and CuO nano-particles was investigated on some growth and physiological parameters of *Brassica napus* L. cultivar SLM046. The seedlings were grown in 1/2 strength Murashige and Skoog medium supplemented with either ZnO or CuO NPs @ 0, 10, 100 and 1000 mg l^{-1} for 10 days. The results indicated that each nano-particle induced the growth responses at 10 mg l^{-1} concentration. The decreases were observed in root and shoot elongation and root dry weights under higher concentrations (100 and 1000 mg l^{-1}) treatments. Imposing canola seedlings to each nano-particles lead to increase and decrease in sugar and protein contents, respectively. The expression of four genes involved in signal transduction pathway including *Auxin Responsive Protein*, *Protein Kinase*, *MPK3* and *MPK4* was also investigated using semi quantitative RT-PCR. Exposure of canola germinated seeds to each nano-particle caused changes in transcript levels of all four genes in root and shoot tissues. Our data indicate that at 10 mg l^{-1} concentration, ZnO and CuO nano-particles supplementation promoted the growth response in *B. napus*, while higher concentrations resulted in toxic responses.

Keywords *Brassica napus* · Copper oxide · Gene expression · Growth parameters · Nano-particles · Zinc oxide

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Introduction

Recently nanotechnology has received an increasing interest in consumer products. This widespread application has raised concern over the impact of nano-particles (NPs) on the environment and on biota. NPs are released into the ecosystem inevitably and contaminate aquatic, terrestrial and atmospheric environments (Colvin 2003). Hence, plants have a critical role in the sustenance of ecosystem, and also may experience greater exposure to NPs.

NPs can enter into plant cells via either endocytosis or non-endocytic penetration. They can be taken up by roots and transported to shoots through vascular systems. However, their impacts on plant metabolism and development depend on the hydrodynamic size, surface chemistry, concentration, and the chemical milieu of the sub-cellular sites of NPs deposition (Dietz and Herth 2011). Moreover, inhibition effect on growth varies greatly among NPs and plants (Faisal et al. 2013). Most likely, the NPs interact with biological systems through, (a) the chemical effects as metal ions in solution; (b) mechanical effects due to hard spheres and defined interfaces; (c) catalytic effects on surfaces; (d) binding with macro-molecules either by non-covalent or covalent mechanisms; and (e) intracellular oxidative stress (Dietz and Herth 2011). Toxic metals bind to cell components such as DNA and proteins, and modify their activities. Interference with cellular processes often causes redox imbalances and oxidative stress in metal exposed plants (Sharma and Dietz 2009).

Plant hormone, auxin activates many early response genes that are thought to be responsible for diverse aspects of plant growth and development. It has been proposed that auxin signal transduction is mediated by a conserved signaling cascade consisting of three protein kinases: the mitogen-activated protein kinase (MAPK), MAPK kinase

(MAPKK) and MAPKK kinase (MAPKKK) (Yang et al. 2009). As a conserved signaling pathway, MAPK cascades have been implicated in plant signal transduction including response to wounding, pathogens, abiotic stresses, plant hormones, and elicitors (Pedley and Martin 2005). Protein kinases constitute one of the major classes of signal transducers involved in mediating a cell's response to external stimuli. These important regulatory enzymes can be comprised of several integral components in a signaling cascade, from the initial receptor protein to the final effector protein, and form the basis for controlling cellular events in response to environmental, metabolic and/or developmental cues (Stone and Walker 1995; Satterlee and Sussman 1998).

The genus *Brassica*, belonging to the family *Cruciferae* (*Brassicaceae*), includes the largest number of economic plants which are cultivated in a wide range of environments. Today, oilseed rape (*Brassica napus*) is the most important source of vegetable oil in Europe and the second most important oilseed crop in the world after soybean (Ashraf and McNeilly 2004).

The present study, therefore, was undertaken to determine the effects of ZnO and CuO NPs on growth and transcript abundance of four genes involved in signal transduction pathway in *B. napus* L.

Materials and methods

Plant material and treatments

Seeds of *B. napus* cultivar SLM046 were obtained from seed Research Center of Karaj, Iran. Seeds were surface sterilized with 70 % ethanol for 2 min and 1.5 % (w/v) sodium hypochlorite solution for 15 min. The seeds were washed with sterilized distilled water three times and germinated on plates with ½ strength Murashige and Skoog (MS) medium (Duchefa Biochemie, Haarlem, The Netherlands), pH 5.8, containing 0.8 % agar under a 16 h light/8 h dark cycle at 22 ± 1 °C. After germination, seeds were transferred to bottles containing ½ strength MS medium, 0.8 % agar and appropriate concentration of either ZnO or CuO NPs. Bottles were transferred to growth chamber and incubated under conditions of 16 ± 8 h of light/dark, with light intensity of 100 mE m^{-2} s^{-1} at 22 ± 1 °C for 10 days. The preparation of NPs was as follows: Zinc chloride and copper chloride, oxalic acid and NaCl were used as the main raw materials. $ZnCl_2$, $CuCl_2$ and NaCl were thoroughly dried in vacuum oven at 120 °C for 24 h to remove the residual or absorbed water. In a typical synthesis, 2 g of dried $ZnCl_2$ and oxalic acid were put in an agate mortar in a molar ratio of 2:1, followed by addition of 4 g of NaCl as a diluent to form the mixture. The starting materials were mixed and milled for 45 min at room temperature. The precursor was calcinated at 450 °C in air in a porcelain crucible for 30 min to prepare the ZnO-NPs. Removal of the salt by-product was carried out by washing the powder with deionized double distilled water. Finally, ZnO nano-particles were obtained after drying of washed powders (Biparva et al. 2014). The CuO NPs were synthesized by the same method. The size of NPs was determined 20 nm by XRD. The ZnO and CuO solutions were prepared at 0, 10, 100 and 1000 mg l^{-1} concentrations, separately and sonicated for 20 min by Coleparmer 750 watt ultrasonic homogenizer probe (USA) to break up agglomerates. The NPs were directly added to the medium after autoclaving and shaken vigorously in order to break the agglomerates. The medium was solidified immediately by keeping at 4 °C, avoiding precipitation of NPs (Nair and Chung 2014).

Samples harvesting

Seedlings were removed from culture medium after 10 days and physiological parameters including root and shoot lengths, dry weights, sugar and protein contents were examined. Roots and shoots were dried in an oven at 60 °C for 3 days and the weight of biomass was determined.

Total soluble sugar and protein estimation

Soluble sugars were measured by phenol–sulfuric acid method based on acid hydrolysis of soluble sugars and formation of furfural compounds that produce a colored complex with phenol (Kochert 1978).

Total protein content was determined according to the Lowry method using Folin reagent (Lowry et al. 1951).

RNA isolation and RTPCR analysis

Root and shoot samples were frozen in liquid nitrogen for RNA isolation. Total RNA was extracted using Trizol and first-strand cDNA was synthesized according to the manufacturer's instructions (Fermentas) with Oligo dT primers. PCR reaction were conducted in 25 µl volume containing 12.5 µl master mix (Fermentas), 1.0 µl cDNA, 0.75 µM of each of the primers (Table 1) and 10 µl double distilled water. The reactions were initiated at 95 °C for 3 min, followed by 28–30 cycle of: 95 °C for 25 s, 58–62 °C for 20 s, 72 °C for 25 s and a final extension at 72 °C for 7 min. The amplification products were electrophoresed on 2 % agarose gel at 120 V in TBE buffer (0.045 M Tris–borate, 0.001 M EDTA, pH 8.0) using known concentration of DNA ladders. Gels were stained with ethidium bromide and visualized using gel documentation system (Gel Logic 212 Pro Imaging System, USA).

Table 1 list of primers used in RT-PCR study

Genes	Primer sequence	References
Protein Kinase Protein	F:5′-GTTGCCCAACATCATTATCAAGC-3′	Liang et al. (2010)
	R:5′-AGAAGAAAACAGAGCACTGCTCC-3′	
Auxin-Responsive Protein	F:5′-AACCATCCAGTCTTCGTCGGAC-3′	Liang et al. (2010)
	R:5′-AGCTGAGAAAACATCATCAGCAG-3′	
BnMPK3	F:5′-ATGAACAACGCCGGCGGCCA-3′	Yu et al. (2005)
	R:5′-CTAAGCATATGTTGGATTGAGTGC-3′	
BnMPK4	F:5′-TTGTATCAGTTGTTGCGTGGG-3′	Wang et al. (2009)
	R:5′-TGAAGTCAGTCTCGGATTTGGTC-3′	
Actin2	F:5′-GTGACAATGGAACTGGAATGG-3′	Yu et al. (2005)

Statistical analysis

Statistical analysis was performed using one-way and two-way Analysis of Variance (ANOVA), followed by LSD tests. Lower case letters ($p \leq 0.05$) were considered as statistically significant.

Results and discussion

The application of NPs significantly affected plant growth components of canola plant. At 10 mg l^{-1} both the NPs improved root and shoot lengths, and exerted adverse effects at higher applications in a concentration dependent manner (Table 2). After 10 days of exposure at 1000 mg l^{-1} of ZnO NPs, root and shoot lengths were significantly decreased by 1.8 and 1.7 folds, respectively, compared to control plants (Table 2), however, at 1000 mg l^{-1} CuO-NPs caused a greater decrease in root and shoot length, by 3.3 and 2.5 times, respectively compared to control plants (Table 2).

However, the magnitude of plant growth responses to ZnO and CuO NP exposures differed, and CuO caused more toxic effect on canola seedlings (Table 2). Our results indicated that the root growth was more sensitive to the applied exposures than the shoot growth. Since, the uptake of NPs occurs along with the uptake of moisture and nutrients from root parts to the shoots, roots are the first target tissues to confront pollutants and toxic effects appear more strongly in roots rather than in shoots. Earlier studies also indicated phytotoxic effect of Zinc oxide NPs on radish (*Raphanus sativus*), rape (*B. napus*), ryegrass

Table 2 Effect of ZnO and CuO nano-particles on some growth factors of 10 days of *B. napus* seedlings grown in ½ strength MS medium with 0, 10, 100 and 1000 mg $l^{-1}$of each nanoparticles

Treatments (mg l^{-1})	Root length (cm)	Shoot length (cm)	Root dry weight (g)	Shoot dry weight (g)	Sugar content (mg g^{-1} dry wt.)	Protein content (mg g^{-1} dry wt.)
ZnO						
0 (Control)	5.40	12.40	0.013	0.04	1.35	0.052
10	5.90	12.70	0.036	0.065	1.50	0.046
100	3.80	9.00	0.015	0.051	1.60	0.040
1000	3.00	7.30	0.008	0.048	1.70	0.035
Mean	4.52	10.35	0.018	0.051	1.54	0.043
CuO						
0 (Control)	5.60	12.30	0.012	0.040	1.35	0.052
10	7.80	12.90	0.018	0.056	1.65	0.045
100	3.80	7.50	0.007	0.04	1.80	0.039
1000	1.70	4.90	0.004	0.03	1.90	0.031
Mean	4.72	9.40	0.010	0.041	1.68	0.041
Grand mean	4.62	9.88	0.014	0.046	1.61	0.042
LSD ($p \leq 0.05$)						
Nano-particles (NP)	5.08	24.14	0.20	0.244	3.49	0.038
Concentration (C)	12.50	20.64	0.058	0.063	1.23	0.052
NP × C	4.18	3.87	0.025	0.027	0.301	0.005

(*Lolium perenne*), lettuce (*Lactuca sativa*), Corn (*Zea mays*), cucumber (*Cucumis sativus*) (Lin and Xing 2007), soybean (*Glycine max*) (Lopez-Moreno et al. 2010) and *Arabidopsis thaliana* (Lee et al. 2010), with greater decline in root length. Greater toxicity of Cu has also been reported for several species, including *L. perenne* L. (Wong and Bradshaw 1982), *Brassica chinensis* L. (Wong et al. 1986), mung bean (*Phaseolus radiatus*) and wheat (*Triticum aestivum*) (Lee et al. 2008).

Exposing canola seedlings to the two NPs increased root dry weight at 10 mg l^{-1} concentration. This induction appeared to be much greater for ZnO (0.036 g) than CuO (0.018 g). The root dry weight in untreated control seedlings was 0.012–0.013 g. Application of ZnO and CuO NPs at 1000 mg l^{-1} significantly decreased the root dry weight of canola seedlings by 60 and 30 %, respectively compared to control (Table 2). The ZnO-NPs did not exert any negative effect on shoot dry weight in canola and even increased it slightly (Table 2). However, CuO-NPs at 1000 mg l^{-1} concentration decreased the shoot dry weight to 0.03 g compared to 0.04 g of control seedlings, however, the values were statistically non-significant ($p \leq 0.05$). Copper and zinc have been shown to decrease root growth, nutrient absorption and chlorophyll content in plants (Lee et al.2008; Van Assche et al. 1988). Therefore, the observed growth reduction in our experiment might be due to decrease in nutrient absorption by roots. According to Kazemi-Shahandashti et al. (2013) generation of ROS and oxidative stress also play a crucial role in heavy metal toxicity.

The sugar content analysis revealed a concentration dependent increase up to the highest level (1000 mg l^{-1}) at each of NPs application (Table 2). Upon exposure to either of the two NPs, sugar content increased linearly from 1.35 mg g^{-1} dry wt. in control plants to 1.65 mg g^{-1} dry wt. at 1000 mg l^{-1} in case of ZnO and to 1.9 mg g^{-1} dry wt. in case of CuO. However, the magnitude of increase was always greater in case of CuO than ZnO. There are reports indicating that heavy metals cause changes in osmotic balance of plants (Glenn et al. 1997). Our finding is in line with previous study reporting a considerable increase in sugar levels under abiotic stresses such as NaCl, PEG, Cold and heat (Gill et al. 2001).

Supplementation of each of the NPs to the medium caused reduction in total protein level with increasing concentration and the greater adverse effect was exerted at 1000 mg l^{-1} (Table 2). Comparison of protein content shows that CuO was more toxic than ZnO for *B. napus* plants. CuO lowered the protein content from 0.052 mg g^{-1} dry wt. in control seedlings to 0.031 mg g^{-1} dry wt. compared to ZnO, which decreased it to 0.035 mg g^{-1} dry wt. at 1000 mg l^{-1}. The decrease in protein content under high ion concentrations can be due to decrease in synthesis of some proteins or probably increase in activities of proteolytic enzymes (Khudsar et al. 2001; Panda and Choudhury 2005).

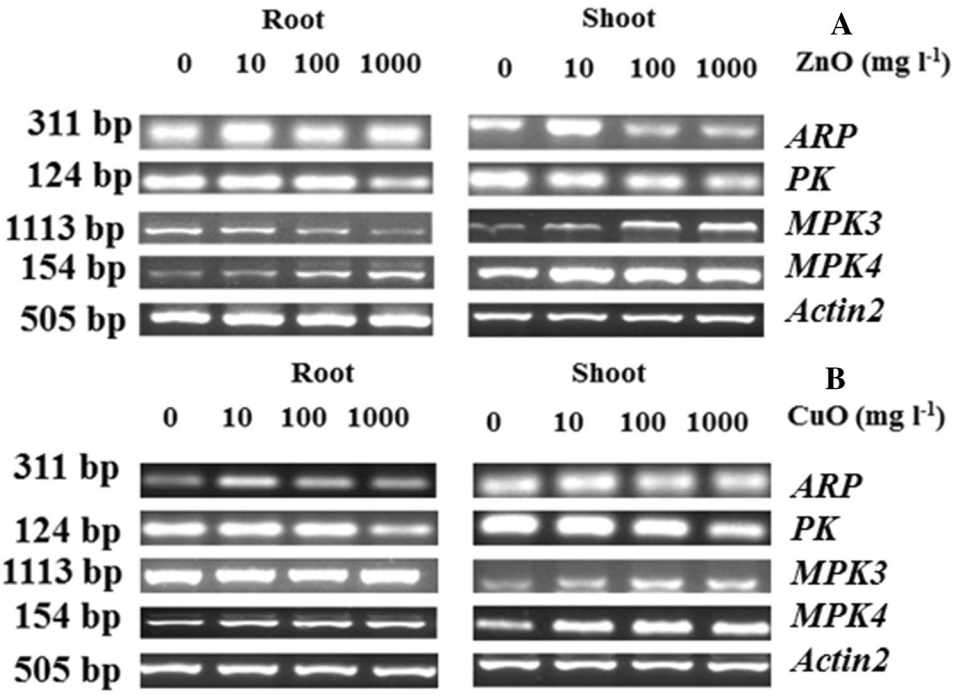

Fig. 1 Expression profile of *Auxin Responsive Protein* (*ARP*), *Protein kinase* (*PK*), *MPK3* and *MPK4* in root and shoot tissues of *Brassica napus* under ZnO (**A**) and CuO (**B**) exposures. Seedlings were grown in ½ MS medium with 0, 10, 100 and 1000 mg l^{-1} of either ZnO or CuO NPs for 10 days

Gene expression

Adverse environmental conditions, especially extreme temperature, drought, salinity, pollution, contamination with NPs, pathogens and insects adversely affects plants growth and yield (Mizoguchi et al. 1996). Landa et al. (2012) reported up and down regulations of an array of genes upon exposure of *Arabidopsis thaliana* plants to ZnO NPs, which are generally induced in response to stresses, including both abiotic, viz., oxidative, salt, water deficit, and biotic stresses such as wounding, and insect and pathogen attack. The *Auxin-responsive* genes have been

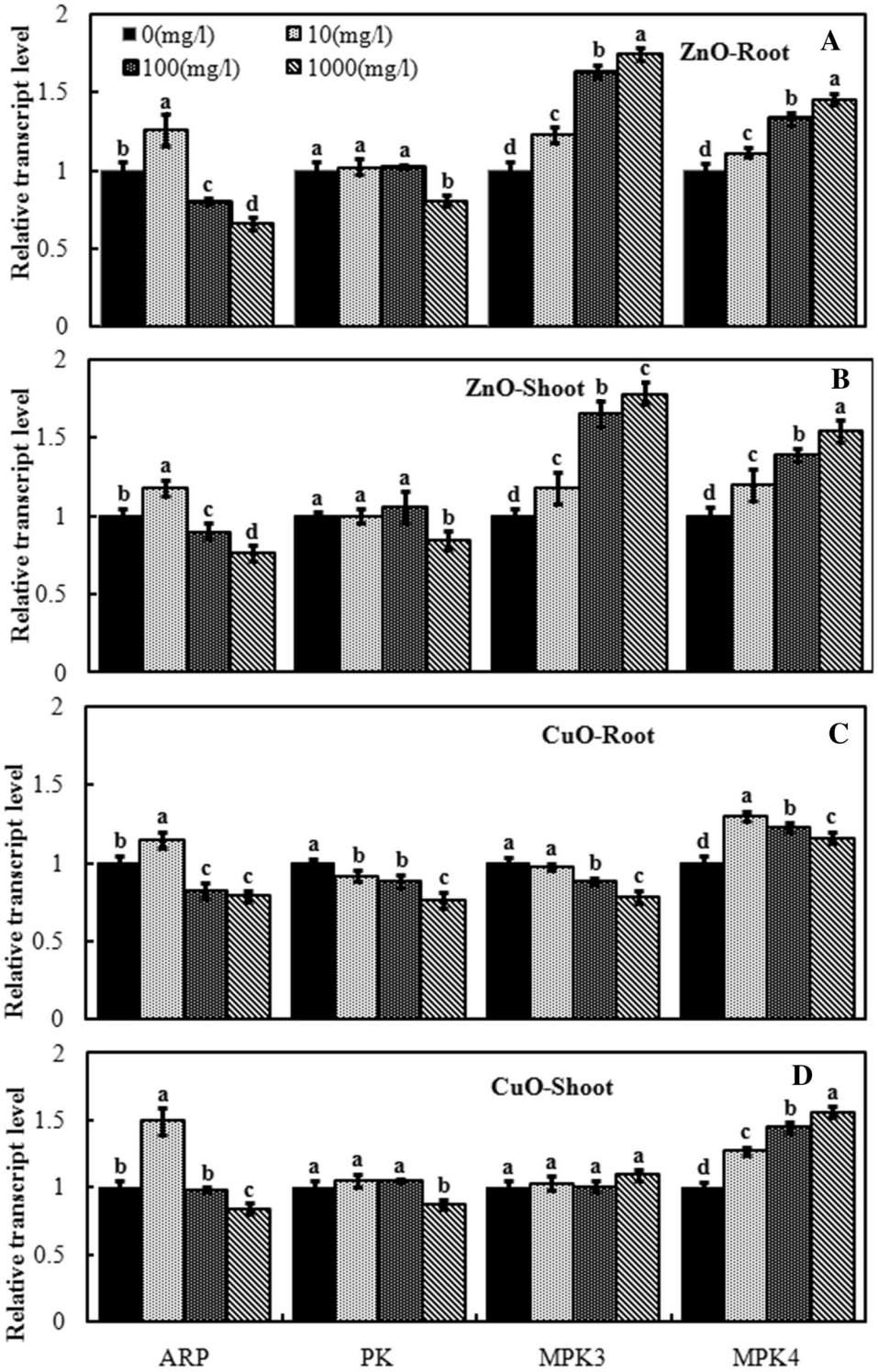

Fig. 2 Relative transcript levels of *Auxin Responsive Protein* (*ARP*), *Protein Kinase* (*PK*), *MPK3* and *MPK4* using semi-quantitative RT-PCR under ZnO in roots (**A**), shoots (**B**), and under CuO in roots (**C**), shoots (**D**). Seedlings were grown in ½ strength MS medium with 0, 10, 100 and 1000 mg l^{-1} of either ZnO or CuO NPs for 10 days. Values were compared with Actin2 gene and experiments carried out with three replications. Values were normalized to control (0 mg l^{-1}). Different letters show significant differences

known to play important role in the adaptation of plants to abiotic stresses (Park et al. 2007) and in various stages of vegetative and reproductive developmental processes (Jain and Khurana 2009). The expression profile of the *Auxin Responsive Protein* showed highest level of expression under both the NPs treatment at 10 mg l^{-1}, both in root (Figs. 1A, B, 2A, C) and shoot (Figs. 1A, B, 2B, D). The gene expression decreased with increasing ZnO and CuO concentrations at 100 and 1000 mg l^{-1}in both roots and shoots. In the case of each NPs, a parallelism was observed between *Auxin-responsive protein* expression, and root and shoot growth data (Table 2) (Fig. 1). Since, the auxin is one of the factors that can influence growth and development in plants by altering gene expression, it can be concluded that the growth induction at 10 mg l^{-1} might be due to generation of auxin hormone in *B. napus* plant.

The highest transcript abundance of *Protein kinase* occurred in the roots (Figs. 1A, B, 2A, C) and shoots (Figs. 1A, B, 2B, D) of control (0 mg l^{-1}) plants under each NPs treatment, which decreased with increasing concentration up to 1000 mg l^{-1} in both the tissues. In plants, protein phosphorylation is suggested as one of the major signaling events occurring in response to environmental stresses (Umezawa et al. 2004). *Protein kinase* contributes in the regulation of cellular functions including cell division, metabolism and response to environmental stimuli (Chen et al. 2010). The expression of *Protein kinase* was reduced in our experiment, which is consistent with previous study reporting severe down regulation of *Protein kinase* under 24 h incubation of canola seedlings to 150 mM NaCl stress. The results suggest that the gene may play important roles in signal transduction related to abiotic stresses in *B. napus* (Liang et al. 2010).

MPKs are key enzymes activated by various stresses, including pathogen infection, wounds, temperature, heavy metals, drought, salinity, osmolarity, ultraviolet radiation, ozone and reactive oxygen species (Mizoguchi et al. 1996). To investigate whether *BnMPK3* and *BnMPK4* expression changes under NPs treatments, their transcription level was studied using semi-quantitative RT-PCR. The highest *MPK3* gene expression was observed in roots of control (0 mg l^{-1}) plants under the exposure of either of the two NPs (Fig. 1). Application of higher concentrations of ZnO (Figs. 1A, 2A) decreased *MPK3* transcript level in roots, while CuO did not affect its mRNA expression at higher concentrations (Figs. 1B, 2C). In shoots, the expression of the *MPK3* was up regulated by both the treatments, and the highest *MPK3* transcript abundance was observed at 1000 mg l^{-1} (Figs. 1A, B, 2B, D). Earlier studies have also shown that expression of *ZmMPK3* is activated under different stresses such as mannitol, NaCl, cold, H_2O_2, abscisic acid, salicylic acid and methyl jasmonate (Fu et al. 2002). In our study, both the NPs could activate *MPK3* only in shoot tissues.

The ZnO and CuO imposed similar effects on *MPK4* expression with activation both in roots (Figs. 1A, B, 2A, C) and shoots (Figs. 1A, B, 2B, D). The highest relative *MPK4* expression level was observed under 1000 mg l^{-1} of both the NPs. There are reports indicating that environmental stresses such as low temperature, low humidity, high salinity, and injury can activate *BnMPK4* (Wang et al. 2014). Abiotic stimuli such as cold and salt stresses could also lead to *OsMPK4* activation at the transcriptional level in rice (Jonak et al. 2004). We observed changes in *MPK3* and *MPK4* mRNA levels in roots and shoots of plants exposed to NPs stress. These results suggest that the two genes may play important roles in signal transduction related to NPs in *B. napus.*

Conclusion

ZnO and CuO NPs at 10 mg l^{-1} concentrations were optimum for inducing the growth response and expression of various genes in *B. napus*. Supplementation of growth media at higher concentrations caused undesirable responses. Interestingly, between two nano-particles, CuO had greater negative impact than ZnO. The study will facilitate understanding of genes involved in *B. napus* responses to ZnO and CuO nano particles. Investigation on studied genes in detail will further help in understanding the molecular mechanism of *B. napus* in response to NPs exposures.

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Compliance with ethical standards

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

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