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Research article

Nickel oxide nanoparticles cause substantial physiological, phytochemical, and molecular-level changes in Chinese cabbage seedlings

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ABSTRACT

Nickel oxide nanoparticles (NiO NPs) are utilized in various industries and their release into the environment may lead to the pollution of agricultural areas. However, assessing the toxicity of NiO NPs in major food crops is difficult due to the limited information available on their toxicity. The present investigation was carried out to evaluate how NiO NPs affect plant growth, photosynthetic efficiency, and phytochemical content, as well as changes at the transcriptional level of these phytochemicals in Chinese cabbage seedlings. Chlorophyll, carotenoid, and sugar contents were reduced, while proline and the anthocyanins were significantly upregulated in NiO NPs-treated seedlings. The levels of malondialdehyde, hydrogen peroxide, and reactive oxygen species, as well as peroxidase (POD) enzyme activity, were all enhanced in seedlings exposed to NiO NPs. The levels of glucosinolates and phenolic compounds were also significantly increased in NiO NPs-treated seedlings compared to control seedlings. The expression of genes related to oxidative stress (*CAT*, *POD*, and *GST*), MYB transcription factors (*BrMYB28, BrMYB29*, *BrMYB34*, and *BrMYB51*), and phenolic compounds (*ANS*, *PAP1,* and *PAL*) were significantly upregulated. We suggest that NiO NPs application stimulates toxic effects and enhances the levels of phytochemicals (glucosinolates and phenolic compounds) in Chinese cabbage seedlings.

1. Introduction

The use of nanoparticles (NPs) is of increasing significance due to their great potential, but also their adverse effects on human health and the environment (Khan et al., 2017). Nanomaterials (NMs) have been defined as materials comprising particles either in the unbound state or as aggregates with a diameter range of 1–100 nm. In addition to their tiny size, these particles are characterized by their large surface areas, high aspect ratios, and unusual surface properties. Nanotechnology has been adopted for diverse applications including the production of nano-fertilizers, nano-pesticides, for nutrient management, plant disease treatment, genetic improvement, and promotion of plant growth (De la Rosa et al., 2017). Nanoparticles elicit both beneficial as well as adverse effects in exposed plants (Peralta-Videa and Sahi, 2017). Nickel (Ni) is a profuse metal in the Earth's crust, and its uses in various manufacturing processes have resulted in increased Ni accumulation in the environment; consequently, it is now considered a large-scale contaminant worldwide (Hussain et al., 2013). Nickel oxide nanoparticles (NiO NPs) have extensive applications, including as a gas sensor and catalyst, and are widely found in alkaline battery cathodes, magnetic material, electrochromic film, fuel cells, stainless steel, artificial jewelry, and electrical goods (Manna and Bandyopadhyay, 2017). Wide-ranging production and usage of NiO NPs have raised concerns regarding their release into the environment and interactions with living organisms (Gong et al., 2011). High levels of NiO NPs are toxic to plants, inducing numerous symptoms of injury like chlorosis, necrosis, growth inhibition, and wilting (Lin and Xing, 2007; Stampoulis et al., 2009). A limited number of studies have analyzed cellular and molecular changes in microalgae, aquatic organisms, and plants resulting from NiO NPs toxicity (Gong et al., 2011; Oukarroum et al., 2015; Soares et al., 2018a; Pinto et al., 2019). NiO NPs were shown to be naturally transported into plants, producing cytotoxic and genotoxic effects (Magaye and Zhao, 2012; Pinto et al., 2019). Moreover, Faisal et al. (2013) reported that NiO NPs triggered apoptosis in tomato root cells and induced the release of caspase-3 proteases from mitochondria. Earlier results had suggested the extensive production of reactive oxygen species (ROS) and oxidative stress as possible mechanisms of NPs toxicity (Siddiqui et al., 2012). ROS acts as signaling molecules at lower concentrations whereas cause oxidative damages to intracellular components at higher concentrations (Foyer, 2018). The highly evolved antioxidant systems in plants such as enzymatic (superoxide dismutase, catalase, guaiacol peroxidase, glutathione S-transferase, ascorbate peroxidase, glutathione

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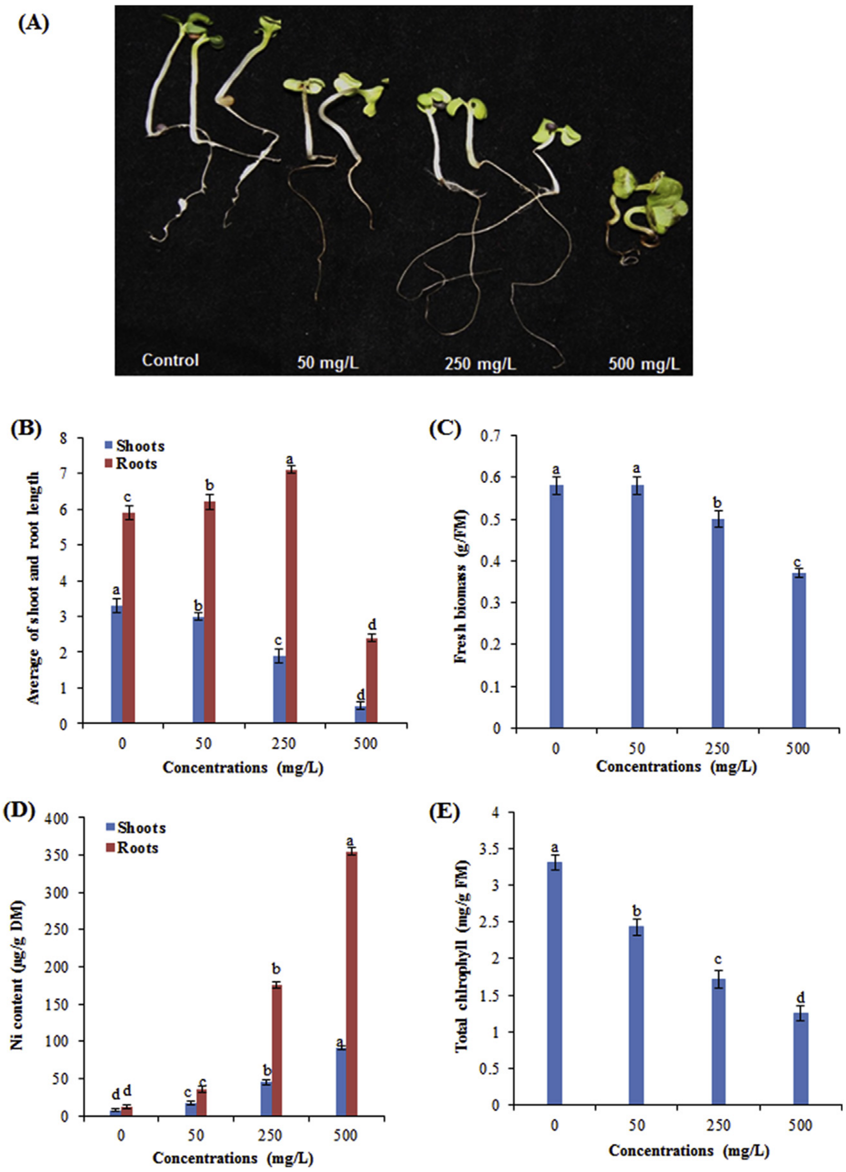

Fig. 1. (A) Phenotypes of *in vitro*-grown Chinese cabbage seedlings (left to right: control, 50, 250, and 500 mg/L) exposed to the designated concentrations of NiO NPs for 15 days, (**B)** average shoot and root length, (**C)** fresh mass (FM) of the seedlings, (**D)** nickel and **(E)** total chlorophyll contents. Data are presented as means ± SD of three replicates. Different letters indicate a significant difference at $P \leq 0.05$.

reductase, monodehydro ascorbate reductase and dehydro ascorbate reductase) and non-enzymatic (glutathione, ascorbic acid, phenolic compounds and proline) mechanisms were involved in the maintenance of redox homeostasis (Soares et al., 2018b). Additionally, some reports demonstrated that NiO NPs may induce programmed cell death in tomato (Faisal et al., 2013) and *Lemna* spp. (Oukarroum et al., 2015). Finally, exposure to Cu NPs was reported to induce a greater accumulation of bioactive compounds in the tomato (López-Vargas et al., 2018).

Brassicaceae vegetables are used as food as well as in traditional medicine. Chinese cabbage (*Brassica rapa* ssp. *pekinensis*) is a widely-consumed green leafy vegetable in Asia, and an ingredient of Kimchi, a popular Korean dish. Brassicaceae vegetables are characterized as functional foods due to their reported preventive role against some cancers and chronic diseases (Šamec et al., 2017). Moreover, they possess anti-inflammatory, antioxidant, anti-obesity, and gastro-protective properties associated with the presence of different bioactive compounds such as carotenoids, phenolics, and glucosinolates (Šamec et al., 2017). Therefore, this study was undertaken to assess the effects of NiO NPs at various concentrations (50, 250, and 500 mg/L) on the physiological growth and phytochemical variation of *in vitro*-grown Chinese cabbage seedlings. NiO NPs-mediated changes in growth characteristics (biomass, root, and shoot length) and Ni content were recorded for 15 days-old *in vitro*-grown seedlings treated with various concentrations of NiO NPs. Biochemical parameters determined include changes in the levels of photosynthetic pigments (chlorophyll and carotenoids), anthocyanin, sugar, proline, and ROS (MDA and H_2O_2), as well as the activity of peroxidase enzyme. *In situ* ROS levels were analyzed using fluorescence microscopy. Additionally, changes in transcript levels of genes related to oxidative stress (catalase, peroxidase, and glutathione S-transferase) were also evaluated, as were the effects of NiO NPs treatment on phytochemical concentrations (GSLs, phenolics, flavonoids, and anthocyanins) and related biosynthetic gene expression levels in Chinese cabbage seedlings.

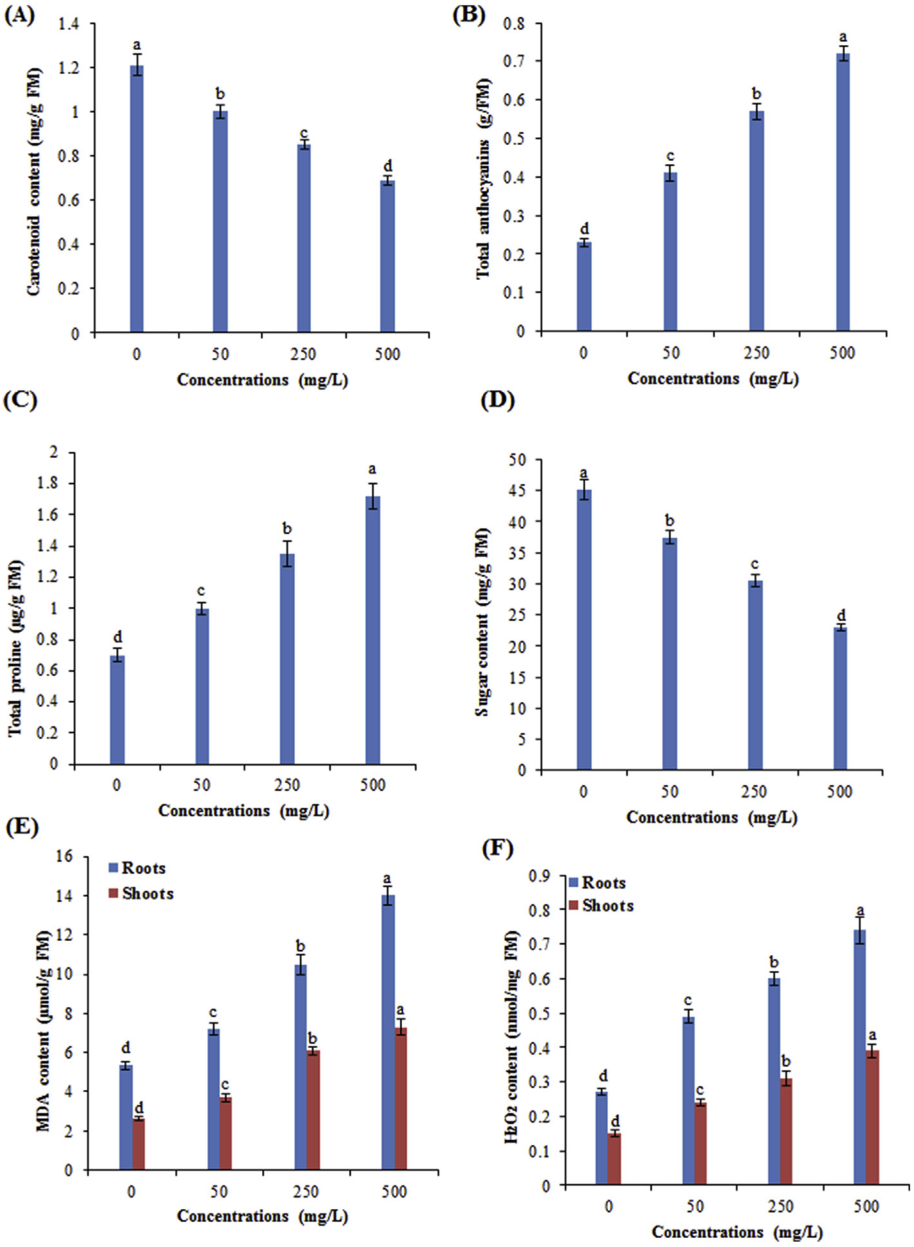

Fig. 2. Effects of treatment with different concentrations of NiO NPs on the contents of, **(A)** carotenoid, **(B)** anthocyanin, **(C)** proline, **(D)** sugar, **(E)** MDA and **(F)** H_2O_2 in 15 days-old *in vitro*-grown Chinese cabbage seedlings. Data are presented as means ± SD of three replicates. Different letters indicate a significant difference at $P \leq 0.05$.

2. Materials and methods

2.1. Seed treatment of NiO NPs

Chinese cabbage (*Brassica rapa* ssp. *Pekinensis* var. Seoul) seeds were sterilized with 70% ethanol for 1 min and then thoroughly washed in distilled water. NiO NPs (10–20 nm, 99%) were obtained from US Research Nanomaterials in Houston, TX, USA. The different concentrations (50, 250, and 500 mg/L) of NiO NPs were selected based on previous reports and our primary trials; bulk particles of $NiSO_4$ inhibited seedling germination, even at low concentrations, while exposure to low concentrations of NiO NPs (< 50 mg/L) had no significant effect on seedling growth. For each treatment, approximately 25–30 seeds were inoculated per culture box containing sterile filter paper to which 3–5 mL of NiO NPs solutions were added. Culture boxes were maintained for 15 days, at 25 °C, under a 16-h/8-h photoperiod. Three replicates were used for each concentration. The samples were harvested after 15 days, and the length of the seedlings (shoot and root) was measured using a ruler. All the fresh samples were stored at −80 °C for subsequent experiments.

2.2. Determination of Ni content

Shoots and roots from control and NiO NPs-exposed (50, 250, and 500 mg/L) plants were separated and dried at 75 °C for 48 h in a hot-air oven. The samples (100 mg) of the pulverized tissue were digested with 5 mL concentrated HNO_3 at 110 °C for 1 h in a dry bath. The digest was diluted to 20 mL with deionized water and filtered through 0.2 µm nylon filters. The Ni content was measured using ICP-MS (Varian 820-MS, USA), and the analysis was done in triplicate.

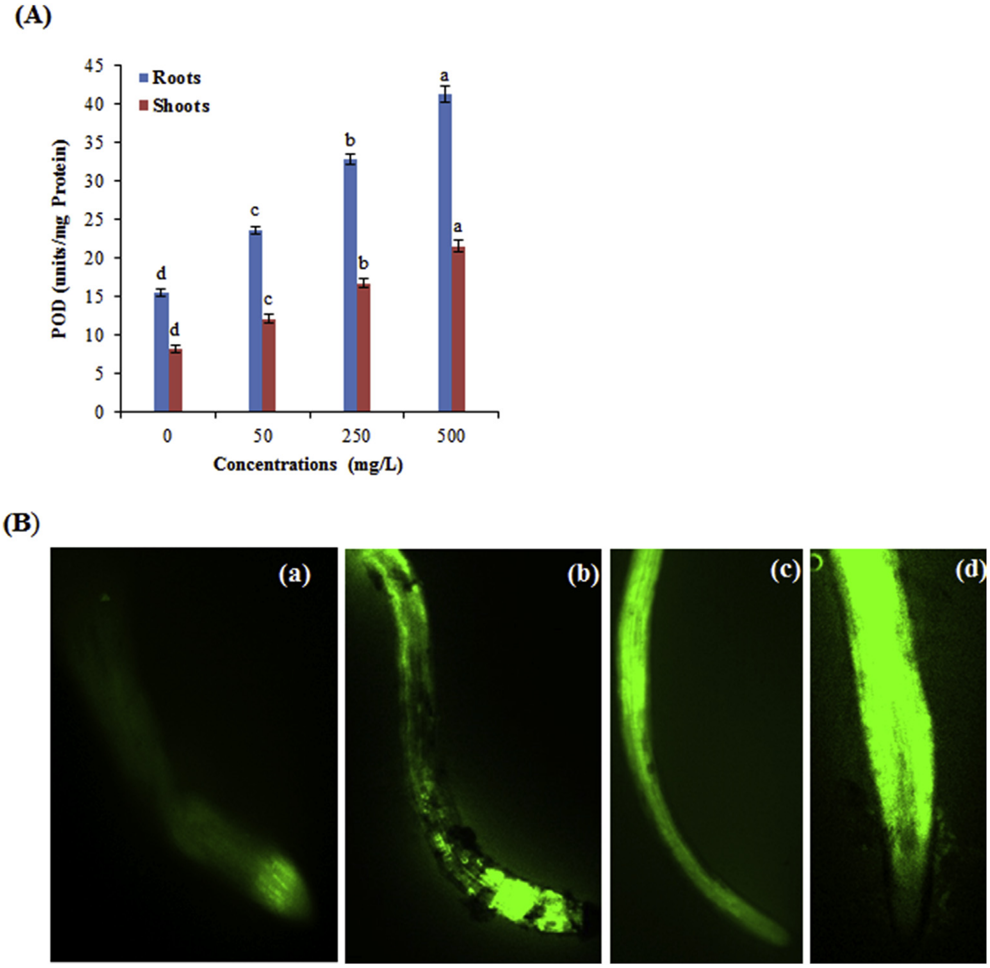

Fig. 3. Effects of NiO NPs treatment on **(A)** POD content and **(B)** ROS generation in the roots of 15 days-old *in vitro*-grown Chinese cabbage seedlings. Green fluorescence indicates the presence of ROS in the roots. **(a)** Control, **(b)** 50 mg/L NiO NPs, **(c)** 250 mg/L NiO NPs, and **(d)** 500 mg/L NiO NPs. Data are presented as means ± SD of three replicates. Different letters indicate a significant difference at $P \leq 0.05$. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

2.3. Estimation of chlorophyll, carotenoid, anthocyanin, proline, and sugar contents

Total chlorophyll and carotenoid contents of NiO NPs-treated and control Chinese cabbage seedlings were assessed as reported by Lichtenthaler (1987). The level of anthocyanin was evaluated as described previously by Thiruvengadam and Chung (2015). Proline content was estimated by spectrometry (Bates, 1973). Total soluble sugar content was assessed using a previously described method (Hedge and Hofreiter, 1962).

2.4. Estimation of lipid peroxidation, H_2O_2 content, and ROS generation

Lipid peroxidation assays were performed on root and shoot samples of NiO NPs-treated and non-treated seedlings (Heath and Packer, 1968). The H_2O_2 content was determined as previously reported (Brennan and Frenkel, 1977). Total ROS was measured qualitatively in control and NiO NPs-treated roots using the DCFH-DA staining procedure, as reported by Faisal et al. (2013).

2.5. Estimation of peroxidase activity

Peroxidase (POD; EC 1.11.1.7) enzymatic activity was measured according to the method of Fang and Kao (2000). Samples (100 mg) were homogenized in 1 mL 10 mM phosphate buffer (pH 6.8) and centrifuged at 12 000 × *g* for 15 min. The supernatant (25 μL) was mixed with 50 mM H_2O_2, 5 μL 250 mM guaiacol, and 195 μL 12.5 mM 3,3-dimethylglutaric acid (pH 6.0). All the extraction protocol was performed at 4 °C. POD activity was estimated by measuring the increase in absorbance at 470 nm after a 15 min incubation at room temperature. Enzyme activity was calculated as μM of guaiacol oxidized per min per gram of fresh mass (FM).

2.6. Estimation of phenolic compounds

2.6.1. Total phenolic and flavonoid contents

The total phenolic content (TPC) and the total flavonoid content (TFC) of NiO NPs-treated and non-treated seedlings were determined by spectrophotometry using Folin-Ciocalteu reagent and by using calorimetry, respectively, according to Thiruvengadam et al. (2014).

2.6.2. Individual phenolic compounds

The method used for the extraction of NiO NPs-treated and non-treated seedlings was performed as previously reported (Thiruvengadam and Chung, 2015). Sixteen phenolic compounds (PCs) were measured using UHPLC (Accela system, Thermo, USA) with a reverse phase column (C18, 2.1 × 100 mm, 2.6 mm) in NiO NPs-treated and non-treated seedlings. The solvent, standard, and gradient procedure followed the method previously described by Thiruvengadam and Chung (2015).

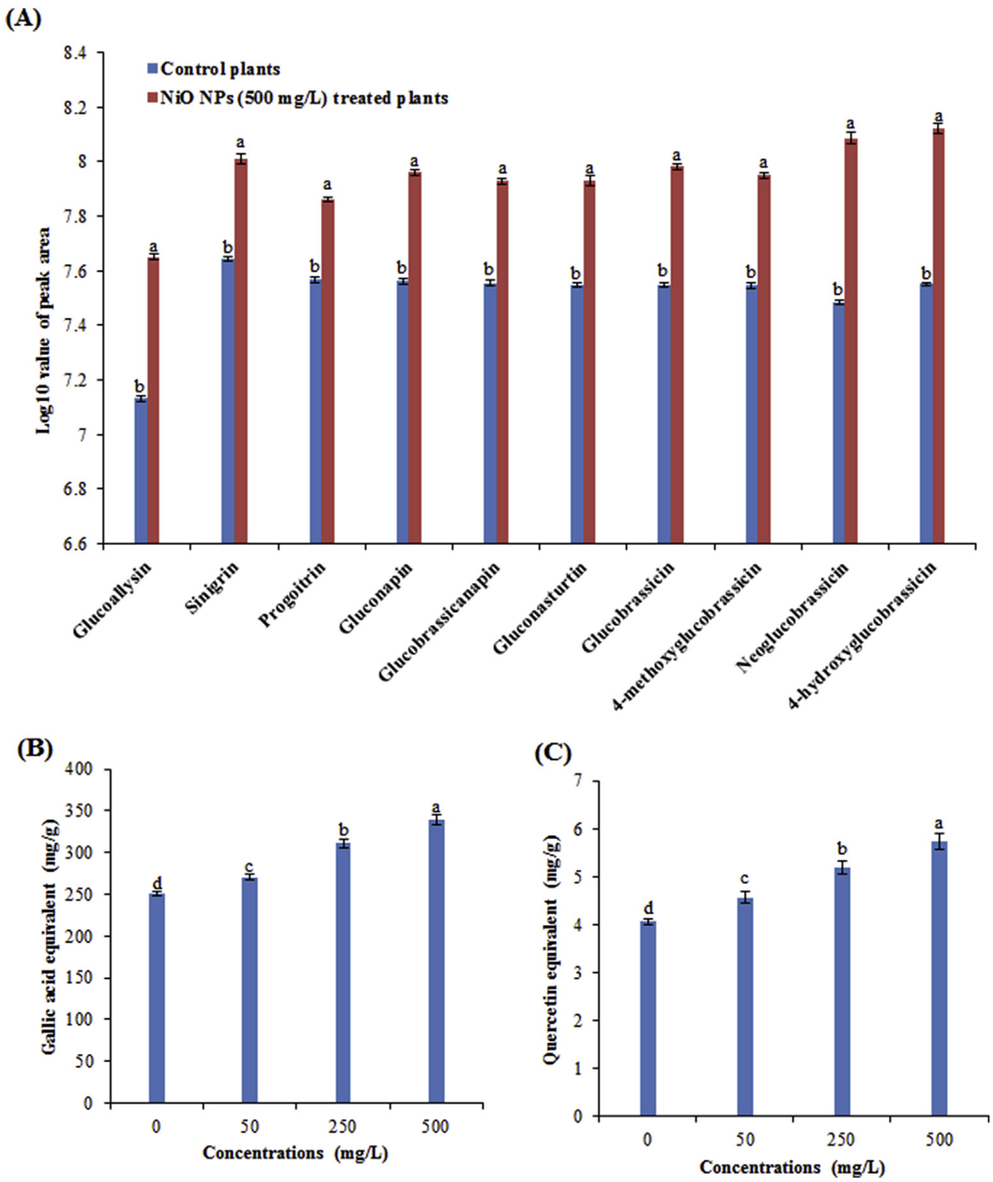

Fig. 4. Effect of NiO NPs treatment on phytochemical content. **(A)** GSLs identified by UHPLC-TQMS, **(B)** TPC, and **(C)** TFC in 15 days-old *in vitro*-grown Chinese cabbage seedlings. Data are presented as means ± SD of three replicates. Different letters indicate a significant difference at $P \leq 0.05$.

2.6.3. *Estimation of glucosinolates (GSLs)*

NiO NPs-treated and non-treated seedlings were extracted as reported previously (Thiruvengadam and Chung, 2015). GSLs were estimated using an EVOQ advanced UHPLC –TQMS (CTC PAL-xt Autosampler, Bruker, USA) system and samples were separated on a column (C18, 50 mm × 2 mm × 3 µm). The solvent and gradient procedures were according to Thiruvengadam and Chung (2015).

2.7. *Real-time polymerase chain reaction (RT-PCR)*

Total RNA was extracted from NiO NPs-treated and non-treated seedlings using an RNeasy plant Miniprep Kit (Qiagen, USA) according to the manufacturer's protocol. 2 µg of total RNA was used for the first-strand cDNA synthesis and the reaction was carried out according to the manufacturer's instructions. The primer sequences are presented in Supplementary Table 1. 100 ng of freshly synthesized cDNA was used as a template for RT-PCR. The RT-PCR was performed with a CFX 96 RT-PCR Detection System, using a SYBR® Green PCR Kit (Bio-Rad, USA). The PCR conditions, comparative threshold values, and relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method (Thiruvengadam and Chung, 2015).

2.8. *Data analysis*

All the experiments were done in triplicate and are expressed as the mean ± standard deviation (SD). Each experiment was repeated twice. The level of significance was determined at $P \leq 0.05$), and the means were separated using Duncan's multiple range test (DMRT).

3. Results and discussion

3.1. *Effects of NiO NPs on physiological parameters*

The effect of NiO NPs (50, 250, and 500 mg/L) on the growth profile of Chinese cabbage was examined for seed germination and the lengths of the shoots and roots. Seed germination and root elongation can be considered as signs of toxicity (Rao and Shekhawat, 2014). Compared to control seedlings, the root and shoot lengths decreased with increasing concentrations of NiO NPs (Fig. 1A–C). The morphological features indicated that treatment with the higher concentrations of NiO

Table 1

Effect of NiO NPs-treated on phenolic compounds identified by UHPLC analysis in 15 days-old *in vitro* grown seedlings of Chinese cabbage.

Compounds	Concentration (μg/g dry mass)	
	Control	NiO NPs (500 mg/L)
Flavonols		
Myricetin	98.06 ± 5.10^{g}	136.27 ± 5.25^{f}
Quercetin	274.56 ± 4.26^{c}	494.20 ± 8.90^{c}
Kaempferol	58.28 ± 0.23^{h}	95.70 ± 1.45^{g}
Catechin	151.1 ± 1.85^{e}	197.16 ± 8.34^{e}
Naringenin	9.63 ± 1.90^{l}	36.20 ± 0.92^{l}
Rutin	177.33 ± 4.22^{d}	219.39 ± 2.75^{d}
Hesperidin	24.88 ± 1.55^{k}	49.26 ± 1.11^{k}
Total	**793.84**	**1228.18**
Hydroxy-cinnamic acid		
p-Coumaric acid	142.29 ± 3.50^{f}	220.26 ± 2.10^{d}
Ferulic acid	501.99 ± 1.14^{b}	568.66 ± 1.52^{b}
Chlorogenic	56.94 ± 0.31^{h}	85.93 ± 3.06^{h}
t-cinnamic acid	5.40 ± 0.10^{m}	15.53 ± 0.63^{m}
Total	**706.62**	**890.38**
Hydroxy-benzoic acid		
p-hydroxy-benzoic acid	44.58 ± 1.53^{i}	84.95 ± 2.10^{h}
Protocatechuic acid	27.37 ± 1.09^{k}	35.21 ± 1.11^{l}
Syringic acid	35.71 ± 0.39^{j}	78.17 ± 0.35^{i}
Gentisic acid	514.15 ± 2.19^{a}	666.51 ± 7.95^{a}
Vanillin	37.60 ± 0.277^{j}	56.81 ± 4.66^{j}
Total	**659.41**	**921.65**

Means ± standard deviation of three replicates within a column followed by the same letter are not significantly different according Duncan's multiple range test at $P \leq 0.05$.

NPs (250 and 500 mg/L) significantly suppressed the growth of the plants (Fig. 1A–C). Biomass production was seen to be clearly and significantly affected by treatment with NiO NPs in *Hordeum vulgare* (Soares et al., 2018c) and in *Lycium barbarum* shoots grown under *in vitro* conditions (Pinto et al., 2019). Moreover, Faisal et al. (2013) revealed concentration-dependent suppression of root length in tomato treated with NiO NPs. In agreement with this, we observed a strong reduction in seedling length with the higher NiO NPs concentrations (250 and 500 mg/L). Treatment with the different concentrations of NiO NPs significantly enhanced Ni levels in the seedlings (Fig. 1D). However, the Ni content was higher in the roots than the shoots (Fig. 1D). Entry of dissolved metal NPs into cells was shown to be enhanced as a result of decreased pH and interaction with organic acids and proteins inside the plant tissues (Shi et al., 2014). Greater accumulation of NPs in the roots of Chinese cabbage could be easily elucidated by its root appearance (Fig. 1A).

3.2. Effect of NiO NPs on photosynthetic pigment content

Nanoparticles have been reported to induce chloroplast disorganization due to a decrease in the levels of thylakoid and grana that can result in the loss of chlorophyll content (Ebbs and Uchil, 2008). The total chlorophyll and carotenoid contents decreased gradually with NiO NPs treatment at 50, 250, and 500 mg/L compared to control plants (Figs. 1E and 2A). A substantial reduction in chlorophyll and carotenoid levels were observed at the higher concentration of NiO NPs (Figs. 1E and 2A). In accordance with our results, NiO NPs treatment resulted in a concentration-dependent reduction in chlorophyll and carotenoid contents in aquatic plants (Gong et al., 2011; Torbati, 2018). NiO NPs-treated plants exhibited injured photosynthetic pigments, while a significant reduction in both chlorophyll and carotenoid contents was observed in *Hordeum vulgare* (Soares et al., 2016, 2018c), *Solanum melongena* (Baskar et al., 2018) and *Lycium barbarum* (Pinto et al., 2019). The dissolution of Ni ions may play an important role in the toxicity of nanoparticles, such as altered chloroplast activity, in response to surplus Ni^{2+} (Appenroth et al., 2010).

3.3. Effect of NiO NPs on anthocyanin content

Anthocyanins are stress-responsive compounds, protecting plants from injury induced by ROS. Anthocyanin levels were measured in NiO NPs-treated and untreated Chinese cabbage seedlings. The total anthocyanin content with exposure to NiO NPs (50, 250 and 500 mg/L) was significantly increased compared to non-treated seedlings (Fig. 2B). Correspondingly, a greater amount of anthocyanin was also recorded in *Solanum melongena* and *Arabidopsis* plants exposed to NiO NPs (Baskar et al., 2018) and CuO NPs (Nair and Chung, 2014). Anthocyanins act as non-enzymatic antioxidants in plants, defending cells against ROS-induced oxidative stress (Gill and Tuteja, 2010), suggesting that the observed increase in anthocyanin concentration in Chinese cabbage treated with NiO NPs may have been due to oxidative stress.

3.4. Effect of NiO NPs on proline and sugar contents

Proline has been described as improving plant resistance to oxidative stress by scavenging free radicals and increasing the activity of anti-oxidative enzymes, thereby helping to sustain redox homeostasis (Matysik et al., 2002). Preeti and Tripathi (2011) reported that a reduction in sugar levels observed in *Albizia procera* seedlings exposed to different NPs might result from the decreased synthesis of storage sugars and increased synthesis of other phytochemicals. We measured the proline and sugar contents in the NiO NPs (50, 250 and 500 mg/L) treated and non-treated seedlings and found a significant increase in proline content in the seedlings treated with NiO NPs compared to non-treated seedlings (Fig. 2C). Similarly, Rao and Shekhawat (2016) found higher levels of proline in CuO and TiO_2 NPs-treated *B. juncea* plants than in control plants. In this study, we observed a significant decrease in sugar content with NiO NPs treatment (50, 250 and 500 mg/L; Fig. 2D), possibly due to reduced rates of photosynthesis resulting from a reduction in photosynthetic pigments under NiO NPs stress. The observed proline accumulation may be a plant protective mechanism to prevent protein denaturation and ROS-induced injury to cellular structures (Chiang and Dandekar, 1995).

3.5. Effect of NiO NPs on MDA, H_2O_2 content, and antioxidant enzymes

Levels of lipid peroxidation is a potential indicator of cellular membrane injury caused by oxidative stress. Tuteja et al. (2001) demonstrated that stress induces membrane lipid peroxidation, resulting in membrane degradation and production of thiobarbituric acid reactive species, particularly MDA, that can degrade DNA. Quantification of lipid peroxidation and H_2O_2 content in seedlings exposed to different concentrations of NiO NPs (50, 250 and 500 mg/L) showed significant increases in both parameters compared to non-treated seedlings (Fig. 2E and F). However, a considerable increase in lipid peroxidation and H_2O_2 content was observed with NiO NPs treatment (50, 250 and 500 mg/L) in the shoot and roots, but with higher levels in the roots than the shoots. A gradual, dose-dependent increase in MDA and H_2O_2 concentrations was seen with NiO NPs treatment, indicating NPs-mediated induction of oxidative stress. Higher accumulation of MDA is indicative of increased ROS production. As in this study, MDA levels were seen to increase with exposure to NiO NPs in *Allium* spp. (Manna and Bandyopadhyay, 2017), *Solanum melongena* (Baskar et al., 2018) and *Lycium barbarum* (Pinto et al., 2019). Moreover, treatment with NiO NPs also significantly increased MDA and H_2O_2 levels in *Hordeum vulgare* (Soares et al., 2016). The enhancement of MDA production indicates membrane damage in roots exposed to NiO NPs. Peroxidase (POD) involved in several crucial functions such as auxin catabolism, polymerization of lignin in the presence of H_2O_2, growth regulation and cross-linking of pectins (Gaspar et al., 1991; del Rıo et al., 2006). POD enzyme activity was measured to investigate the level of oxidative stress in Chinese cabbage seedlings treated with NiO NPs. The activity of POD was higher in NiO NPs-treated plants than control plants

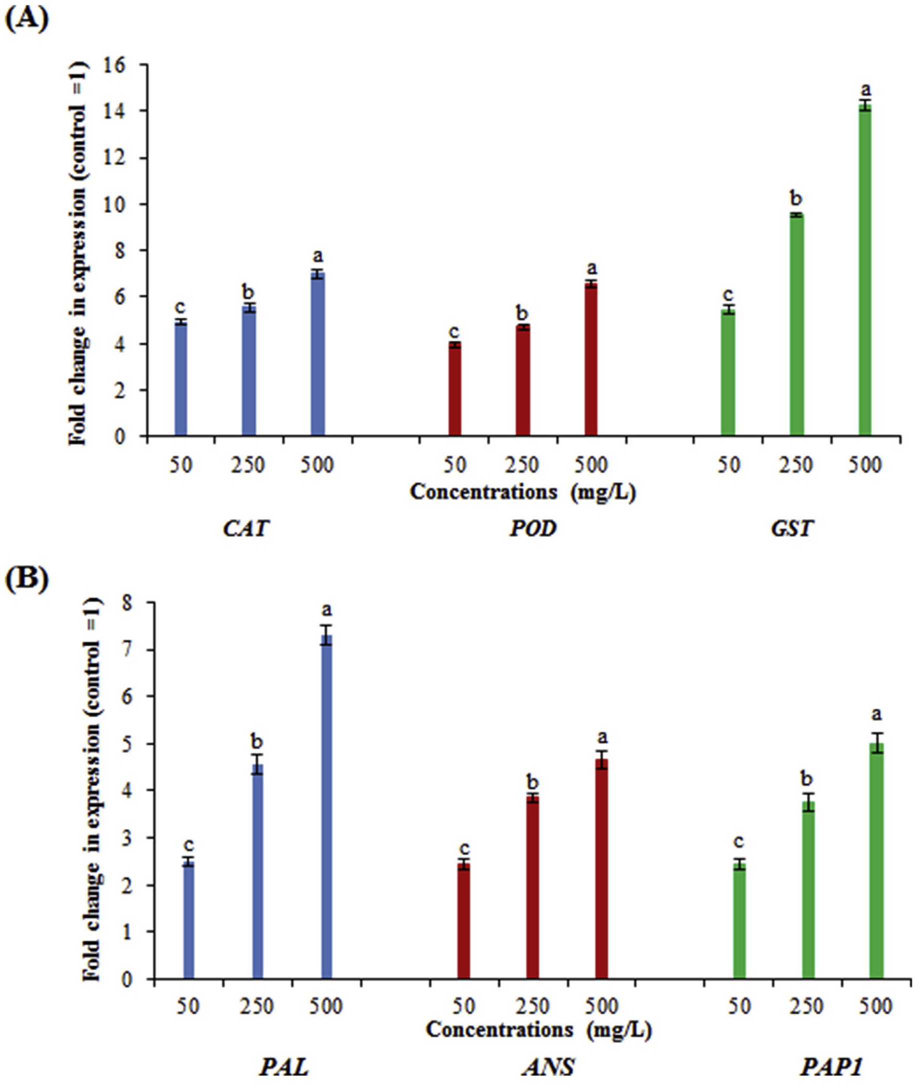

Fig. 5. Effect of treatment with different concentrations of NiO NPs on the mRNA expression levels of **(A)** antioxidant enzymes (*CAT*, *POD*, and *GST*), **(B)** phenolic biosynthetic genes (*PAL ANS*, and *PAP1*) in 15 days-old *in vitro*-grown Chinese cabbage seedlings. Data are presented as means ± SD of three replicates. Different letters indicate a significant difference at $P \leq 0.05$.

(Fig. 3A), similar to the results already documented in *Allium* spp. (Manna and Bandyopadhyay, 2017).

3.6. Effect of NiO NPs on intracellular ROS formation

Measurement of DCF fluorescence is widely used as an optimistic test for evaluation of oxidative stress in living organisms. Microscopic analysis revealed that NiO NPs-treated root tips exhibited significantly increased fluorescence (Fig. 3B b–d) compared to control root tips (Fig. 3B a). Previous studies have confirmed that higher NiO NPs concentrations resulted in higher levels of ROS generation in tomato (Faisal et al., 2013) and *Lemna gibba* (Oukarroum et al., 2015). Exposure to NiO NPs may result in programmed cell death via cellular damage in which ROS play a major role. To alleviate the opposing effects of ROS generated under abiotic stress, plant cells increase the activity of different antioxidant enzymes, establishing a first line of defense against oxidative stress and toxicity inside the cell (Gill and Tuteja, 2010; Soares et al., 2018b).

3.7. Effect of NiO NPs on the levels of phytochemicals

3.7.1. Estimation of GSLs content

GSLs are one of the known plant defense compounds that responds to various biotic and abiotic stresses in plants (Baskar et al., 2012). In the present study, we evaluated the influence of NiO NPs, a metal oxide NPs on the profile of individual GSLs in Chinese cabbage seedlings (Fig. 4A). UHPLC-TQ-MS identified five aliphatic GSLs (gluconapin, glucoallysin, glucobrassicanapin sinigrin, and progoitrin), four indolic GSLs (4-methoxyglucobrassicin, 4-hydroxyglucobrassicin, neoglucobrassicin, and glucobrassicin) and one aromatic GSL (gluconasturtiin). All types of GSLs (aromatic GSLs, indolic GSLs, and aliphatic GSLs) were significantly increased in the NiO NPs (500 mg/L) treated seedlings compared to control seedlings. In accordance with this, Asad et al. (2013) reported that Ni treatment induced the production of total GSLs in *Thlaspi caerulescens* (hyperaccumulator species). The exposure of *Arabidopsis halleri* to zinc and cadmium resulted in a significant increase in the total GSLs compared to control plants. In addition, the GSLs accumulation was higher in the initial growth period compared to later stages (Kazemi-Dinan et al., 2015). Similarly, in our previous study, we also have shown that the cadmium chloride ($CdCl_2$) induced the production of various types of GSLs in *B. rapa* seedlings (Thiruvengadam

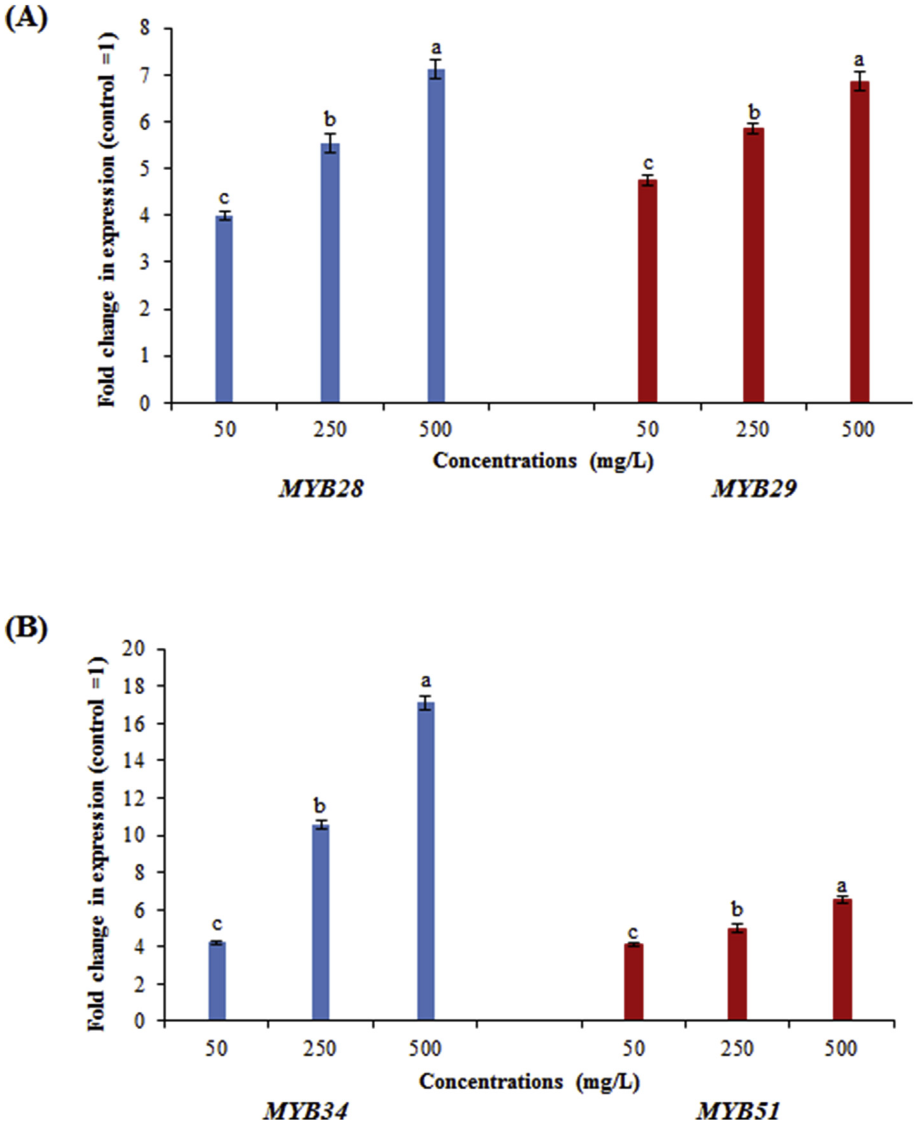

Fig. 6. Effect of treatment with different concentrations of NiO NPs on the mRNA expression levels of **(A)** aliphatic GSL biosynthesis (*MYB28* and *MYB29*) and **(B)** indolic GSL (*MYB34* and *MYB51*) transcription factor genes in 15 days-old *in vitro*-grown Chinese cabbage seedlings. Data are presented as means ± SD of three replicates. Different letters indicate a significant difference at $P \leq 0.05$.

and Chung, 2015). Among them, gluconasturtiin concentration was significantly increased in $CdCl_2$ treated *B. rapa* seedlings (Thiruvengadam and Chung, 2015).

3.7.2. *Estimation of total phenolic and flavonoid contents*

Phenolic compounds (PCs) and organic acids are thought to regulate enzymes and the cell cycle and contribute to organoleptic characteristics (Pulido et al., 2000). Polyphenols can also enhance plant defenses against heavy metal stress by chelating metals and scavenging reactive oxygen species (Oloumi et al., 2015). In this study, we analyzed TPC and TFC in NiO NPs-treated and control samples. A gradual increase in TPC and TFC concentrations was seen with the NiO NPs treatment, and the seedlings treated with 500 mg/L of NiO NPs accumulated higher TPC and TFC concentrations (Fig. 4B and C). Similar to our observations, some earlier studies indicated that NPs could increase TPC and TFC contents in seedlings of different plant species in a concentration-dependent manner (Oloumi et al., 2015; Zafar et al., 2016; Baskar et al., 2018).

3.7.3. *Estimation of individual phenolic compounds*

Heavy metals at a higher concentration can induce toxicity through the generation of ROS in plants. Plants can respond to ROS through the production of antioxidants (enzymatic and non-enzymatic) that can suppress ROS molecules. Phenolic acids are one of the non-enzymatic antioxidant molecules in plants and provide tolerance to various abiotic stresses. We explored the influence of the NiO NPs-treated and control seedling samples detected by UHPLC (Table 1). We identified sixteen PCs, consisting of seven flavonols (kaempferol, myricetin, quercetin, catechin, naringenin, rutin, and hesperidin), four hydroxycinnamic acids (*p*-coumaric, *t*-cinnamic, chlorogenic, and ferulic acids), and five hydroxybenzoic acids (vanillin, *p*-hydroxybenzoic, protocatechuic, syringic, and gentisic acids). The NiO NPs (500 mg/L) treated seedlings contained considerably higher hydroxybenzoic acid (921.65 μg/g), hydroxycinnamic acid (890.38 μg/g), and flavonol (1228.18 μg/g) contents than control seedling, at 659.41, 706.62, and 659.41 μg/g, respectively (Table 1). In accordance with our study, the foliar application of Ni on the *Fagopyrum esculentum* results in the elevation of ROS, total phenolics and phenolic acids (Sytar et al., 2015). Time and concentration-dependent enhancement of phenolic acids was observed in Ni-treated *F. esculentum* plants. The significant increase in the *p*-hydroxybenzoic, chlorogenic, *p*-anisic, hesperetic, and caffeic acids at post 24 & 48 h of Ni treatment, while hesperetic, *p*-hydroxybenzoic, *p*-anisic, caffeic acids and *p*-coumaric contents were increased after 72 h of exposure. Similarly, the levels of ferulic acid and isovitexin were elevated with cadmium oxide NPs treatment in barley plants (Večeřová et al., 2016), while Jasim et al. (2017) reported a significant enhancement of diosgenin levels in fenugreek treated with Ag NPs. We conclude that the increased amounts of phenolic compounds could be correlated with NiO NPs induced ROS generation which is evidenced through enhanced MDA formation and H_2O_2 generation.

3.8. Effect of NiO NPs on the antioxidant enzyme and phytochemical (phenolics and GSLs)-related gene expression

We used RT-PCR to study the effects of NiO NPs exposure on Chinese cabbage seedlings by measuring the relative transcript levels of genes involved in the biosynthesis of antioxidant enzymes (*CAT*, *POD*, and *GST*), phenolic compounds (*PAP1*, *ANS*, and *PAL*), and glucosinolates (*BrMYB28*, *BrMYB29*, *BrMYB34,* and *BrMYB51*). Antioxidant enzymes are biomarkers that specify the level of oxidative stress (Gill and Tuteja, 2010). Catalases (CAT) are involved in maintaining cellular redox status in cells by scavenging excess H_2O_2 (Du et al., 2008), and we found that *CAT*, *POD*, and *GST* transcript levels were higher with increasing NiO NPs concentrations of up to 500 mg/L (Fig. 5A). Similar to our results, *CAT* expression was higher in *Oryza sataiva* exposed to Ag NPs compared to controls (Nair and Chung, 2014). Correspondingly, in the present investigation, substantial transcriptional up-regulation of antioxidant genes was observed in treated seedlings, which correlates with increased H_2O_2 generation. It is known that SOD catalyses the conversion of superoxide to H_2O_2, whereas CAT and POD catalyze H_2O_2 breakdown (Bowler et al., 1992). Exposure to CuO NPs significantly increased the expression levels of antioxidant genes such as *CAT*, *SOD,* and *POD* in chick pea (Nair and Chung, 2014). The transcript levels of *ANS* and *PAP1* were progressively up-regulated in seedlings treated with higher concentrations (250 and 500 mg/L) of NiO NPs (Fig. 5B). These observations reflect the expected effects of NiO NPs on anthocyanin levels reported in section 3.3 above; meanwhile, the expression of the *PAP1* gene has been associated with anthocyanin levels (Borevitz et al., 2000). Flavonoid and anthocyanin biosynthetic genes in *A. thaliana* were shown to be upregulated with Ag NPs treatment (Garcia-Sanchez et al., 2015). *PAL* expression was substantially higher in the NiO NPs-exposed than non-treated seedlings (Fig. 5B). Transcriptional activation of *PAL* has been shown to influence several environmental stress factors in various plant species (Winkel-Shirley, 2002). GSLs are nitrogen- and sulfur-containing secondary metabolites involved in biotic and abiotic stress resistance (Martinez-Ballesta et al., 2013). Increased GSL accumulation has been linked to the higher expression of genes involved in GSL biosynthesis pathway regulation of aliphatic GSL (*MYB28* and *MYB29*) and indolic GSL (*MYB51* and *MYB34*) genes (Thiruvengadam and Chung, 2015). AGSLs (*BrMYB28* and *BrMYB29*) and IGSLs (*BrMYB34* and *BrMYB51*) transcripts were higher in seedlings treated with higher concentrations (250 and 500 mg/L) of NiO NPs (Fig. 6A and B).

4. Conclusions

This investigation confirmed that treatment with NiO NPs elicited a toxic response in Chinese cabbage seedlings. NiO NPs induced a substantial reduction in plant biomass and inhibition of shoot and root growth in a dose-dependent manner. Analysis of metal uptake revealed a high Ni content, indicating the uptake and dissolution of NiO NPs in the plants. Chlorophyll, carotenoid, and sugar contents declined sharply in the NiO NPs treated seedlings, whereas the levels of anthocyanin and proline were significantly enhanced. High Ni accumulation elicited significant lipid peroxidation, increased levels of H_2O_2 and total ROS, and antioxidant enzyme (POD) activity in the roots. Furthermore, NiO NPs treatment resulted in increased transcript levels of genes coding for antioxidant enzymes (*CAT*, *POD*, and *GST*) and phytochemicals (GSLs, phenolics, and flavonoids). Our results demonstrate that high concentrations of NiO NPs induce toxic effects, as well as increased phytochemical contents, in Chinese cabbage seedlings.

Author contributions

M. Thiruvengadam carried out the experiments and also wrote the manuscript. V. Baskar carried out molecular analyses and helped to write the manuscript. I. M. Chung designed the experiments and also wrote the manuscript the experiments.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2019.03.010.

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