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Research Article

Effects of cobalt oxide nanoparticles (Co_3O_4 NPs) on ion leakage, total phenol, antioxidant enzymes activities and cobalt accumulation in *Brassica napus* L.

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Abstract

Interaction of nanoparticles (NPs) as a significant threat to ecosystems with biological processes of plants is very important. Here, the effects of cobalt oxide (Co_3O_4) NPs on some physio-biochemical characteristics of *Brassica napus* L. were investigated. The two-weeks seedlings were sprayed with different concentrations of Co_3O_4 NPs (0, 50, 100, 250, 500, 1000, 2000, and 4000 mg L^{-1}). The results showed that this treatment significantly affected the fresh and dry weights, area, relative water content (RWC) and relative chlorophyll value (SPAD) of leaves. The highest reduction of growth and biomass indexes occurred at 4000 mg L^{-1} NPs. The content of H_2O_2 and electrolyte leakage (EL) increased respectively, after 100 and 250 mg L^{-1} of Co_3O_4 NPs and showed a maximum level at 4000 mg L^{-1}. The activities of phenylalanine ammonia lyase (PAL), ascorbate peroxidase (APX) and superoxide dismutase (SOD) increased after 100 mg L^{-1} of Co_3O_4 NPs. However, tyrosine ammonia lyase (TAL) activity enhanced after 500 mg L^{-1}. The catalase (CAT) activity and protein content decreased after 1000 mg L^{-1} of Co_3O_4 NPs. Application of concentrations higher than 500 mg L^{-1} of Co_3O_4 NPs induced polyphenol oxidase (PPO) activity but reduced glutathione reductase (GR). The activities of guaiacol peroxidase (GPX) and glutathione S-transferase (GST) increased at 250-1000 mg L^{-1} of Co_3O_4 NPs and then decreased. These results suggested that low concentrations of Co_3O_4 NPs induced a positive effect on growth parameters but high levels caused extensive oxidative damage and mediated defense responses by organization of phenolic compounds and antioxidative system.

Keywords: antioxidant defense system; environmental concerns; glutathione S-transferase; nanotoxicity; oxidative stress; phenolic compounds; phenylalanine and tyrosine ammonia lyase

Introduction

More a last decade, nanotechnology has gained a huge research notice because of its applications in public health, medicine, industry, and agriculture. Nanometals stimulate plant growth and activate metabolic processes in plant (Rizwan *et al.*, 2017). Due to their small size, nanoparticles (NPs) can enter into cell membrane (Chichiriccò and Poma, 2015). Thus, interaction between NPs and plants is one of the most

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promising areas of research in nanoscience. In previous studies, the biological effects of NPs, as well as their useful and dangerous effects on plants have determined (Lee *et al.*, 2010).

Cobalt (Co) as a transition metal and magnetic element with atomic number 27 and atomic weight 58.9 g mol^{-1} has properties similar to iron and nickel (Gál *et al.*, 2008). The low concentration of Co has been showed positive effect on plants. In legumes nutrition, Co has necessary function for the atmospheric nitrogen fixing in microorganisms (Minz *et al.*, 2018). However, its effect to the rest of the plant species is still unclear. In during the photosynthetic process, respiration and cell growth of crops, the sufficient supply of Co is important (Palit *et al.*, 1994; Minz *et al.*, 2018). In different cellular processes of human and animal, including the oxidation of fatty acids and the synthesis of DNA, Co acts as a coenzyme or is important for the synthesis of different enzymes. However, in agricultural crops with critical role in the human food chain, Co accumulation may be resulted in toxic effects depend on plant species, type and chemistry of soil (Bakkaus *et al.*, 2005). Relatively higher concentrations of Co have toxic effects on plants, including leaf fall, bleached veins, closure of premature leaf, inhibition of active transport and greening, as well as disruption in chlorophyll biosynthesis (Ayeni *et al.*, 2010).

Cobalt oxide is a significant material that finds applications in diverse fields such as catalysis, different types of sensors and electrochromic and other devices. Cobalt NPs are the most interesting chemical elements for biomedical applications e.g. $CoFe_2O_4$ NPs for drug delivery or $CoFe_2O_4/SiO_2/Ag$ NPs composite for antibacterial activity (Kooti *et al.*, 2015). Different plants species can uptake and accumulate NPs in their tissues depend on the size, composition, and accessibility of NPs in the media (Lee *et al.*, 2010; Schwab *et al.*, 2011; Rico *et al.*, 2013; Rizwan *et al.*, 2017).

The excess reactive oxygen species (ROS) in plants under various stressful environmental conditions such as NPs exposure can cause oxidative stress (Sharma *et al.*, 2012). Antioxidant defense system of plants contain non-enzymatic antioxidants (thiols, glutathione (GSH), phenolics, ascorbate (AsA)) and enzymatic components (catalase (CAT), superoxide dismutase (SOD), guaiacol peroxidase (GPX), and glutathione reductase (GR)) (Ma *et al.*, 2015). It has been noted that NPs influence the plant defense enzymes (Rico *et al.*, 2013). Cobalt ferrite ($CoFe_2O_4$) NPs decreased CAT activity in tomato, (López-Moreno *et al.*, 2016). Also, in *Brassica juncea* root, CuO NPs increased SOD activity and decreased APX activity (Nair and Chung, 2015).

Rapeseed (*Brassica napus* L.) is a member of family Brassicaceae and it is one of the most commonly cultivated oil crops in the world because of the healthy fatty acid composition (Nath *et al.*, 2016). To the best of the authors' knowledge, there is no study on the effects of cobalt oxide NPs in rapeseed.

Fast development of nanotechnology has enabled the production of NPs in several industries as well as in agriculture. This has raised ecotoxicological concerns due to the release of NPs to the environment. On the other hand, plants are the entry point of NPs into the food chain. Therefore, this study was conducted to determine the effects of Co_3O_4 NPs spraying application on morphological characteristics, relative water content, oxidative stress, phenolic compounds and enzymatic defense responses of *B. napus*.

Materials and Methods

Preparation of cobalt oxide nanoparticles

The Co_3O_4 NPs were purchased from US Research Nanomaterials Company (Houston, TX, USA). The NPs stock suspension in deionized water were ultrasonically (Ultrasonic Cleaner, Fungilab, model S.A. 160 W-40 Hz) dispersed for 45 min before use. Then, the NPs were assessed for their particle mean diameter and size distribution by dynamic light scattering (DLS) method using a particle size analyser (model VASCO 3, Cordouan, Pessac, France) at 25 °C. Moreover, zeta potential was measured at pH 6.30 by a zetasizer (Nano-ZS, model ZEN3600). The particles size was evaluated using a transmission electron microscopy (TEM; model LEO 912 AB, Zeiss, Germany) and also by a field emission scanning electron microscopy (FE-SEM; model Mira 3-XMU, Tescan, Czech Republic). Furthermore, the structure of NPs was determined by an X-ray

diffractometer (XRD; model EXPLORER, GNR, Italy, 40 kV, 30 mA). Also, the elemental analysis of NPs was assessed by energy dispersive X-ray (EDX; SAMx, Germany).

Plant growth and nanoparticles treatment

This experiment was conducted in a completely randomized design with four replications. Pots (18 cm height × 19 cm diameter) were filled with proper soil (a mixture of loam, clay, and sand (2:2:1 ratio)) and in each pot, five seeds of rapeseed (*Brassica napus* L. cv. 'Zarfam') were sown. Pots were placed in a growth chamber with 16/8 h photoperiod, 25±5 °C day/night temperatures and 30 ± 5% relative humidity. After two weeks; seedlings were treated with different concentrations (0, 50, 100, 250, 500, 1000, 2000, and 4000 mg L^{-1}) of Co_3O_4NPs with foliar spray method. Treatments were carried out for five weeks and in treatment period, five times spray were done on leaves (Figure 1). After the treatment period, the harvested plants were carefully washed with distilled water and some morphological traits including fresh and dry weights of leaves were measured. Remained samples were frozen in liquid nitrogen for later examinations. All chemicals and reagents used in this study were of analytical grade.

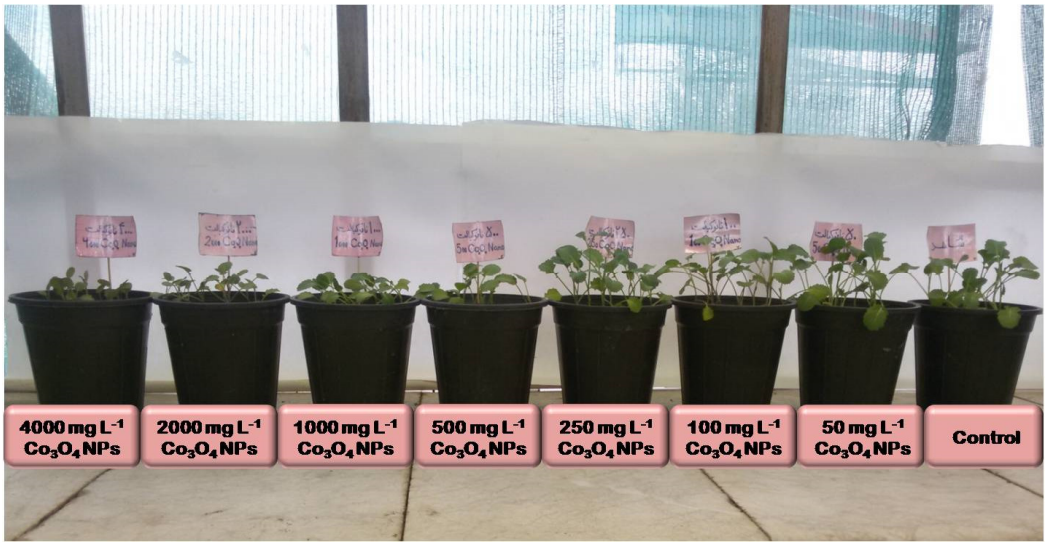

Figure 1. Impact of different concentrations of Co_3O_4 NPs on *Brassica napus* grown under controlled conditions in a growth chamber

Number, area, relative chlorophyll value (SPAD) and relative water content (RWC) of leaves

Number of leaves was considered. Leaf area was calculated according to the method of Pandey and Singh (2011). Relative chlorophyll value was calculated indirectly and without degradation in leaves, by a chlorophyll meter (SPAD 502, Minolta Co. Ltd., Japan) after the treatment and before harvesting. The RWC of leaves was assessed according to the method of Wheatherley (1950).

Hydrogen peroxide content and electrolyte leakage (EL)

The hydrogen peroxide (H_2O_2) content was assayed at 390 nm based on the reaction between potassium iodide (KI) and H_2O_2 in an acidic environment, as described by Alexieva *et al.* (2001).

EL was measured for each sample by a conductivity meter (CM-115, Kyoto Electronics, Japan) before and after autoclaving (121 °C for 20 min) (Dionisio-Sese and Tobita, 1998).

Total phenolic and anthocyanin contents

Total phenolic content was measured at 765 nm and gallic acid was used as a standard for the calibration curve (Singleton and Rossi, 1965).

The anthocyanin content of leaves was measured based on the method of Wagner (1979). The concentration was determined at 550 nm using the extinction coefficient of 33,000 M^{-1} cm^{-1}.

Phenylalanine ammonia lyase (PAL) and tyrosine ammonia lyase (TAL) activities assay

PAL (EC 4.3.1.24) activity was assayed by determining the rate of cinnamic acid production at 290 nm. One unit of enzyme activity was expressed as μmol cinnamic acid mg^{-1} protein min^{-1} (Beaudoin-Eagan and Thorpe, 1985).

TAL (EC 4.3.1.23) activity was determined by determining the rate of coumaric acid production at 333 nm. One unit of enzyme activity was expressed as μmol coumaric acid mg^{-1} protein min^{-1} (Beaudoin and Thorpe, 1985).

Activities of antioxidant enzymes (APX, SOD, CAT, GPX, PPO, GR and GST) and protein content

Total protein content was assayed with bovine serum albumin (BSA) as a standard using the dye-binding method of Bradford (1976).

Ascorbate peroxidase (APX; EC 1.11.1.11) activity was estimated by a spectrophotometric method as described by Nakano and Asada (1981) and APX was assayed by recording the decrease in optical density due to ascorbic acid at 290 nm. One unit of enzyme determines the amount needed to decompose 1 μmol of ascorbate per min.

Superoxide dismutase (SOD; EC 1.15.1.1) activity was determined by the method of Giannopolitis and Ries (1977) and one unit of enzyme is the amount of SOD that inhibits the rate of nitro blue tetrazolium (NBT) formation by 50% at 560 nm.

Catalase (CAT; EC 1.11.1.6) activity was measured using the extinction coefficient of 40 mM^{-1} cm^{-1} at 240 nm and expressed as μmol of H_2O_2 min^{-1} mg^{-1} protein (Aebi, 1984).

Guaiacol peroxidase (GPX; EC 1.11.1.7) activity was determined according to the method of MacAdam *et al.* (1992) by measuring the increase in absorbance for 3 min at 436 nm.

Polyphenol oxidase (PPO; EC 1.14.18.1) activity was estimated by a spectrophotometric method as described by Raymond *et al.* (1993). The activity was expressed as change in absorbance at 430 nm for 4 min.

Glutathione reductase (GR; EC 1.6.4.2) activity was calculated based on an extinction coefficient of 6.2 mM^{-1} cm^{-1} for NADPH at 340 nm for 3 min (Foyer and Halliwell, 1976).

Glutathione S-transferase (GST; EC 2.5.1.18) activity was determined according to Habig *et al.* (1974) and was assayed spectrophotometrically with reduced glutathione (GSH) and 1-chloro-2,4-dinitrobenzene (CDNB) as substrates. This was done by watching an increase in absorbance at 340 nm. The activity was calculated based on an extinction coefficient of 9.6 mM^{-1} cm^{-1}.

Leaf Co accumulation

Cobalt content of leaf was determined by inductively coupled plasma optical emission spectrometry (ICP-OES; SPECTRO ARCOS 76004555, Germany). Oven-dried leaves (0.5 g) were ground to a fine powder. Powdered samples were digested with 5:1 (v/v) solution of concentrated HNO_3:30% H_2O_2. Prepared digests were heated at 90 °C. After the sample digest was clear, heating was continued until near dryness. Then, the digest was brought to a final volume of 25 mL with deionized water; Co concentration of leaf was defined by ICP-OES (Kalra, 1998).

Statistical analysis

The experiment was conducted in a completely randomized design. The data were subjected to one-way analysis of variance (ANOVA) using SPSS version 22.0 (IBM Corp, Armonk, NY, USA, 2013). Duncan's

multiple range test was used to compare the means at 5% probability level. All the errors were expressed as standard deviation of four replicates. The graphs were plotted in Excel (Microsoft Office).

Results

Characteristics of Co_3O_4 NPs

Figure 2A-C showed the size of Co_3O_4 NPs were <50 nm by TEM and FE-SEM measurements. Figure 2D showed the XRD form of Co_3O_4 NPs among 2θ° angles of 10°-80°. The diffraction peaks at 2θ°: 19.25°, 31.54°, 37.09°, 38.81°, 45.07°, 56.01°, 59.61° and 65.51° corresponded to (111), (220), (311), (222), (400), (422), (511) and (440) planes, respectively and were readily indexed to a pure cubic phase structure (JCPDS file No. 01-074-2120) (Figure 2D). The data of DLS revealed that hydrodynamic size of Co_3O_4 NPs based on number, intensity, and volume was respectively equal to 26.01, 81.64 and 47.24 nm and also, the average of hydrodynamic diameter was equal to 71.84 with a polydispersity index (PDI) of 0.212 (Figure 2E-G). Zeta potential of Co_3O_4 NPs was -21.37 mV at pH 6.30 with the mobility of -1.61 $\mu m^{-1} s^{-1} V^{-1}$ cm (Figure 2H-I). EDX analysis confirmed that all of the detected particles contained cobalt and oxygen (Figure 2J).

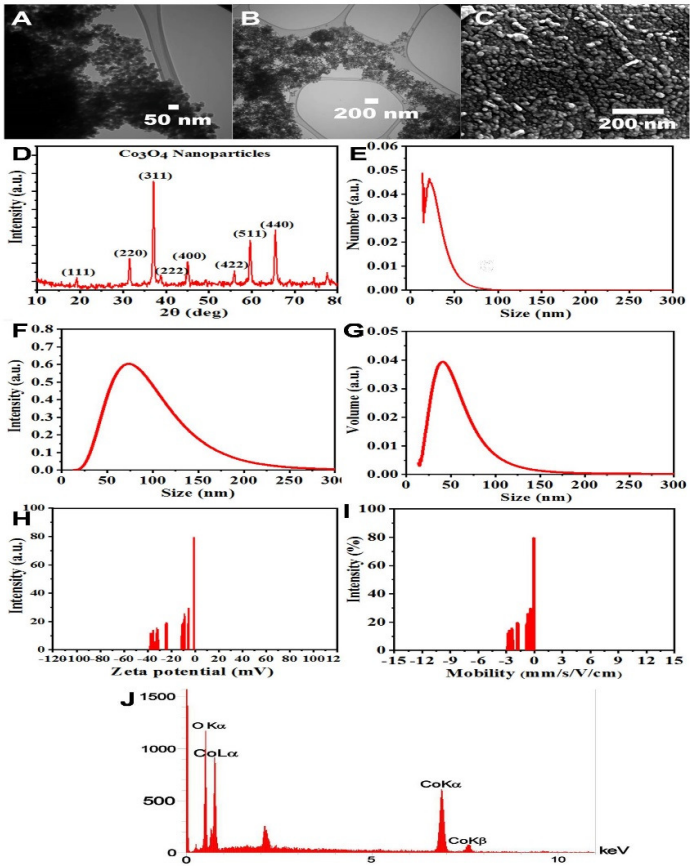

Figure 2. Images of TEM (scale bars: 50 and 200 nm) (A-B), FE-SEM (scale bar: 200 nm) (C), XRD (D), hydrodynamic diameter based on number, intensity, and volume (E-G), zeta potential (H) mobility (I) and EDX (J) of Co_3O_4 NPs

Growth parameters, RWC, and SPAD of leaves

Effects of Co_3O_4 NPs on the growth parameters and RWC of *B. napus* leaves are presented in Figure 3A-E. In comparison to the control, low concentrations of Co_3O_4 NPs (50 and 100 mg L^{-1}) increased fresh

weight (FW) of leaves but high concentrations (500-4000 mg L^{-1}) decreased it (Figure 3A). The concentration of 50 mg L^{-1} of NPs increased the dry weight (DW) of leaves by 9.5% of the control (Figure 3B). The lowest DW were observed at 2000 and 4000 mg L^{-1} of Co_3O_4 NPs treatment (Figure 3B). On the other hand, Co_3O_4 NPs did not affect leaf number at 50-1000 mg L^{-1}, but a ~26.9% reduction was observed at 4000 mg L^{-1} of NPs (Figure 3C). Leaf area and RWC significantly increased at 50 and 100 mg L^{-1} of Co_3O_4 NPs (Figure 3D-E). The lowest levels of these parameters were observed at 2000 and 4000 mg L^{-1} of treatment (Figure 3D-E). The SPAD value was significantly increased by ~9.01% at 50 and 100 mg L^{-1} compared to the control (Figure 3F). By increasing Co_3O_4 NPs concentration, SPAD was decreased in the leaves and the lowest amount of this parameter was obtained at 2000 and 4000 mg L^{-1}, which was ~40.20% lower than the control (Figure 3F).

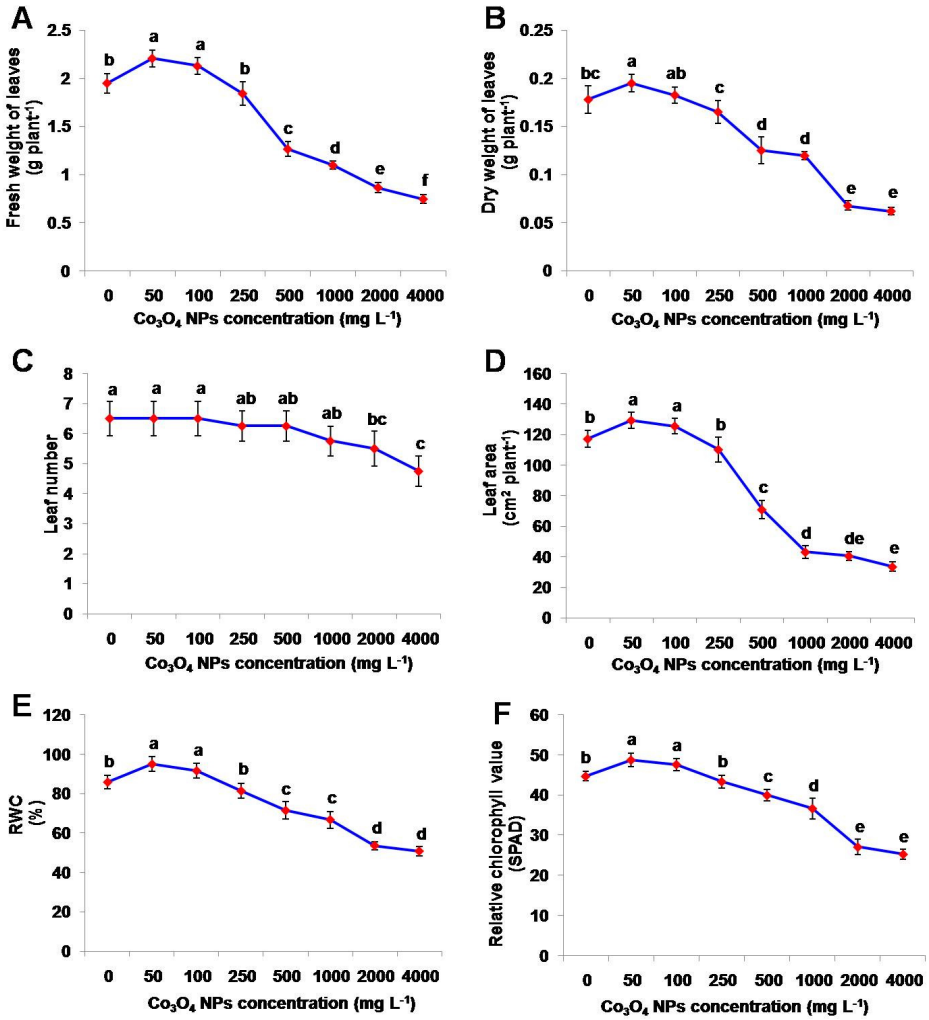

Figure 3. Impacts of different concentrations of Co_3O_4 NPs on FW (A), DW (B), number (C), area (D), RWC (E), and relative chlorophyll value (SPAD) (F) of *Brassica napus* leaves. Data represents mean ± SD of four replicates. Means with different letters are significantly different at $p \leq 0.05$ as determined by the Duncan test

H_2O_2, EL and cobalt accumulation of leaves

The effect of Co_3O_4 NPs on H_2O_2 and EL in the leaves of *B. napus* is shown in Figure 4. Results showed that H_2O_2 content significantly increased after applying 250 mg L^{-1} of Co_3O_4 NPs and reached a peak at 4000 mg L^{-1} by 2.1-fold (21.87 μM g^{-1} FW) increment over the control (Figure 4A). Also, EL enhanced at 500 mg L^{-}

[1] of Co_3O_4 NPs, and presented a maximum level at 2000 and 4000 mg L^{-1} by ~3.2-fold increase in comparison to the control (Figure 4B). Cobalt content in *B. napus* leaves showed a clear dose-dependent effect and maximum level was exhibited at 4000 mg L^{-1}, which was 889.12 mg kg^{-1} DW (Figure 4C).

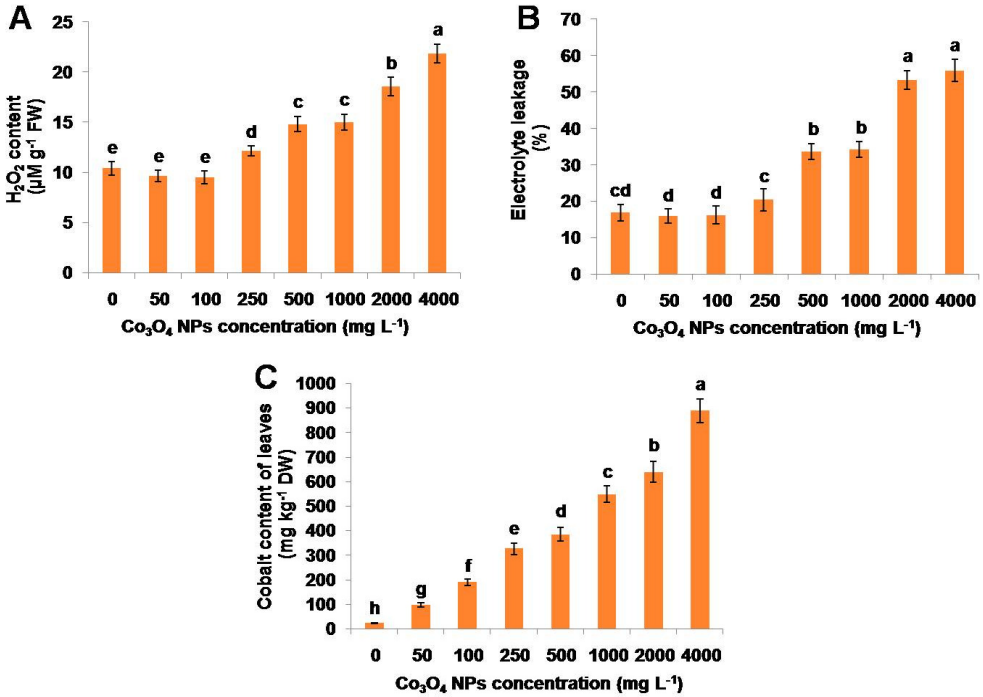

Figure 4. Impacts of different concentrations of Co_3O_4 NPs on H_2O_2 (A), EL (B), and cobalt content (C) in the leaves of *Brassica napus*. Data represents mean ± SD of four replicates. Means with different letters are significantly different at $p \leq 0.05$ as determined by the Duncan test

Total phenol, anthocyanin and PAL and TAL activities

Total phenol was elevated at 500 mg L^{-1} of Co_3O_4 NPs and showed the highest level at 2000 mg L^{-1}, which was ~1.73-fold over the control (Figure 5A). Anthocyanin content at 50-500 mg L^{-1} of Co_3O_4 NPs showed no significantly difference with the control (Figure 5B). This phenolic pigment reached its highest level at 2000 mg L^{-1} with 1.57-fold increase in comparison to the control (Figure 5B).

The activity of PAL in the leaves was not affected by Co_3O_4 NPs until concentration of 100 mg L^{-1} (Figure 5C). At higher concentrations, PAL activity increased and showed the maximum levels at 1000 and 2000 mg L^{-1} of Co_3O_4 NPs, which was ~62.5% over the control (Figure 5C). Moreover, TAL activity was measured in leaves exposing to Co_3O_4 NPs (Figure 5D). TAL activity did not significantly change up to 500 mg L^{-1} of the treatment, whereas an apparent elevation was observed in its activity at 1000-4000 mg L^{-1} of Co_3O_4 NPs (Figure 5D). The activity of TAL at its highest level was ~32.8% more than the control (Figure 5D).

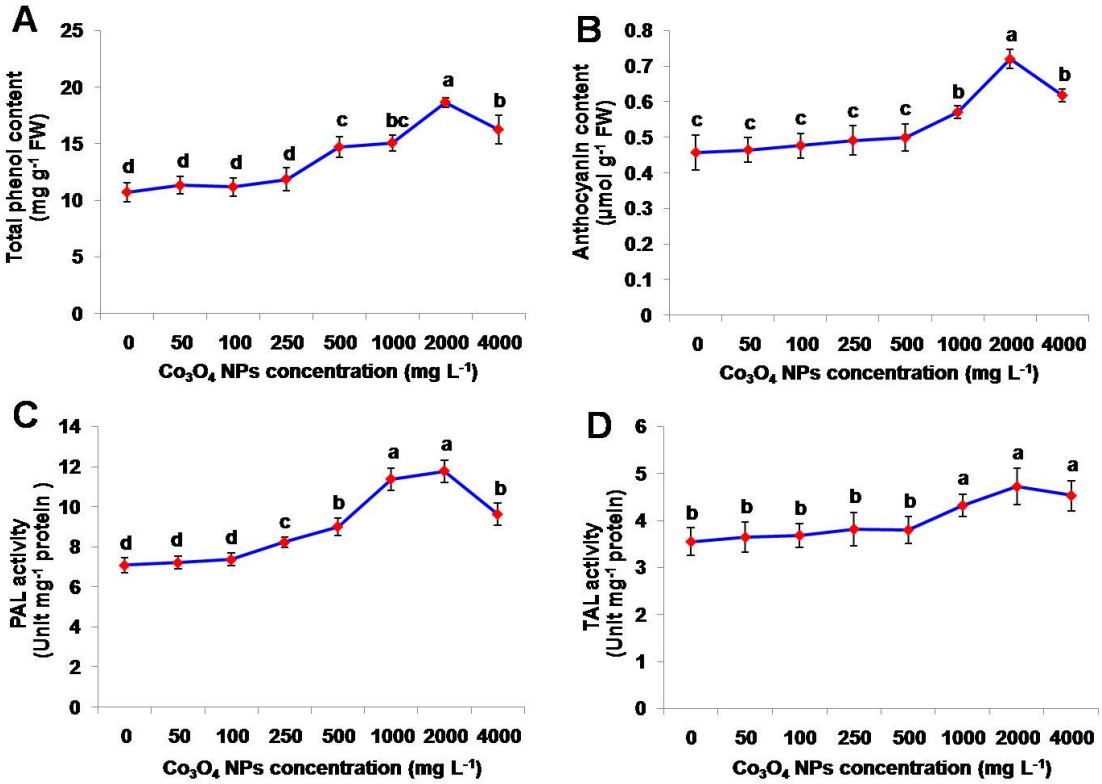

Figure 5. Impacts of different concentrations of Co_3O_4 NPs on total phenol (A), anthocyanin (B) and enzymes activities of PAL (C) and TAL (D) in the leaves of *Brassica napus*. Data represents mean ± SD of four replicates. Means with different letters are significantly different at $p \leq 0.05$ as determined by the Duncan test

Protein content and antioxidant enzymes activities

Changes in protein content and enzymatic antioxidants activities including APX, SOD, CAT, GPX, PPO, GR and GST in leaves treated with Co_3O_4 NPs are shown in Figure 6. Results revealed that protein content did not significantly change up to 1000 mg L^{-1} and then decreased at high concentrations of treatment (2000 and 4000 mg L^{-1}) (Figure 6A). By increasing the concentration of Co_3O_4 NPs, APX activity was significantly enhanced and reached the maximum at 4000 mg L^{-1} (70.2% over the control) (Figure 6B). Also, a notable enhancement in SOD activity was found at 500 and 1000 mg L^{-1} of treatment with ~1.5-fold increase than the control (Figure 6C). Co_3O_4 NPs did not affect CAT activity up to 2000 mg L^{-1}, whereas an apparent reduction was observed at 4000 mg L^{-1} (~19.9% decline in comparison to the control) (Figure 6D). The activity of GPX was enhanced after applying 250 mg L^{-1} of Co_3O_4 NPs and showed the maximum level at 1000 mg L^{-1} (88.9% over the control) (Figure 6E). Although its activity declined at 2000 and 4000 mg L^{-1} compared to lower concentrations, it was still higher than the control (Figure 6E). PPO activity exhibited significant increment at 1000-4000 mg L^{-1} of NPs with ~1.5 time increase than the control (Figure 7F). In contrast, Co_3O_4 NPs decreased GR activity at 1000-4000 mg L^{-1} and the highest reduction was ~40.06% over the control at 4000 mg L^{-1} (Figure 6G). Increment in GST activity occurred by increasing the concentration of Co_3O_4 NPs up to 1000 mg L^{-1}, but a rapid reduction was seen at 2000 and 4000 mg L^{-1} (~1.15-fold decrease in comparison to the control) (Figure 6H).

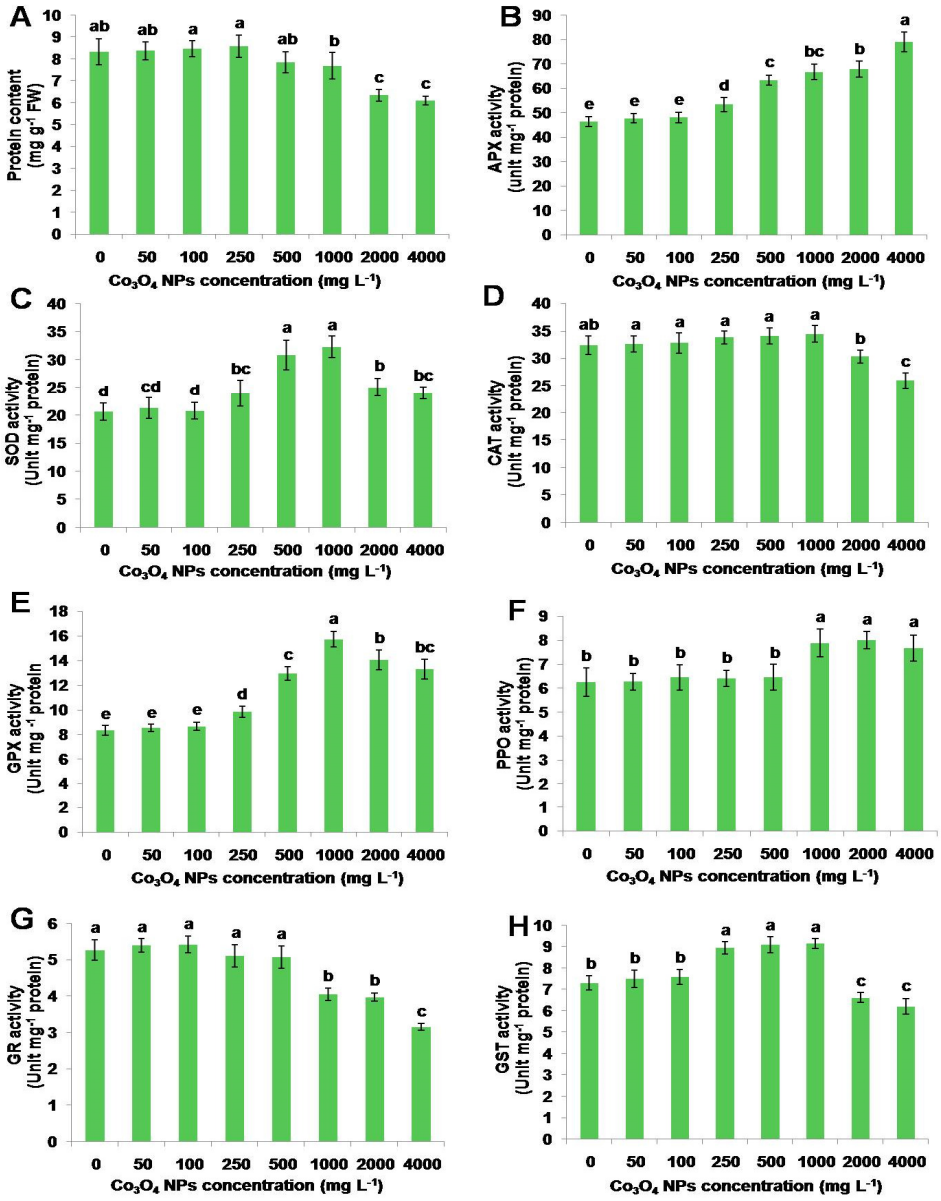

Figure 6. Impacts of different concentrations of Co_3O_4 NPs on protein and antioxidant enzymes activity. Protein (A), APX (B), SOD (C), CAT (D), GPX (E), PPO (F), GR (G), and GST (H) activities in the leaves of *Brassica napus*. Data represents mean ± SD of four replicates. Means with different letters are significantly different at $p \leq 0.05$ as determined by the Duncan test

Discussion

In order to recognize the possible benefits and hazards of NPs in agriculture, it is important to analyse uptake and transport of NPs in plants (Nair *et al.*, 2014; Hajra and Mondal, 2017). The NPs characteristics play a significant role in reactivity and toxicity. Therefore, both positive and negative effects of NPs are observed in plants (Zhu *et al.*, 2019). In recent years, NPs have received considerable attention due to their applications in technology and sciences (Salehi *et al.*, 2018; Hasanabadi *et al.*, 2019; Vicas *et al.*, 2019). So,

before its widespread employment and applications in the life, its probable influences on living beings and environment should be considered.

In this study, spraying application of Co_3O_4 NPs led to reduction in growth factors and biomass of leaves although an increment was shown at 50 and/or 100 mg L^{-1}. These results suggested that doses of more than 250 mg L^{-1} of Co_3O_4 NPs were toxic for growth indexes in *B. napus*. Similarly, the negative effects of nano-sized ceria at more than 250 mg L^{-1} on growth and RWC in bean was reported by Salehi *et al.* (2018). Prakash *et al.* (2017) also reported CuO-based NPs declined shoot and root growth as well as plant biomass in canola. Low doses of ZnO NPs (0.4-0.8 g L^{-1}) could improve grain yield of triticale under salinity condition (Kheirizadeh Arough *et al.*, 2016).

Marchiol *et al.* (2016) revealed that in nanoceria treated plants, leaf area and the number of spikes were reduced at concentrations of 500-1000 mg kg^{-1}. However, almost opposite results were illustrated by Prasad *et al.* (2012). They showed that with increment in the zinc oxide-based NPs concentration from 400 to 1000 mg L^{-1}, the growth of the plant significantly enhanced with respect to the control. Pošćić *et al.* (2016) also demonstrated that nano-sized TiO_2 and CeO_2 at concentrations of 500 and 1000 mg kg^{-1} had no effect on leaf surface of barley. The NPs utilization has been confirmed to damage cell membrane and leaves morphology due to the reduction in RWC (Hong *et al.*, 2014; Salehi *et al.*, 2018).

In this study, relative chlorophyll value (SPAD) was affected under Co_3O_4 NPs. Under various stressful environmental conditions, photosynthesis is influenced (Borowiak *et al.*, 2018; Skórska and Murkowski, 2018; Puła *et al.*, 2019). It was reported that 62.5-1000 mg L^{-1} of cobalt ferrite-based NPs did not show any significant difference in chlorophyll value in tomato leaves (López-Moreno *et al.*, 2016), while 1000 and 2000 mg kg^{-1} nano-sized ceria reduced chlorophyll value in romaine lettuce (Zhang *et al.*, 2017). In parallel, Nair and Chung (2015) reported a significant decrease in chlorophyll value of *B. juncea* subjected to nano-sized CuO. It was suggested that high concentration of NPs may induce structural changes in chloroplast membrane by the heightened production of ROS (Nair and Chung, 2015). On the other hand, it was elucidated chlorophyll is very important for plant growth processing (Eckhardt *et al.*, 2004). Here, reduction in chlorophyll production and photosynthesis may be a cause for decrease in biomass production.

This study showed high values of H_2O_2 and EL by increasing Co_3O_4 NPs concentration. Metal oxide-based NPs induced over-production of ROS as witnessed by reduction in RWC and higher levels of H_2O_2 content (Khan, 2016). Here, excessive accumulation of H_2O_2 caused the disintegration of membrane lipids and eventually led to the high values of EL in Co_3O_4 NPs-treated leaves. The presented results are in line with Gorczyca *et al.* (2015) who demonstrated that nano-sized Ag incremented EL by two-fold in the treated wheat seedlings over the controls. In addition, nano-sized CuO and CeO_2 treatments caused H_2O_2 accumulation in leaves of mung bean and corn, respectively (Zhao *et al.*, 2012; Nair *et al.*, 2014). Similarly, in rice roots, 125 mg L^{-1} of nano-sized ceria (for 10 days) elevated lipoperoxidation and EL; however, H_2O_2 value was elevated at 500 mg L^{-1} (Rico *et al.*, 2013).

Furthermore, increasing in the antioxidant phenolic pigments such as anthocyanin and also total phenol was seen in this investigation. Phenolics play a role in scavenging free radicals, defense against pathogens and reducing membrane damage in chloroplast (Matysik *et al.*, 2005; Agati *et al.*, 2012; Skórska *et al.*, 2019). These compounds can protect plants against NPs toxicity by metal chelation and direct scavenging of ROS (Michalak, 2006). Elevation in anthocyanin and total phenol was reported in Arabidopsis treated with nano-scaled indium and cerium oxide stress (Ma *et al.*, 2016). Besides this, Hajra and Mondal (2017) showed increment of phenol in chickpea under nano-scaled titanium and zinc oxide.

In this study, moreover the phenolic compounds, PAL and TAL activities displayed an increase in treated plants by higher concentrations of Co_3O_4 NPs. These enzymes are major enzymes in the phenolics biosynthesis pathway and their activities regulate these compounds levels in plants (Wang *et al.*, 2000; Kitamura *et al.*, 2002). Similarly, the increment of PAL and TAL activities in marigold leaves sprayed with nano-scaled CeO_2 was reported (Jahani *et al.*, 2018).

Furthermore, here, protein level was altered in response to Co_3O_4 NPs and showed a reduction at higher doses (2000 and 4000 mg L^{-1}). Some studies revealed the reduction in protein value in NPs-treated plants (Du *et al.*, 2015; Majumdar *et al.*, 2015; Ma *et al.*, 2016). This reduction can be due to ROS over-generation, extreme oxidative stress, and proteins structure damaging. Also, it has been reported that the declined levels of protein may be related to the protein oxidation, which is a common result of heavy metal toxicity. Heavy metals can directly interact with proteins because of their affinities for thioyl, histidyl, and carboxyl groups (Hossain *et al.*, 2012).

In this study, based on observation of heightened H_2O_2 as an indicator of ROS in Co_3O_4 NPs-treated *B. napus*, the activities of key enzymes for ROS scavenging including SOD, APX and GPX were observed. SOD can convert $O_2^{\bullet-}$ (superoxide) to H_2O_2. The heightened H_2O_2 should be reduced or scavenged by other antioxidant enzymes such as APX, GPX and CAT (catalyze H_2O_2 to H_2O) to protect plants against oxidative stress (López-Moreno *et al.*, 2016; Ma *et al.*, 2016). In this study, although APX and GPX activities were elevated in the most of Co_3O_4 NPs concentrations, CAT activity was decreased at the highest concentration of treatment. This reduction might be due to decline in protein content at higher doses of Co_3O_4 NPs. The increased-activities of SOD, APX, GPX and CAT in the nano TiO_2-treated spinach was reported by Lei *et al.* (2008). López-Luna *et al.* (2018) stated that the induction of CAT, APX, and GPX activities is indicating of the generation of ROS and oxidative damage induced by cobalt ferrite NPs in wheat seedlings. In addition, Iannone *et al.* (2016) showed that nano-sized Fe_3O_4 raised the levels of SOD, APX, GPX and CAT in wheat. Besides these, in agreement with our results, decline in CAT activity was reported in *Lycopersicon lycopersicum* plant subjected to nano-scaled $CoFe_2O_4$ (López-Moreno *et al.*, 2016). Variation in CAT activity can be because of the metal dose and plant tolerance to the metal (Pandey *et al.*, 2009; Nair and Chung, 2015).

Here, analysis of activities of stress-related antioxidant enzymes in response to Co_3O_4 NPs showed that GST activity was incremented and then decreased at higher doses, while GR activity diminished. GST activity involves in conjugation of glutathione (GSH) to target molecules for detoxifying toxic compounds and heavy metals. By breaking down H_2O_2 in ascorbate-glutathione cycle, GSH is converted to oxidized form by glutathione peroxidase. The latest is reduced to GSH by GR (Pauly *et al.*, 2006; Dalton *et al.*, 2009). Similarly, the heightened activity of GST was shown in *Arabidopsis thaliana* subjected to nano-scaled CeO_2 (Ma *et al.*, 2016). The diminished levels of GR activity were demonstrated in *Vicia faba* under nano-scaled TiO_2 (Foltête *et al.*, 2011). Here, the decrease in GR activity may be related to heightened levels of ROS production and lipoperoxidation.

PPO is another antioxidant enzyme for ROS scavenging and detoxification of metals, which converts phenols into quinones (Kováčik *et al.*, 2009). In this study, PPO activity was elevated at 1000-4000 mg L^{-1} of Co_3O_4 NPs in *B. napus*. Similarly, the heightened activity of PPO was reported in marigold subjected to 800-3200 mg L^{-1} of nano-sized CeO_2 (Jahani *et al.*, 2019).

In this study, cobalt accumulation in dose-dependent pattern in Co_3O_4 NPs-treated plants was seen. Accumulation of cobalt in leaves at higher concentrations was very high, as expected. Several studies have showed that plants can uptake NPs and accumulate them in their tissues (Lee *et al.*, 2010; Schwab *et al.*, 2011; Prasad *et al.*, 2012; Hong *et al.*, 2014). López-Moreno *et al.* (2016) reported cobalt and Fe accumulation in tomato plants subjected to nano-sized $CoFe_2O_4$ NPs. Furthermore, Co content was elevated in wheat under cobalt ferrite-based NPs (López-Luna *et al.*, 2018). It has been reported that size of NPs has critical role in plant uptake, and smaller NPs are more likely to be taken up by plants (Zhang *et al.*, 2015). In summary, a schematic image of the present study is shown in Figure 7.

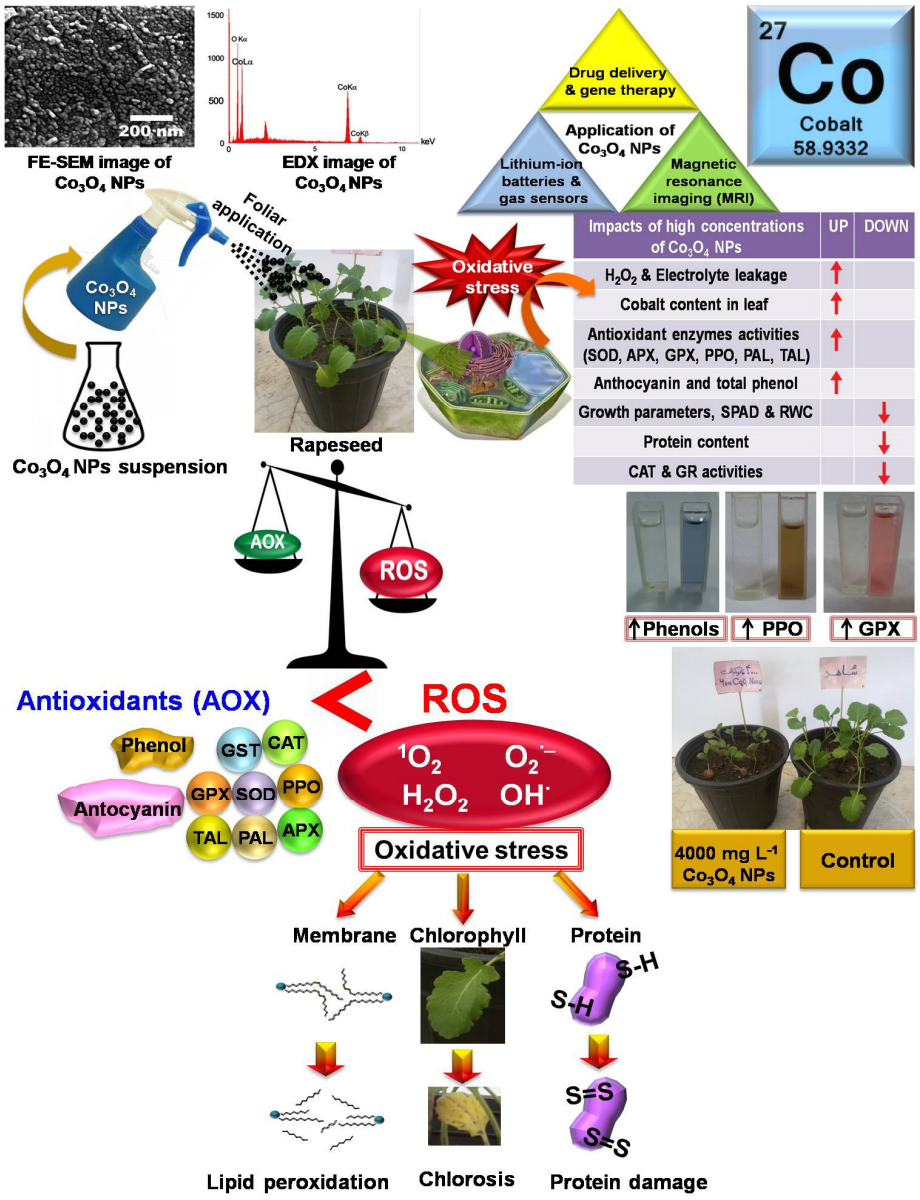

Figure 7. A schematic image of the present study

Conclusions

B. napus is one of the most important oilseed crops worldwide. Considering the presented results, Co_3O_4 NPs spraying application, depending on its dosage, exerted a dual effect on morpho-physiological parameters. Co_3O_4 NPs at low concentrations (50 and 100 mg L^{-1}) stimulated growth and photosynthesis while high concentrations (500-4000 mg L^{-1}) adversely affected and caused morpho-physiological symptoms of phytotoxicity. Probably, the mechanisms of Co_3O_4 NPs toxicity may be associated with the release of Co ions, H_2O_2 over-generation, lipoperoxidation and ion leakage, which eventually led to decline in photosynthesis and biomass. However, the participation of antioxidant enzymes and phenolic compounds (total phenol and anthocyanin) as an activated defense system against extreme oxidative stress caused by high Co_3O_4 NPs concentrations was not enough for ROS scavenging and protecting deleterious impacts. Overall, the results

clearly demonstrated the toxicity of Co_3O_4 NPs at higher concentrations. Therefore, its probable hazards effects on crops and the environment should be taken into account.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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