Anatomical and ultrastructural responses of Brassica napus after long-term exposure to excess zinc

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Abstract: Heavy metal contamination resulting from anthropogenic activities is one of the major environmental problems in the modern world. Progress toward creating modified plants that are more efficient in phytoremediation requires an understanding of the anatomical and ultrastructural changes involved in heavy metal accumulation. A large supply of zinc (Zn) is toxic for plants and can lead to functional and structural disorders. The present study investigates the anatomical and ultrastructural responses of Brassica napus (rapeseed) to excess Zn after long-term exposure. Results showed that Zn bioaccumulation in the roots and leaves of plants treated with 350 µM Zn²⁺ was severely increased, leading to a variety of anatomical and ultrastructural alterations in several cell types. Overall, although B. napus has been reported as a metal-accumulator species, our data revealed that B. napus cultivar Hayola 401 could not tolerate a high concentration of Zn and thus is not a good candidate for Zn phytoremediation.

Key words: Heavy metals, phytoremediation, soil contamination, zinc

1. Introduction
The modern world is facing a major environmental problem, that of heavy metal contamination (Li et al., 2006). The progressive release of heavy metals into the environment resulting from anthropogenic activities, such as mining, smelting of metalliferous ores, and electroplating, can detrimentally affect living components of ecosystems (Maruthi Sridhar et al., 2005; Wang et al., 2009). Heavy metal accumulation in agricultural soils has raised concerns about food safety and subsequent adverse effects on human health (Brunetti et al., 2011). Zinc (Zn) is the second most ample transition metal after iron (Fe) (Wang et al., 2009). Besides industrial activities, the use of Zn-containing fertilizers and pesticides in agriculture and the wide use of Zn in paint, rubber, dye, and wood preservative industries have contributed to environmental contamination with Zn (Maruthi Sridhar et al., 2005).

Zn is an essential element for plants, required for the activity of many plant enzymes and for chlorophyll biosynthesis (Taiz and Zeiger, 2010). Zn is also involved in protein synthesis, carbohydrate metabolism, tryptophan and indole acetic acid synthesis, and membrane integrity. However, a large supply of Zn can be toxic for plants and may lead to functional and structural disorders (Marschner, 1995).

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Use of plants to clean up or extract pollutants from the environment is called phytoremediation (Li et al., 2006; Katayama et al., 2013). In recent years, many plants have been screened to find hyperaccumulator species that accumulate high metal concentrations, have fast growth, produce high biomass, and are fully harvestable (Marchiol et al., 2004). Most hyperaccumulator species produce little biomass and have slow growth rates (Li et al., 2006). Moreover, metal uptake by plants and its bioaccumulation in different parts of the plant are intrinsically limited (Maruthi Sridhar et al., 2005). Progress toward creating modified plants that are more efficient in phytoremediation requires an understanding of the ultrastructural, biochemical, physiological, and molecular mechanisms involved in heavy metal accumulation in different parts of the plant (Li et al., 2006). Since anatomical and ultrastructural changes can reflect and/or lead to biochemical, physiological, and molecular alterations in the plant (Todeschini et al., 2011), investigating these changes can help to understand the overall process of phytoremediation (Maruthi Sridhar et al., 2005).

Fast-growing and high biomass-accumulating plants, such as the Brassicaceae species, are good candidates for phytoremediation (Brunetti et al., 2011). Brassica napus (rapeseed), the third most important source of edible
vegetable oil after soybean and palm (Burbulis et al., 2008), is mainly used in food applications (Grispen et al., 2006), but it is also used as a medicinal food plant in Central Asia, North Africa, and West Europe (Saeidinia and Gohari, 2012). In recent years, it has also been used in the production of biofuel. Thus, the cultivation of rapeseed in heavy metal-contaminated soils is beneficial both for phytoremediation and for biofuel production, in addition to its importance in producing edible and healthy oil (Grispen et al., 2006). Since, to the authors’ knowledge, simultaneous studies on the long-term anatomical and ultrastructural responses of plants to excess heavy metals are scarce, the present study investigates the responses of *B. napus* to Zn²⁺.

2. Materials and methods

2.1. Plant culture and experimental design

Seeds of rapeseed cultivar Hayola 401 were prepared by the Agricultural and Natural Resources Research Center of Khorasan-Razavi, Mashhad, Iran. Seeds were sterilized in 10% sodium hypochlorite solution for 10 min, rinsed thoroughly three times with deionized water, soaked in deionized water for about 2 h, and then cultured hydroponically with perlite as a supporting material. Seeds were sown in 1.5-L plastic pots filled with perlite and arranged in two groups. In the control group, pots were fed with 10%-strength Hoagland nutrient solution (Taiz and Zeiger, 2010). In the other group, pots were fed with same strength of Hoagland nutrient solution containing 350 μM Zn²⁺, prepared by dissolving appropriate amounts of ZnSO₄·7H₂O in deionized water. The treatment concentration (350 μM) was selected after a primary study to find a first estimate of toxicity thresholds. For this purpose, the effect of different concentrations of Zn²⁺ on the growth of rapeseed was investigated, and then the maximum Zn²⁺ concentration tolerated by rapeseed was selected according to the growth parameters. Moreover, our previous study, investigating the effect of a wide range of concentrations of Zn²⁺ on some growth parameters of rapeseed seedlings, provided a good estimation of Zn toxicity (Mousavi Kouhi et al., 2014).

Plants were grown at 25 ± 1 °C under a 16/8-h photoperiod. The nutrient solution of each group was renewed every 10 days. For this purpose, the pots were fully rinsed with distilled water and then fed with a relevant nutrient solution, where the rinsing water was removed by leaching. Nutrient solution strength was increased to 25% and 50% in the second and subsequent renewals, respectively. Pots were weighed daily and weight loss was reversed by adding nutrient solution. Two months after planting, the plants were harvested for subsequent analyses.

2.2. Microscopic analyses

Root segments of about 2 mm in length were cut 1 cm above the root tip and about 1 cm above the junction between branch and taproot. Leaf rectangular segments (1 × 2 mm) were sectioned from the sixth leaf, between the third and the fourth principal lateral veins (Stefanowska et al., 1999), and immediately transferred to 4% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) at 4 °C for overnight fixation. After rinsing three times with the same buffer for 15 min each, the samples were postfixed in 1% OsO₄ in the same buffer for 2 h, followed by washing three times in the same buffer. Segments were dehydrated in a graded series of ethanol (30%, 50%, 70%, 90%, and 100%) and were then infiltrated with resin overnight. Semithin (1 μm) and ultrathin (80 nm) sections were cut on an ultramicrotome (Ultracut UCT, Leica, Austria). Semithin sections were observed with a light microscope (Olympus BX 51, Japan) connected to a digital camera (Olympus DP 71) after staining with 1% toluidine blue in 1% borax. Ultrathin sections were mounted on copper grids and then observed with transmission electron microscopy (TEM; LEO 912 AB, Zeiss, UK) (Mousavi Kouhi et al., 2015).

Different sections were prepared in the present study. However, the best section, being a good representative, was selected to study anatomical and ultrastructural modifications. The modifications that were completely distinct from those of the control plant were descriptively investigated without quantitative analysis. For instance, although the size of starch grains is a qualitative parameter, their modifications relative to the control were so obvious that they did not need to be measured quantitatively. However, parameters such as cell size, number of chloroplasts, and size of mitochondria were investigated quantitatively. In order to measure the cell size, the diameter of ten cells was determined and then the mean size was calculated. To measure the size of cell organelles, the diameter of ten organelles from a given cell was determined and the mean size was calculated. The number of chloroplasts was counted in more than 20 mesophyll cells and the mean number was recorded for each cell.

2.3. Determination of Zn content of roots and leaves

Plant roots and leaves were dried at 70 °C for 48 h. The dried samples were ground to a fine powder and 0.5 g of each powder was digested with 5 mL of HNO₃, and 1 mL of 30% H₂O₂, followed by heating at 90 °C. The two latter stages were repeated until the digest was clear. After clearing the sample digest, heating was continued until near dryness. Finally, digest residue was dissolved with diluted HNO₃ and brought to a final volume of 25 mL with deionized water (Kalra, 1998). The Zn content of root and leaf extracts was determined with atomic absorption spectrometry (AA-670, Shimadzu, Japan).
2.4. Statistical analyses
There were three replicates for each group, arranged in a completely randomized design. A one-way analysis of variance (ANOVA), followed by Duncan's multiple range test, was used to test significant differences and means comparisons with MSTAT-C. Data were reported as mean ± standard deviation. Significant difference was defined as \( P < 0.05 \).

3. Results
3.1. Zn bioaccumulation in roots and leaves
Zn bioaccumulation in the roots and leaves of the plants treated with 350 \( \mu \text{M Zn}^{2+} \) was severely increased compared to the control (Figure 1). The results showed that the Zn content of roots and leaves of Zn-treated plants was 5.7 and 6.42 times higher than that of the control plants, respectively.

3.2. Root anatomy and ultrastructure
Cross-sections of the root tips of Zn-treated plants showed a decreased number of cortical and stellar cells and a decreased diameter of root tip compared to the control (Figures 2a and 2b). Light micrographs revealed that the most vulnerable tissue of the root tip cells under Zn treatment was the epidermis. Root epidermal cells of Zn-treated plants were deformed and shrunken, having a smaller size than that of the control (Figures 2c and 2d). The stele diameter and size of the pericycle cells also decreased under Zn stress (Figures 2e and 2f). Although the number of vascular cells appeared lower compared to the control, their size seemed to be larger (Figure 2f). Light micrographs of about 1 cm above the junction between the branch and the taproot revealed that under excess Zn, the stele diameter and its cell numbers were decreased, and conducting cells of the xylem and phloem were undersized compared to the control (Figure 3a–3d).

TEM micrographs of the cortical cells of the root tips of Zn-treated plants revealed Zn deposition in the cell walls. These cell walls had different thicknesses at different sites and were deformed at certain sites (Figures 4a and 4b). Other detectable and prominent ultrastructure alterations in the cortical cells of Zn-treated plants were not observed. The TEM image of the vascular tissue of the root tip of the control plants showed a large starch grain in the plastids of several vascular cells (Figure 5a). However, this feature was not observed in Zn-treated plants. Moreover, several plastids of later cells were deformed and misshaped in Zn-treated plants. Zn deposition was observed in vacuoles and intercellular spaces of vascular tissue (Figures 5b and 5c). The stellar cells of the root tips revealed excess Zn-induced disintegration of mitochondria (Figure 5c).

3.3. Leaf anatomy and ultrastructure
The leaves of the plants treated with high Zn showed general chlorosis and had a small leaf area compared to the control plants (data not shown). Light micrographs of the leaf section of Zn-treated plants revealed that the number and sizes of chloroplasts decreased and the size of the epidermal cells was larger than in the control (Figure 6). Moreover, disorganization of spongy parenchyma cells (mainly their chloroplasts) and decreased size of vascular bundles were also observed in Zn-treated plants (Figure 6a–6d).

TEM micrographs of leaf mesophyll cells revealed certain ultrastructural changes induced by excess Zn, including breakdown of cell walls, structural disorder of chloroplasts including decreased number of thylakoid and grana, increase in size and number of plastoglobuli in the chloroplasts, decrease in the size of the nucleus, and Zn deposition in vacuoles (Figure 7).

4. Discussion
The Zn content of soils is between 60 and 89 mg/kg (Kabata-Pendias, 2011). The critical toxicity level of Zn in the leaves of crop plants is approximately 300 mg/kg dry weight (Marschner, 1995). In the present study, the Zn content of leaves was 1600 mg/kg dry weight. Many studies have reported that high Zn accumulation can result in functional and structural changes in different parts of the plant (Maruthi Sridhar et al., 2005; Jiang et al., 2007; Todeschini et al., 2011). Since the root is the first target tissue exposed to high concentrations of heavy metals, it seems that functional and structural disorders appear more often in roots than in the aboveground parts of the plant.

It has long been known that Zn toxicity can lead to stunted growth and leaf chlorosis (Zeng et al., 2011). Leaf chlorosis may result from induced deficiency of some
ions with the same electrical charge or ion radius such as magnesium and iron (Marschner, 1995). It has also been known for many years that interference of Zn with Fe metabolism can lead to chlorosis (Zeng et al., 2011) due to competition between Zn and Fe, and/or interference with the chelation processes of Fe during root uptake (Kabata-Pendias, 2011).

The most vulnerable root tip tissue under Zn treatment was the epidermis. For this reason, root epidermal cells are the first cells exposed to excess Zn; thus, they are likely to be the most negatively affected cells. Decreased diameter of the root tip may result from a decreased number of cortical and stellar cells, which may subsequently result from the fact that metal-induced toxicity can lead to arrest of cell division (Gangwar et al., 2014). Decreased number of vascular cells of the root tip and their increased size in Zn-treated plants may also be due to decreased cell division. Decreased cell division induced by heavy metals (Hossain et al. 2012) may allow preexisting vascular cells to grow further as a result of potential competition.

A study by Sresty and Madhava Rao (1999) on the root cells of pigeonpea under excess Zn and nickel revealed many ultrastructural alterations, including major changes in the nucleus, highly condensed chromatin, disruption and dilation of nuclear membranes in several cortical cells, withdrawal of the plasma membrane from cell walls.

Figure 2. Light micrographs of cross semithin sections of the root tip of B. napus. a, c, and e: Control plants; b, d, and f: plants treated with 350 µM Zn²⁺; pc: pericycle; en: endoderm.
structureless cytoplasm, disintegration of cell organelles, and development of vacuoles.

Deposition of metals in different cell compartments is one of the processes used by plants to tolerate the excess concentration of heavy metals. Sites of deposition depend on the metal and plant species. In tolerant species, Zn accumulates in the vacuole, whereas in nontolerant species it accumulates in the cytosol (Marschner, 1995). However,
Zn may also be sequestrated in chloroplasts (Kabata-Pendias, 2011). Jiang et al. (2007), using TEM microscopy, reported the deposition of Zn with P in the vacuoles of roots. Zn deposition as electron-dense globules in vacuoles may play a role in the tolerance of excess Zn by decreasing its levels in the cytoplasm and nucleus (Sresty and Madhava Rao, 1999).

Cell walls with certain electronegative ions, such as $\text{HPO}_4^{2-}$, $\text{H}_2\text{PO}_4^-$, $\text{Cl}^-$, and $\text{SO}_4^{2-}$, can sequestrate metals and thus prevent their translocation from root to shoot (Jiang et al., 2007). It has been reported that metal sequestration in cell walls may lead to a decrease in their elasticity and then to deformity or breakdown. On the other hand, under heavy metals, decreased cell turgidity due to reduced vacuole size can lead to deformity or breakdown of cell walls, resulting in irregular cell alignments (Pokhrel and Dubey, 2013).

Similarly to the present study, a breakdown of spongy parenchyma cells was reported in *Brassica juncea* treated with high concentrations of Zn. However, other symptoms observed in *B. juncea*, including reduction in the palisade and epidermal cells, breakdown in the palisade parenchyma cells, loss of cell shape, decrease in intercellular spaces, shrinkage of epidermal cells, and decrease in the starch content of leaves (Maruthi Sridhar et al., 2005), were not observed in *B. napus* exposed to toxic levels of Zn in the present study. In another study, consistent with the present study, increased size of leaf epidermal cells and decreased numbers of chloroplasts of mesophyll cells were reported in poplar treated with high Zn concentration (Todeschini et al., 2011).

As with the present study, several studies on the *Brassica* species have reported negative effects of excess Zn on the disorganization of chloroplasts and a decreased number of thylakoid and grana (Ebbs and Kochian, 1997; Jiang et al., 2007; Ebbs and Uchil, 2008; Wang et al., 2009) that can induce chlorosis (Wang et al., 2009). The decreased number of chloroplasts in the present study was also reported in *Brassica oleracea* treated with nickel (Molas, 2002). Furthermore, it was reported that the chloroplasts of high Zn-treated plants are undersized. Disintegration of double-membrane organelles, such as chloroplasts and mitochondria, was also reported in poplar leaves treated with excess Zn, which may be considered a sign

![Figure 5. TEM micrograph of cross ultrathin sections of the root vascular tissue cells of *B. napus*. a and b: Control plants; c, d, e, and f: plants treated with 350 µM Zn$^{2+}$; cw: cell wall; mc: mitochondrion; v: vacuole; dp: metal deposition; N: nucleus; pl: plastid; s: starch grain; ER: endoplasmic reticulum.](image-url)
Figure 6. Light micrographs of cross semithin sections of leaf of *B. napus*. a and c: Control plants; b and d: plants treated with 350 µM Zn²⁺. c and d are higher magnifications of the frames in a and b, respectively. ep: epidermal cell; pl: palisade cell; sp: spongy mesophyll cell.
Figure 7. TEM micrograph of cross ultrathin sections of the leaf mesophyll cells of *B. napus*. a, b and c: Control plants; d, e, and f: plants treated with 350 µM Zn<sup>2+</sup>; cw: cell wall; mc: mitochondrion; v: vacuole; N: nucleus; nu: nucleolus; Cp: chloroplast; pG: plastoglobule; s: starch grain; dp: metal deposition

of advanced induced senescence (Todeschini et al., 2011).

Consistent with our study, Todeschini et al. (2011) reported an increase in the number of plastoglobuli of chloroplasts induced by high Zn concentration. Occurring more frequently during senescence, increased plastoglobuli of chloroplasts is one of the ultrastructural changes under abiotic stresses such as heavy metals (Brehelin et al., 2007). Besides sequestration in root cells, Zn can also be deposited in leaf cells. Zn sequestration in the vacuoles of leaf epidermal (*Thlaspi caerulescens*) and mesophyll (*Arabidopsis halleri*) cells has been reported (Todeschini et al., 2011).

Overall, our data revealed that *B. napus* cultivar Hayola 401 could not tolerate a high concentration of Zn. Under 350 µM Zn, *B. napus* showed many anatomical and ultrastructural toxicity symptoms. However, some species accumulate more than 10,000 mg of Zn per kilogram of dry weight without any toxicity symptoms. For instance, it has been reported that *Arabis paniculata* could tolerate 2000 µM Zn, accumulating more than 12,000 and 6000 mg Zn/kg dry weight of root and shoot, respectively. Interestingly, excess Zn up to 500 µM increased the biomass of *A. paniculata* (Zeng et al., 2011). In conclusion, although *B. napus* has been reported as a metal-accumulator species (Grispen et al., 2006), we suggest that *B. napus* cultivar Hayola 401 is not a good candidate for the phytoremediation of excess Zn.

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References


