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Genotypic variation of the responses to chromium toxicity in four oilseed rape cultivars

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Abstract

Heavy metal toxicity in soils has been considered as major constraints for oilseed rape (*Brassica napus* L.) production. In the present study, toxic effects of chromium (Cr) were studied in 6-d-old seedlings of four different cultivars of *B. napus* (ZS 758, Zheda 619, ZY 50, and Zheda 622). The elevated content of Cr inhibited seedling growth, decreased the content of photosynthetic pigments, and activities of antioxidant enzymes, as well as increased the content of malondialdehyde and reactive oxygen species in all the cultivars. The Cr content in different parts of plants was higher in Zheda 622 than in other cultivars. The electron microscopic study showed changes in ultrastructure of leaf mesophyll and root tip cells at 400 μmol Cr, and these changes were more prominent in Zheda 622. An increased size and number of starch grains and number of plastoglobuli, damaged thylakoid membranes, and immature nucleoli and mitochondria were observed in leaves. In roots, enlarged vacuoles, disrupted cell walls and cell membranes, an increased number of mitochondria and a size of nucleolus, as well as plasmolysis (in Zheda 622) were observed. On the basis of these findings, it can be concluded that cv. Zheda 622 was more sensitive to Cr as compared to other three cultivars.

Additional key words: antioxidants enzyme activities, cell ultrastructures, chlorophyll content, seedling growth, reactive oxygen species.

Introduction

Heavy metals like lead, cadmium, and chromium are biologically non-essential for plants and toxic above the certain levels (Dixit *et al.* 2002). The Cr-toxicity can reduce seed germination (Panda *et al.* 2002) and plant growth and development (Shanker *et al.* 2005). There is a marked difference in Cr tolerance among plant species and even among genotypes within a species (Shahandeh and Hossner 2002). For example, Qiu *et al.* (2010) marked differences among 127 rice doubled haploid lines.

An elevated Cr content disturbs the physiological, biochemical, and especially photosynthetic processes in several plant species (Panda and Patra 2000). Bera and Kanta-Bokaria (1999) studied the toxicity of Cr in 6-d-old mungbean seedlings and found the decline in chlorophyll content. Further, plants growing at Cr stress face potential risks caused by Cr-induced production of reactive oxygen species (ROS) which cause oxidative damage and cell death (Panda and Choudhury 2005). To scavenge ROS and to avoid oxidative damage, plants have developed antioxidants enzymes (Tian *et al.* 2012) which include ascorbate peroxidase (APX), catalase (CAT), superoxide dismutase (SOD), and guiacol

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Abbreviations: APX - ascorbate peroxidase; Car - carotenoids; CAT - catalase; Chl - chlorophyll; GR - glutathione reductase; MDA - malondialdehyde; POD - guaiacol peroxidase; ROS - reactive oxygen species; SOD - superoxide dismutase; TSP - total soluble protein.
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peroxidase (POD) (Bočová *et al.* 2012, Martin *et al.* 2012). The accumulation of Cr in plants can reduce growth, induce chlorosis in young leaves, reduce pigment content, alter enzyme functions, damage root cells, and cause ultrastructural modification of chloroplast and cell membranes (Han *et al.* 2004). However, as far as we know, there are few reports about assessing the Cr-induced morphological and ultrastructural changes in oilseed rape. Therefore in the present study, four leading cultivars of *B. napus* were tested against different Cr concentrations in relation to growth, biochemical, and ultrastructural changes in seedlings.

Materials and methods

Seeds of four oilseed rape (*Brassica napus* L.) cultivars (ZS 758, Zheda 619, ZY 50, and Zheda 622) which show different tolerance against heavy metals were obtained from the College of Agriculture and Biotechnology, Zhejiang University. Mature seeds were treated with 70 % (v/v) ethanol for 3 min, 0.1 % (m/v) $HgCl_2$ for 8 min, and then rinsed with deionized water. In every Petri dish, 50 seeds were placed on a wet filter paper. After germination, 20 seedlings were selected randomly for each treatment and transferred to Petri dishes filled with two pieces of filter paper to which 6 cm^3 of chromium (Cr) solutions (0, 100, 200 and 400 μM) had been added. After 24 h, the excess solutions were discarded, and then the seedlings were treated with half-strength Hoagland's nutrient solution. Potassium dichromate ($K_2Cr_2O_7$) was used to maintain different Cr concentrations and treatments were replicated four times. The seeds germinated in a growth chamber under day/night temperatures of 25/20 °C, a 16-h photoperiod, an irradiance of 300 μmol m^{-2} s^{-1}, and a relative humidity of 60 - 70 %. After 6 d, plants were divided into shoots and roots for determinations of dry masses, and morphological, ultrastructural, physiological, and biochemical characteristics.

At the termination of the experiment, shoot length and root length were measured. A dry mass was estimated according to Zhang *et al.* (2008). For determination of Cr content in shoots and roots, samples were dried at 65 °C for 24 h, and then ashed in a muffle furnace at 550 °C for 20 h. After that, the ash was incubated with 31 % (m/v) HNO_3 and 17.5 % (v/v) H_2O_2 at 70 °C for about 2 h and dissolved in distilled water. The Cr concentration in the digest was determined using an atomic absorption spectrophotometer (*PE-100*, *Perkin Elmer*, New York, USA). Chlorophyll (Chl) and carotenoid (Car) content in cotyledons were determined according to Porra *et al.* (1989). Lipid peroxidation in the cotyledons, measured in terms of malondialdehyde (MDA) content, was analyzed according to Zhou and Leul (1999). For determination of hydrogen peroxide content, cotyledons (0.5 g) were extracted with 5.0 cm^3 of trichloroacetic acid (0.1 %, m/v) in an ice bath, and the homogenate was centrifuged at 12 000 *g* for 15 min (Velikova *et al.* 2000). The supernatant (0.5 cm^3) was mixed with 0.5 cm^3 of 10 mM potassium phosphate buffer (pH 7.0) and 1 cm^3 of 1 M KI was added. The absorbance was read at 390 nm on a spectrophotometer (*PE-100*, *Perkin Elmer*) and the H_2O_2 content was calculated by using a standard curve.

The superoxide radical was determined according to Jiang and Zhang (2001) with some modifications. The fresh cotyledons (0.5 g) were homogenized in 3 cm^3 of 65 mM potassium phosphate buffer (pH 7.8) and then the homogenate was centrifuged at 5 000 *g* and 4 °C for 10 min. After that, the supernatant (1 cm^3) was mixed with 0.9 cm^3 of 65 mM potassium phosphate buffer (pH 7.8) and 0.1 cm^3 of 10 mM hydroxylamine hydrochloride, and then incubated at 25 °C for 24 h. After incubation, 1 cm^3 of 17 mM sulphanilamide and 1 cm^3 of 7 mM α-naphthylamine were were added and left at 25 °C for further 20 min. After incubation, *n*-butanol in the same volume was added and the mixture was centrifuged at 1 500 *g* for 5 min. The absorbance of the supernatant was read at 530 nm. A standard curve was used to calculate the generation rate of O_2^-. For estimation of extracellular hydroxyl radicals according to Halliwell *et al.* (1987), 0.5 g of leaves was incubated in 1 cm^3 of 10 mM Na-phosphate buffer (pH 7.4) consisting 15 mM 2-deoxy-D-ribose (*SRL*, Mumbai, India) at 37 °C for 2 h. Following incubation, an aliquot of 0.7 cm^3 from the above mixture was added to a reaction mixture containing 3 cm^3 of 0.5 % (m/v) thiobarbituric acid (TBA, 1 % stock solution made in 5 mM NaOH) and 1 cm^3 of glacial acetic acid, heated at 100 °C in a water bath for 30 min, and cooled down to 41 °C for 10 min before the measurement of absorbance at 532 nm.

For determination of enzyme activities, cotyledons (0.5 g) were homogenized in 8 cm^3 of 50 mM potassium phosphate buffer (pH 7.8) under ice cold conditions. This homogenate was centrifuged at 10 000 *g* and 4 °C for 20 min and the supernatant was used for the determination of following enzyme activities. The glutathione reductase (GR, EC 1.6.4.2) activity was assayed according to Jiang and Zhang (2002) as oxidation of NADPH measured at 340 nm for 1 min (the coefficient of absorbance of 6.2 mM cm^{-1}). The reaction mixture contained 50 mM potassium phosphate buffer (pH 7.0), 2 mM EDTA-Na_2, 0.15 mM NADPH, 0.5 mM oxidized glutathione (GSSG), and 0.1 cm^3 of the enzyme extract in a 1 cm^3 volume. The reaction was started by using NADPH. The ascorbate peroxidase (APX, EC 1.11.1.11) activity was measured in 3 cm^3 of reaction mixture containing 100 mM phosphate buffer (pH 7), 0.1 mM EDTA-Na_2, 0.3 mM ascorbic acid, 0.06 mM H_2O_2, and 0.1 cm^3 of the enzyme extract. The change in absorption

was taken at 290 nm for 30 s after the addition of H_2O_2 (Nakano and Asada 1981). The catalase (CAT, EC 1.11.1.6) activity was measured according to Aebi (1984) at 240 nm for 1 min (the coefficient of absorbance of 39.4 mM cm^{-1}) in 3 cm^3 of reaction mixture containing 50 mM potassium phosphate buffer (pH 7.0), 2 mM EDTA-Na_2, 10 mM H_2O_2, and 0.1 cm^3 of the enzyme extract. The peroxidase (POD, EC 1.11.1.7) activity was assayed by the method of Zhou and Leul (1999) with some modifications. The reaction mixture contained 50 mM potassium phosphate buffer (pH 7.0), 1 % (m/v) guaiacol, 0.4 % (v/v) H_2O_2, and 0.1 cm^3 of the enzyme extract. A variation in absorbance was measured at 470 nm. The total superoxide dismutase (SOD, EC 1.15.1.1) activity was determined using the method of Zhang *et al.* (2008) following the inhibition of photochemical reduction of nitroblue tetrazolium (NBT). The reaction mixture comprised 50 mM potassium phosphate buffer (pH 7.8), 13 mM methionine, 75 μM NBT, 2 μM riboflavin, 0.1 mM EDTA, and 0.1 cm^3 of the enzyme extract in a 3 cm^3 volume. One unit of SOD activity was the amount of the enzyme required to cause 50 % inhibition of the NBT reduction measured at 560 nm. The total soluble protein content was determined according to the method of Bradford (1976) by using bovine serum albumin as standard.

For microscopy, leaf fragments without veins (about 1 mm^2) and root tips (about 2 - 3 mm) were fixed in 4 % (v/v) glutaraldehyde in 0.1 M sodium phosphate buffer (PBS, pH 7.4) overnight and then washed three times with PBS. The samples were post fixed in 1 % (m/v) OsO_4 for 1 h and washed again three times with PBS. After that, the samples were dehydrated in a graded series of ethanol (50, 60, 70, 80, 90, 95, and 100 %, v/v) for 15 - 20 min each and then in absolute acetone for 20 min. After dehydration, the samples were embedded in Spurr's resin overnight. After heating the specimens at 70 °C for 9 h, ultrathin sections (80 nm) were cut and mounted on copper grids for observation in a transmission electron microscope (*TEM 1230EX*, *Jeol*, Tokyo, Japan) at 60.0 kV.

The data were analyzed using the statistical package *SPSS v. 16.0* (*SPSS*, Chicago, IL, USA). Two-way *ANOVA* was carried out followed by the Duncan's multiple range test.

Results

In the present study, 6-d-old seedlings of *B. napus* cultivars (ZS 758, Zheda 619, ZY 50, and Zheda 622) were exposed to 0, 100, 200, 400 μM Cr and the maximum shoot length was observed under the control conditions (Table 1). The shoot length of all the cultivars reduced as we increased the Cr concentration in the solution and the minimum shoot length was observed under 400 μM Cr. Zheda 622 was more affected as compared to the other three cultivars. The root, shoot, and leaf dry masses were significantly lower at 400 μM Cr as compared to their respective controls in all the cultivars and minima were again observed in Zheda 622 (Table 1).

Table 1. The effects of different Cr concentrations on shoot and root lengths, and leaf, stem, and root dry masess of four cultivars of *Brassica napus*. Means ± SD, $n = 4$. Values with different letters in individual columns are statistically different at $P \leq 0.05$.

Cultivar	Cr conc. [μM]	Shoot length [cm]	Root length [cm]	Leaf dry mass [mg $plant^{-1}$]	Stem dry mass [mg $plant^{-1}$]	Root dry mass [mg $plant^{-1}$]
ZS 758	0	3.46 ± 0.11a	3.13 ± 0.09a	3.7 ± 0.1a	1.4 ± 0.1a	1.6 ± 0.1a
	100	3.33 ± 0.09a	2.99 ± 0.13ab	3.6 ± 0.1c	1.3 ± 0.1bc	1.4 ± 0.1bc
	200	2.78 ± 0.09cd	2.64 ± 0.09de	3.3 ± 0.1e	1.1 ± 0.1ef	1.3 ± 0.1de
	400	2.19 ± 0.09f	1.89 ± 0.07h	2.6 ± 0.1i	0.8 ± 0.1i	1.1 ± 0.1gh
Zheda 619	0	3.45 ± 0.10a	2.98 ± 0.10ab	3.7 ± 0.1ab	1.3 ± 0.1b	1.5 ± 0.1abc
	100	3.38 ± 0.08a	2.72 ± 0.10cde	3.3 ± 0.1e	1.2 ± 0.1cde	1.4 ± 0.1cd
	200	2.69 ± 0.08cd	2.56 ± 0.10ef	2.7 ± 0.1h	1.0 ± 0.1g	1.3 ± 0.1ef
	400	2.04 ± 0.14fg	1.81 ± 0.10hi	2.3 ± 0.1j	0.7 ± 0.1jk	1.0 ± 0.1h
ZY 50	0	3.43 ± 0.11a	2.97 ± 0.11ab	3.6 ± 0.1c	1.3 ± 0.1bc	1.5 ± 0.1ab
	100	2.98 ± 0.12b	2.86 ± 0.09bc	3.5 ± 0.1d	1.2 ± 0.1bcd	1.4 ± 0.1c
	200	2.65 ± 0.14de	2.37 ± 0.09g	3.2 ± 0.1f	0.9 ± 0.1h	1.2 ± 0.1f
	400	1.98 ± 0.11g	1.66 ± 0.12i	2.2 ± 0.1k	0.6 ± 0.1k	1.0 ± 0.1h
Zheda 622	0	3.37 ± 0.10a	2.82 ± 0.12bcd	3.6 ± 0.1bc	1.3 ± 0.1bcd	1.5 ± 0.1bc
	100	2.85 ± 0.08bc	2.44 ± 0.08fg	3.2 ± 0.1f	1.1 ± 0.1fg	1.3 ± 0.1de
	200	2.47 ± 0.13e	2.31 ± 0.11g	3.1 ± 0.1g	0.7 ± 0.1ij	1.1 ± 0.1g
	400	1.62 ± 0.11h	1.43 ± 0.09 j	2.1 ± 0.1l	0.5 ± 0.1l	0.8 ± 0.1i

Table 2. The effects of different Cr concentrations on Cr content in roots and shoots, and on chlorophyll and carotenoid content in cotyledons of four cultivars of *Brassica napus*. Means ± SD, $n = 4$. Values with different letters in individual columns are statistically different at $P \leq 0.05$.

Cultivar	Cr conc. [μM]	Root Cr content [mg kg^{-1}(d.m.)]	Shoot Cr content [mg kg^{-1}(d.m.)]	Chl *a* content [mg g^{-1}(f.m.)]	Chl *b* content [mg g^{-1}(f.m.)]	Car content [mg g^{-1}(f.m.)]
ZS 758	0	0.03 ± 0.00j	0.01 ± 0.00i	4.461 ± 0.730a	1.213 ± 0.106a	0.30 ± 0.020a
	100	336.45 ± 16.64i	8.56 ± 1.52h	3.812 ± 0.088c	1.057 ± 0.112a-d	0.28 ± 0.013b
	200	610.33 ± 23.71f	15.42 ± 1.58fg	3.615 ± 0.090de	0.908 ± 0.083c-e	0.25 ± 0.008c-f
	400	1286.01 ± 22.65c	27.06 ± 2.21c	3.281 ± 0.091f	0.589 ± 0.044f	0.23 ± 0.010c-f
Zheda 619	0	0.02 ± 0.00 j	0.02 ± 0.00i	4.335 ± 0.108a	1.155 ± 0.092ab	0.28 ± 0.009b
	100	409.66 ± 20.56gh	12.08 ± 1.00g	3.846 ± 0.112c	0.980 ± 0.104b-d	0.26 ± 0.011b-e
	200	721.57 ± 56.96e	19.22 ± 2.64e	3.776 ± 0.082cd	0.757 ± 0.087e	0.24 ± 0.009c-f
	400	1376.34 ± 14.64b	33.71 ± 3.21b	3.193 ± 0.860fg	0.537 ± 0.111fg	0.21 ± 0.011c-f
ZY 50	0	0.04 ± 0.00 j	0.02 ± 0.00i	4.426 ± 0.095a	1.074 ± 0.095a-c	0.30 ± 0.007a
	100	392.46 ± 23.32h	12.54 ± 1.04g	3.846 ± 0.102c	1.011 ± 0.093b-d	0.27 ± 0.005bc
	200	711.45 ± 36.36e	20.45 ± 1.11e	3.616 ± 0.096de	0.960 ± 0.117cd	0.26 ± 0.013b-e
	400	1382.48 ± 33.08b	34.24 ± 3.52b	3.088 ± 0.088g	0.510 ± 0.090fg	0.24 ± 0.010c-f
Zheda 622	0	0.03 ± 0.00 j	0.01 ± 0.00i	4.163 ± 0.096b	1.046 ± 0.112a-d	0.27 ± 0.012b-d
	100	451.87 ± 29.46g	15.66 ± 1.15f	3.827 ± 0.106c	0.970 ± 0.130b-d	0.25 ± 0.011c-f
	200	798.86 ± 36.16d	23.66 ± 2.51d	3.472 ± 0.096e	0.876 ± 0.102de	0.25 ± 0.005c-f
	400	1530.73 ± 55.47a	46.33 ± 3.05a	2.823 ± 0.120h	0.373 ± 0.094g	0.11 ± 0.022c-f

Table 3. The effects of different Cr concentrations on TSP and MDA content, and on ROS accumulation in cotyledons of four cultivars of *Brassica napus*. Means ± SD, $n = 4$. Values with different letters in individual columns are statistically different at $P \leq 0.05$.

Cultivar	Cr conc. [μM]	TSP content [mg g^{-1}(f.m.)]	MDA content [nmol mg^{-1}(protein)]	H_2O_2 [μmol g^{-1}(d.m.)]	$O_2^{\cdot-}$ [nmol g^{-1}(d.m.) min^{-1}]	$^{\cdot}OH$ [μmol g^{-1}(f.m.)]
ZS 758	0	324.51 ± 9.96a	20.35 ± 1.18h-j	1.50 ± 0.09j	7.34 ± 0.97i	0.13 ± 0.01f
	100	295.79 ± 11.42b-d	15.65 ± 1.00k	1.52 ± 0.10j	9.41 ± 1.07gh	0.15 ± 0.02f
	200	278.62 ± 10.51d-f	20.53 ± 1.27g-I	2.55 ± 0.10j	10.92 ± 0.92e-g	0.13 ± 0.01f
	400	271.70 ± 7.68ef	29.38 ± 1.34c	3.53 ± 0.07e	15.53 ± 0.85b	0.14 ± 0.01f
Zheda 619	0	321.80 ± 11.16a	22.42 ± 1.03fg	1.51 ± 0.09j	7.60 ± 1.00i	0.13 ± 0.01f
	100	322.88 ± 10.03a	25.21 ± 1.16de	1.71 ± 0.08i	9.71 ± 0.88gh	0.13 ± 0.01f
	200	303.77 ± 7.93b	30.39 ± 1.29c	1.93 ± 0.07h	11.71 ± 0.93ef	0.15 ± 0.01ef
	400	292.42 ± 7.69b-d	33.54 ± 1.14b	2.51 ± 0.09g	14.69 ± 1.08bc	0.17 ± 0.01e
ZY 50	0	323.42 ± 9.81a	18.70 ± 1.26ij	1.53 ± 0.09j	8.41 ± 1.08hi	0.13 ± 0.01f
	100	299.73 ± 9.95bc	21.92 ± 1.05f-h	2.73 ± 0.11f	10.52 ± 0.86fg	0.25 ± 0.01cd
	200	282.36 ± 8.81c-e	26.40 ± 0.89d	3.85 ± 0.10d	12.30 ± 1.03de	0.24 ± 0.01d
	400	263.81 ± 9.99f	29.46 ± 1.05c	5.22 ± 0.10c	15.48 ± 1.01b	0.26 ± 0.01c
Zheda 622	0	330.89 ± 10.05a	19.99 ± 1.23h-j	1.45 ± 0.10j	7.21 ± 0.81i	0.13 ± 0.01f
	100	323.02 ± 10.72a	18.40 ± 1.08ij	2.88 ± 0.10f	10.34 ± 0.98fg	0.34 ± 0.01b
	200	295.67 ± 9.15b-d	23.58 ± 1.05ef	5.83 ± 0.10b	13.70 ± 1.05cd	0.35 ± 0.02b
	400	266.83 ± 6.89ef	35.52 ± 0.92a	7.75 ± 0.10a	17.27 ± 0.82a	0.44 ± 0.01a

The Cr content in the roots and shoots of all the cultivars increased linearly with the increased Cr concentration in the solution (Table 2). Moreover, the Cr content was higher in the roots than in the shoots in all the cultivars. The Cr content was the highest in Zheda 622 followed by ZY 50, Zheda 619, and ZS 758. The content of Chl and Car gradually decreased with the increasing Cr concentration and a significant reduction was observed in all the cultivars under 400 μM Cr (Table 2). A higher decrease in the content of Chl and Car was observed in Zheda 622 as compared to the other cultivars. In the Cr-treated plants, there was a decline in the protein content in all the cultivars and this reduction was the highest at 400 μM Cr. The soluble protein content decreased with the increasing Cr concentration and this reduction was greater in ZY 50 and Zheda 622

followed by ZS 758 and Zheda 619 (Table 3). The Cr stress significantly increased the production of MDA in the leaves of all the cultivars, and a maximum production of MDA was found at 400 µM Cr. The MDA content was the highest in Zheda 622 and the lowest in ZS 758 under 400 µM Cr. Moreover, the application of Cr increased the ROS production. The amount of H_2O_2 was lower in ZS 758 and Zheda 619 than in ZY 50 and Zheda 622 under 400 µM Cr. The production of $O_2^{\cdot-}$ and $^{\cdot}OH$ was enhanced in all the cultivars as we increased the Cr concentration in the solution and a maximum was found in Zheda 622 at 400 µM Cr (Table 3).

The cultivars ZS 758 and Zheda 619 displayed a maximum GR activity under 400 µM Cr. However, the cvs. ZY 50 and Zheda 622 did not show any significant difference in the GR activity at 400 µM Cr as compared to their respective controls (Table 4). The APX activity decreased as the Cr concentration increased; a minimum APX activity was found in Zheda 622 under 400 µM Cr (Table 4). The CAT activity gradually increased with the increasing Cr concentration in ZS 758 and Zheda 619 and reached a maximum under 400 µM Cr. There was no significant difference in the CAT activity in ZY 50 under the different Cr concentrations. However, the CAT activity gradually decreased in Zheda 622 as the Cr concentration increased (Table 4). The POD activity did

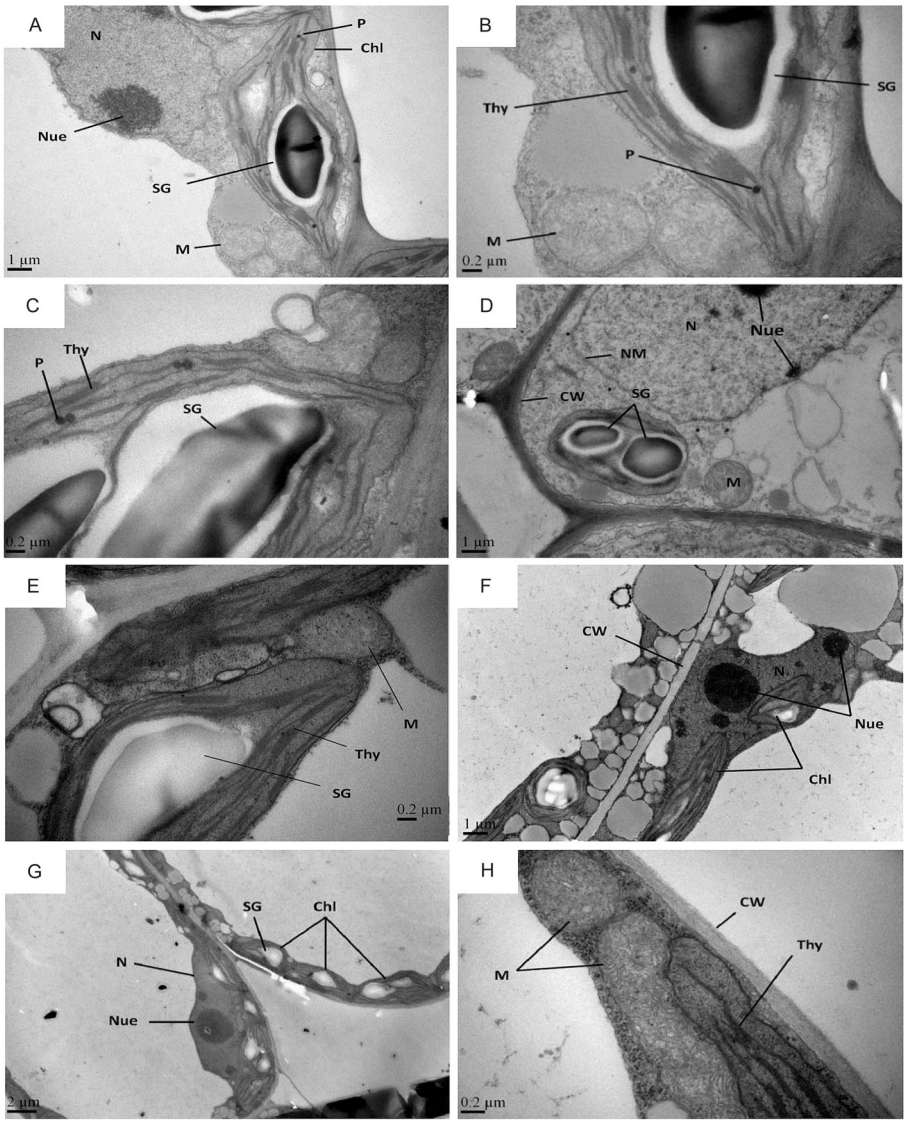

Fig. 1. TEM micrographs of leaf mesophyll cells of 6-d-old seedlings of four cultivars of *Brassica napus* (ZS 758, Zheda 619, ZY 50, and Zheda 622) grown under control conditions. *A,B* - leaf mesophyll cells of ZS 758 show the well-developed nucleus (N) with nucleolus (Nue), starch grain (SG), mitochondrion (M), and chloroplast (Chl). *C,D* - leaf mesophyll cells of Zheda 619 show the cell wall (CW), well developed nucleus (N) with nucleolus (Nue), and mitochondrion (M). *E,F* - leaf mesophyll cells of ZY 50 show the starch grain (SG), mitochondrion (M), and thylakoid membrane (Thy). *G,H* - leaf mesophyll cells of Zheda 622 show the cell wall (CW), mitochondrion (M), chloroplast (Chl), and starch grain (SG).

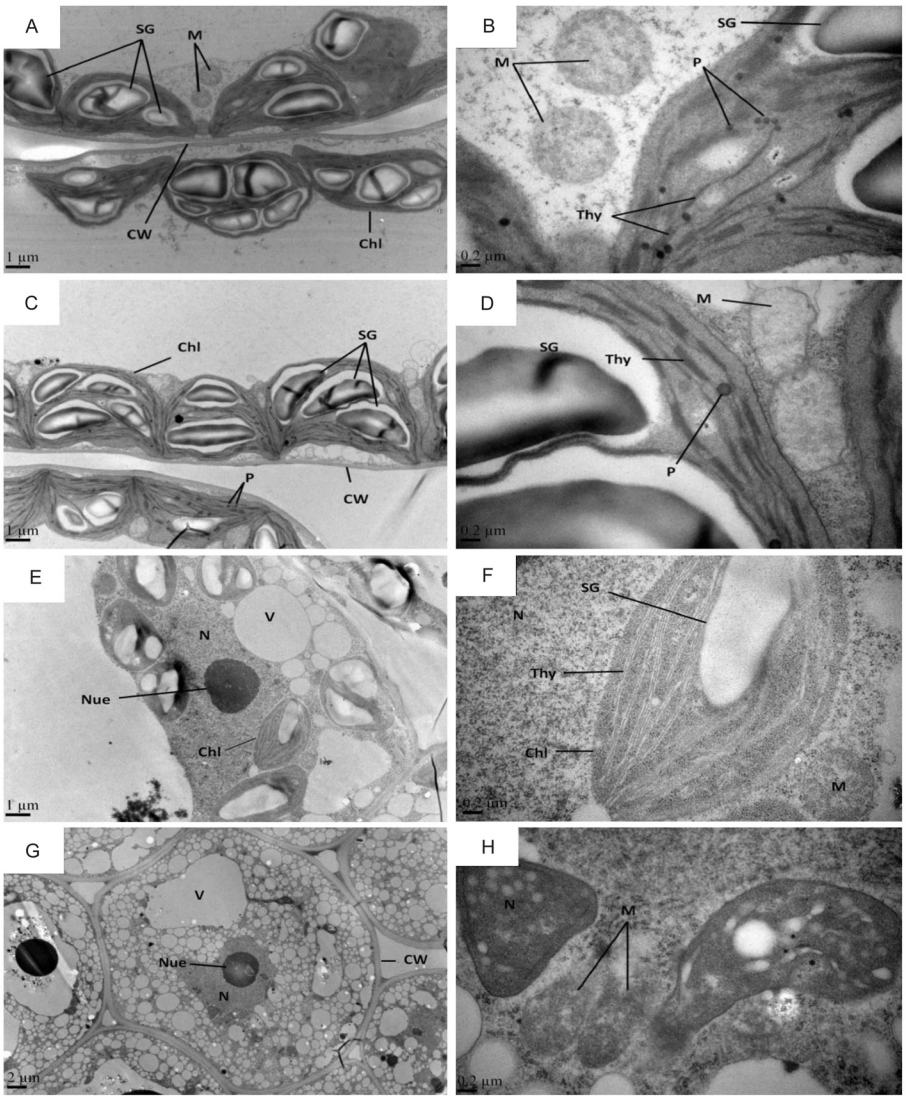

Fig. 2. TEM micrographs of leaf mesophyll cells of 6-d-old seedlings of four cultivars of *Brassica napus* (ZS 758, Zheda 619, ZY 50, and Zheda 622) grown under 400 µM Cr. *A,B* - leaf mesophyll cells of ZS 758 show the well developed chloroplast (Chl), starch grain (SG), mitochondrion (M), and plastoglobule (P). *C,D* - leaf mesophyll cells of Zheda 619 show the chloroplast (Chl), starch grain (SG), thin cell wall (CW), thylakoid membrane (Thy), mitochondrion (M), and plastoglobule (P). *E,F* - leaf mesophyll cells of ZY 50 show the nucleus (N) with nucleolus (Nue), vacuole (V), dissolved thylakoid membrane (Thy), and immature starch grain (SG). *G,H* - leaf mesophyll cells of Zheda 622 show the protein vacuole (V), cell wall (CW), nucleus (N), nucleolus (Nue), and immature mitochondrion (M).

not show any significant difference under the different Cr concentrations except for Zheda 619 in which the POD activity was greater under 400 µM Cr as compared to the control (Table 4). A significant increase was observed in the SOD activity in ZS 758 and Zheda 619 at 400 µM Cr as compared to their respective controls. However, the SOD activity was decreased in ZY 50 and Zheda 622 under 400 µM Cr (Table 4).

As concerns micrographs of leaf mesophyll, cells of control ZS 758 showed thewell-developed chloroplasts with thylakoid membranes and a number of the starch grains. The clear nucleus was present with nucleolus and nuclear membrane as well as a number of the mitochondria (Fig. 1*A*,*B*). There was the well developed nucleus with nucleolus and nucleus membrane, cell wall, large size starch grain, round shape mitochondria, thylakoid membranes with grana and stroma, and visible chloroplast in Zheda 619 under the control conditions (Fig. 1*C*,*D*). Control ZY 50 depicted the large size vacuole, nucleus with two distinct nucleoli, chloroplast, thick and clear cell wall, big starch grain, and mitochondria. Moreover, the well developed thylakoid membranes were found (Fig. 1*E*,*F*). Zheda 622 showed the visible mitochondria, cell wall, thylakoid membrane,

nucleus with nucleolus, and chloroplast with starch grain (Fig. 1*G*,*H*). In ZS 758 and Zheda 619, 400 µM Cr damaged the thylakoid membranes, and a size and number of the starch grains and plastoglobuli increased (Fig. 2*A*,*B*,*C*,*D*). At 400 µM Cr, ZY 50 showed ruptured cells having the immature nucleus and chloroplasts. Moreover, the thylakoid membranes were damaged badly (Fig. 2*E*,*F*). Under the Cr stress, Zheda 622 depicted the ruptured organelles. The vacuole and immature nucleolus were also observed (Fig. 2*G*,*H*).

TEM micrographs of root tip cells of control ZS 758 showed the visible nucleus with a number of the nucleoli. The Golgi bodies and mitochondria were also observed (Fig. 3*A*,*B*). In the case of control Zheda 619, the clear cell wall, mitochondria, nucleus with distinct nuclear membrane and nucleolus, and Golgi bodies were observed (Fig. 3*C*,*D*). Root cells of ZY 50 had the large nucleus, discrete cell wall, and easily distinguishable plastid structures (Fig. 3*E*,*F*). In the case of control Zheda 622, the cell wall, and a number of the Golgi bodies and mitochondria could be seen (Fig. 3*G*,*H*). Under 400 µM Cr, ZS 758 showed the damaged cell wall, nucleoli attained to the large nucleus, and a number of the mitochondria (Fig 4*A*,*B*). At this concentration, Zheda 619 showed the distinct and giant nucleolus in the nucleus, properly developed mitochondria, clear cell wall, and Golgi bodies (Fig. 4*C*,*D*). Under 400 µM Cr, the cell shape of ZY 50 was disturbed and a number of the vacuoles were observed. Furthermore, the oval shape mitochondrion was also noticed (Fig. 4*E*,*F*). Plasmolysis in cells of Zheda 622 was found at 400 µM Cr. All the organelles were damaged and were not clearly seen in the micrographs (Fig. 4*G*,*H*).

Table 4. The effects of different Cr concentrations [µM] on activities of glutathione reductase, ascorbate peroxidase, catalase, guaiacol peroxidase [µmol mg^{-1}(protein) min^{-1}], and superoxide dismutase [U mg^{-1} (protein)] in cotyledons of four cultivars of *Brassica napus*. Means ± S.D, $n = 4$. Values with different letters in individual columns are statistically different at $P \leq 0.05$.

Cultivar	Cr conc.	GR	APX	CAT	POD	SOD
ZS 758	0	39.34 ± 3.76e-h	0.83 ± 0.01c	0.34 ± 0.02fg	1.05 ± 0.14d	1051.77 ± 9.59c
	100	34.90 ± 3.63h	0.86 ± 0.02b	0.38 ± 0.02de	1.14 ± 0.10d	951.62 ± 11.48h
	200	42.07 ± 3.79d-g	0.72 ± 0.02f	0.42 ± 0.02c	1.13 ± 0.11d	1021.46 ± 9.53d
	400	68.20 ± 3.01a	0.65 ± 0.01gh	0.47 ± 0.01b	1.24 ± 0.11cd	1216.15 ± 11.56a
Zheda 619	0	38.66 ± 3.76f-h	0.86 ± 0.02b	0.36 ± 0.02ef	1.19 ± 0.11cd	1016.40 ± 8.89d
	100	36.00 ± 3.49gh	0.85 ± 0.02b	0.39 ± 0.01d	1.16 ± 0.01cd	987.69 ± 10.17ef
	200	46.73 ± 3.11b-d	0.76 ± 0.01e	0.47 ± 0.01b	1.18 ± 0.01cd	1125.13 ± 9.71b
	400	50.43 ± 3.60b	0.65 ± 0.01gh	0.52 ± 0.02a	1.56 ± 0.10b	1202.45 ± 10.54a
ZY 50	0	43.32 ± 3.61c-f	0.89 ± 0.02a	0.36 ± 0.01ef	1.08 ± 0.07d	1004.48 ± 10.72de
	100	44.44 ± 3.87b-f	0.67 ± 0.01g	0.34 ± 0.02fg	1.04 ± 0.14d	966.64 ± 9.45gh
	200	47.92 ± 3.38b-d	0.56 ± 0.02j	0.29 ± 0.01h	1.36 ± 0.08c	962.27 ± 12.49h
	400	50.09 ± 3.59bc	0.62 ± 0.02i	0.36 ± 0.02ef	1.24 ± 0.10cd	753.85 ± 9.81j
Zheda 622	0	41.25 ± 3.82d-h	0.85 ± 0.01b	0.34 ± 0.01fg	1.04 ± 0.13d	981.46 ± 11.01fg
	100	45.69 ± 3.70b-e	0.89 ± 0.02a	0.33 ± 0.01g	1.19 ± 0.12cd	852.21 ± 9.18i
	200	42.16 ± 4.76d-g	0.79 ± 0.01d	0.27 ± 0.02h	1.85 ± 0.09a	766.90 ± 13.43j
	400	46.58 ± 2.52b-d	0.55 ± 0.01j	0.17 ± 0.01i	1.11 ± 0.10d	564.92 ± 8.39k

Discussion

Enrichment of cultivated soils with toxic metals like Cr, Cd, and Pb has become an alarming situation in all over the world (Fernandez *et al.* 2008). Heavy metals can cause abnormalities in the functions of cell organelles, cell membranes, and also in metabolic activities (Sinha *et al.* 2009). In the present study, the shoot and root lengths were differently affected by the Cr stress in four *B. napus* cultivars (Table 1). Similarly in wheat, shoot length decreases by 44 % and root length by 63 % under Cr stress (Dey *et al.* 2009). Nevertheless, roots grow faster than the shoots and higher length of root helped to Cr absorption (Panda *et al.* 2002). However, Samantary (2002) observed a reduction in the development of lateral root growth, an inhibition of root cell division and cell elongation which was probably caused by the deterioration of water and nutrient uptake under Cr toxicity. In this study, the root, stem, and leaf dry masses significantly decreased with the increase in Cr concentration, especially in cv. Zheda 622 (Table 1). The Cr content increased in the roots and shoots of all the genotypes as we increased the Cr concentration in the solution (Table 2). Roots are usually more affected by Cr as a rather small amount of Cr is translocated to shoots (Paiva *et al.* 2009). However, findings of Tiwari *et al.*

(2009) support our results that Cr content increases in all plant parts.

It is well documented that Cr disturbs many physiological and biochemical pathways (Sundaramoorthy *et al.* 2010). The Cr stress reduced the content of Chl and Car in four studied *B. napus* cultivars (Table 2). Similarly, Cr shows an adverse impact on photosynthetic parameters (Ganesh *et al.* 2008, Subrahmanyam 2008). The reduction in Chl content might be due to disturbance in the supply of Fe^{2+}, Zn^{2+}, and Mg^{2+} ions under heavy metal stress (Kupper *et al*. 2000, Ali *et al*. 2013b). MDA is considered as final decomposition product of polyunsaturated fatty acids and it is used to determine oxidative damage (Gunes *et al.* 2007). In the present study, the MDA content increased at 400 μM Cr in all the cultivars (Table 3). Oxidative stress is considered as intrinsic feature of senescence in plants (Delrio *et al.* 1998). It has been considered that chloroplasts are chief source for the generation of ROS in plants (Purvis 1997, Apel and Hirt 2004). In this study, a higher content of ROS was observed under the Cr stress in four cultivars (Table 3) similarly as under other heavy metal stress (Dietz *et al.* 1999, Pandey *et al.* 2009, Ali *et al.* 2013c, 2014). In addition, the cultivar Zheda 622 was proved to be more sensitive to the Cr stress, due to a higher content of MDA, H_2O_2, $^{\cdot}OH$, and $O_2^{\cdot-}$, than other cultivars.

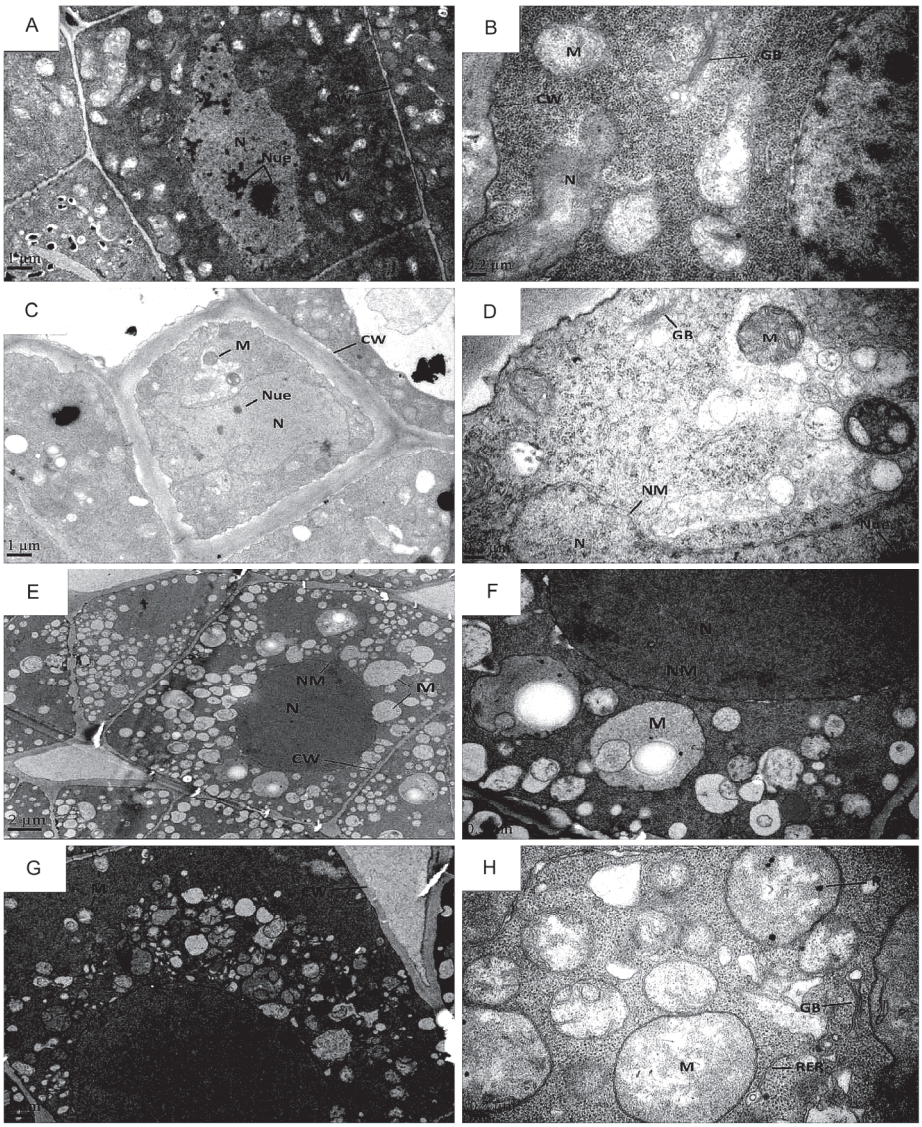

Fig. 3. TEM micrographs of root tip cells of 6-d-old seedlings of four cultivars of *Brassica napus* (ZS 758, Zheda 619, ZY 50, and Zheda 622) grown under control conditions. *A,B* - root tip cells of ZS 758 show the elongated nucleus (N) with a number of the nucleoli (Nue), well developed mitochondria (M), cell wall (CW), and Golgi bodies (GB). *C,D* - root tip cells of Zheda 619 show the mitochondria (M), longitudinal nucleus (*N*) with clear nuclear membrane (NM), small size nucleolus (Nue), and Golgi bodies (GB). *E,F* - root tip cells of ZY 50 show the nucleus (N), nuclear membrane (NM), and cell wall (CW). *G,H* - root tip cells of Zheda 622 show the cell wall (CW), roundish mitochondria (M), Golgi bodies (GB), and rough endoplasmatic reticulum (RER).

Plant cells have evolved antioxidant systems to cope with ROS (Reddy *et al.* 2005, Rucinska-Sobkowiak and Pukacki 2006) and we determined the activities of SOD, POD, CAT, APX, and GR. SOD and CAT play an important role in the removal of $O_2^{\cdot-}$ and H_2O_2, and GR ensures the availability of reduced glutathione for the synthesis of heavy metal-binding peptides (Gomes *et al.* 2006). In the present study, the activities of GR and CAT increased but the APX activity decreased under the Cr stress in all the cultivars except Zheda 622 in which CAT gradually decreased under the Cr stress (Table 4). Moreover, the SOD activity decreased in ZS 758 and Zheda 619, whereas increased in ZY 50 and Zheda 622 under 400 μM Cr. In *Helianthus annuus* and *Brassica juncea* GR, APX, SOD, and POD activities increase under Cr stress (Gallego *et al.* 2002, Pandey *et al.* 2005). In contrast, a decrease in SOD and CAT activities in *Cassia angustifolia* may indicate their inactivation by accumulation of H_2O_2 induced by metal stress (Qureshi *et al.* 2007). Some contrasting reports were also found about effects of other heavy metals on activities of antioxidant enzymes, *e.g.*, Gallego *et al.* (1996) revealed that Cd decreases activities of SOD, CAT, APX, and GR in leaves of *Helianthus annus*, however, Shamsi *et al.*

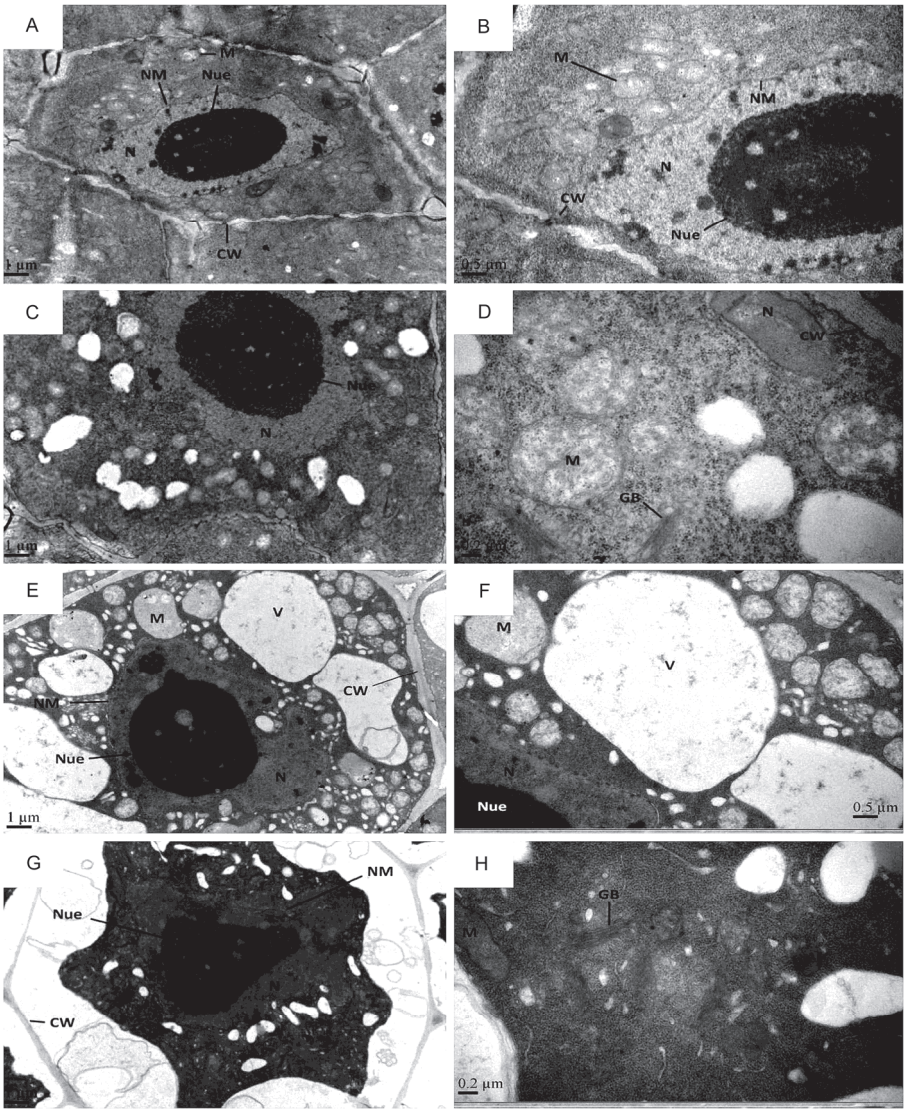

Fig. 4. TEM micrographs of root tip cells of 6-d-old seedlings of four cultivars of *Brassica napus* (ZS 758, Zheda 619, ZY 50, and Zheda 622) grown under 400 μMl Cr. *A,B* - root tip cells of ZS 758 show the nucleus (N) with large size nucleoli (Nue), mitochondria (M), broken cell wall (CW), and nuclear membrane (NM). *C,D* - root tip cells of Zheda 619 show the mitochondria (M), nucleus (N) with clear nuclear membrane (NM), large size nucleolus (Nue), Golgi bodies (GB), and cell wall (CW). *E,F* - root tip cells of ZY 50 show only the large size vacuole (V), nucleus (N), nucleolus (Nue) and immature mitochondria (M). The accumulation of Cr in the form of electron dense granules is present inside the vacuole (V). *G,H* - root tip cells of Zheda 622 show the nucleus (N) with nucleolus (Nue) and immature Golgi bodies (GB), and undeveloped mitochondria (M). Moreover, plasmolysis in the cell could be found. The accumulation of Cr in the form of electron dense granules is present inside the vacuoles.

(2008) proposed that activities of SOD and POD increase under Cd stress in soybean plants. Similarly, it was observed that a CAT activity increased under Al stress in wheat and soybean (Darko *et al.* 2004, Zhen *et al.* 2009). It can be suggested that an activated antioxidant system under heavy metal stress may be beneficial for plants to remove excess ROS and for the inhibition of lipid peroxidation (Ali *et al.* 2013d).

Electron microscopy has been carried out to assess plant damages at tissue and cell levels (Caasilit *et al.* 1997). In leaf mesophyll cells of all the cultivars, the marked effects of Cr were observed on the starch grain and nucleus (Figs. 1, 2). The nucleus has a major role in expression of genes (Jiang *et al.* 2007) which became irregular under Cr stress. Another important organelle disturbed by the Cr stress was the chloroplast. Previously, it was observed that Cr and Pb at different concentrations damage chloroplasts by disruption of chloroplast membranes and disorganization of thylakoids (Chaudary and Panda 2005). Moreover, Ali *et al.* (2013e, 2014) also found these changes in leaf ultrastructure of *B. napus* under heavy metal stress. The chloroplast damage induced by Cr was more obvious in Zheda 622 as compared to the other cultivars. Disappearance of thylakoid membranes and presence of protein bodies in Zheda 622 might be due to a higher uptake of Cr by roots. Similarly, Ali *et al.* (2013c) studied the leaf ultrastructure of barley under Cr toxicity and found that thylakoid membranes disappeared and a number of starch grains increased. The root tip cells also showed significant changes under the Cr stress in all the cultivars (Figs. 3 and 4). The disappearance of the nucleus and immature mitochondria were observed in ZY 50 under the Cr stress. Moreover, the deposition of Cr content was found in the large vacuoles. The cultivar Zheda 622 showed the totally damaged root tip cells under the Cr stress. The disappearance of the mitochondria and endoplasmic reticulum was also observed (Fig. 4*G,H*). Furthermore, these comprehensive changes in Zheda 622 indicate that it was more sensitive to Cr than the other cultivars. Recently, it was observed that Cr stress damages root tip cells of barley by the enlargement of vacuoles and by the disappearance of nuclei (Ali *et al.* 2013c). Ali *et al.* (2014) also found the toxic effect of Cd stress on root tips of 6-d-old *B. napus* seedlings.

According to present study, it can be concluded that the *B. napus* cultivars had different capability to face the Cr toxicity. According to the morphological, biochemical, and ultrastructural findings, it can be suggested that ZS 758 was the most tolerant and Zheda 622 was the most sensitive to the Cr stress. Moreover, our findings would be of great interest to scientists working on phyto-remediation and related area. However, further investigations under field conditions are needed.

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