

Nutrients, ultrastructures, and Cd subcellular localization in the cottonseeds of three upland cotton cultivars under Cd stress

L. Li, J.R. Li, Q.L. He, S.J. Zhu*, J.H. Chen

*Collage of Agriculture and Biotechnology, Zhejiang University, Hangzhou 310058, Zhejiang Province, P.R. China.*Corresponding author: shjzhu@zju.edu.cn*

Abstract

A pot experiment was performed to study the Cd-induced alterations in seed quality at maturity in two transgenic upland cotton cultivars, ZD-90 and SGK3, and the upland cotton standard line, TM-1. The results shown that Cd content in cotton kernels increased linearly with the elevation of Cd stress levels. SGK3 accumulated more Cd than others. Protein content in the kernels was increased under Cd stress generally, but those at 600 μM Cd level were lower than that under 400 μM Cd level. The changes in oil content were inversed to that of protein content. Significant ultra-structural changes in cottonseeds were induced by Cd stress, especially at higher Cd levels, which were more obvious in TM-1, followed by ZD-90 and SGK3. Plasmolytic shrinkage, disintegration of nucleus, cell wall thickening, and eventual cell collapse and disintegration were observed in the cells of cottonseeds under Cd stress. Transmission electron microscope (TEM) observation and energy dispersive X-ray analysis (EDAX) confirmed that Cd existed in the form of rings and crystals as well as electron dense granules, occurred in the intercellular space, the cytoplasm, and the cell wall. SGK3 was a tolerance cultivar to Cd stress with greater Cd accumulation and sequestration in cottonseeds.

Keywords: Cotton, cadmium stress, seed quality, ultrastructure

1. Introduction

Due to the effect of human's activities on the environment such as industrial emissions from smelters, painting, car radiator manufacturing, batteries, as well as the application of sewage sludge and phosphate fertilizers, heavy metals pollution has become a major global environmental problem (Paiva *et al.*, 2009). Cadmium (Cd) is probably one of the most toxic heavy metals in soils for plants (Prasad, 1995) It

can adversely interfere with important plant processes such as water transport, absorption and transport of essential elements, oxidative phosphorylation in mitochondria, photosynthesis, respiration, chlorophyll content, plant growth and reproduction, as well as the yield and quality of the plant products (Prasad, 1995; Cao *et al.*, 2005; Guo *et al.*, 2006; Wu *et al.*, 2006; Huang *et al.*, 2008; Li *et al.*, 2012). As a result, it can

easily enter the food chain (Satarug *et al.*, 2003) and become toxic to human beings and animals. At least 70% of the Cd intake by humans originates from plant foods (Wagner, 1993). More than 1.3×10^5 km² of agricultural soils are contaminated by Cd in China and 1.46×10^8 kg of agricultural products are polluted by Cd every year (Gu and Zhou, 2002). It is urgently necessary to clean Cd from soils where field crops, vegetables, and fruits have been grown, in order to protect ourselves from Cd poisoning.

Plants have developed several different mechanisms to resist the excess of Cd in soils. One of these mechanisms is the compartmentalization of Cd in specific tissues or cellular organelles (Isaure *et al.*, 2006). The epidermis (Wojcik *et al.*, 2005), mesophyll cell (Ma *et al.*, 2005), trichomes (Salt *et al.*, 1995), cell wall (Vazquez *et al.*, 1992; Wojcik *et al.*, 2005), cortex parenchyma cells, endodermis, parenchyma cells of the central cylinder and xylem vessels (Wojcik *et al.*, 2005) were found to be sites that Cd is accumulated or located. Ultra-structural changes in plant organs are known to be some of the worst effects of Cd accumulation (Daud *et al.*, 2009a; Daud *et al.*, 2009b; Küpper *et al.*, 2000; Vitória *et al.*, 2003). However, as far as we are aware, there are few studies conducted on the effect of Cd on seed components and ultrastructure. The hyperaccumulate plants which may have an innate capacity to extract toxic elements from the soil can decontaminate and restore fertility in polluted areas (Krämer, 2005; Zhou and Song, 2004). This phytoremediation process is a cost effective and promising technology for the renovation of Cd contaminated soil (Zhou and Song, 2004). However, the application of phytoremediation technology is still very limited due to the low biomass of hyperaccumulators (Liu, *et al.*, 2008). Thus, the most effective way of reducing soil Cd level is to breed Cd tolerant crop cultivars carrying off more Cd from the contaminated soil.

Cotton (*Gossypium hirsutum* L.), a fiber crop, fulfills various needs for humans and plays an important role in the global economy. Sengalevitch (1999) considered

cotton as a suitable crop for phytoremediation in industrially polluted regions. However, few studies have been conducted on Cd accumulation and the toxic effects of Cd on cottonseeds cultivated under Cd contaminated soils. In the present experiment, three cotton cultivars, namely ZD-90, SGK3 and TM-1, were planted on Cd polluted soils, and their seed components, ultrastructural changes, and Cd subcellular localization in cottonseeds were studied. The main objectives of the study were to find out the toxic effects of Cd on seed components, identify cotton genotypes with higher Cd accumulation, and study the possible mechanism of phytoremediation for Cd contaminated soil through cotton cultivars.

2. Materials and Methods

2.1. Plant materials and Cd-treatment levels

Three cotton cultivars, TM-1, ZD-90, and SGK3, were used in the present experiment. ZD-90 is a transgenic glyphosate tolerant cultivar with EPESP-G6 derived from our laboratory. SGK3 is an insect-resistant cultivar obtained from the Biotechnology Center of the Chinese Academy of Agricultural Sciences. TM-1 is an upland cotton genetic standard line obtained from USDA-ARS, College Station, Texas, USA.

The study was carried out in the experimental station of Zhejiang University. Plants were grown in pots, 35cm in diameter and 40 cm in depth, outdoors in a large and rainproof net house. 15 kg of uncontaminated soil with pH=6.2, 2.99 % organic matter, 1.35 % total N (162.58 mg.kg⁻¹ available N), 0.13 % total P (23.82 mg.kg⁻¹ available P), and 0.83 % total K (112.79 mg.kg⁻¹ available K) was placed in each pot. Five seeds per pot were sown on May 5, 2011 and April 30, 2012, respectively. Only one seedling was remained in whole growth season in each pot after two weeks. Cd in the form of CdCl₂·2.5H₂O was added keeping stable concentrations at 0, 200, 400, and 600 μM, respectively, for the whole growing

season. There were three replications per treatment, which were arranged in a completely randomized experimental design.

2.2. Measurements of components in cottonseeds

The cottonseeds after ginning were delinted with H₂SO₄, then shelled and milled. Powder samples of cottonseed kernel were used to determine protein, oil, and gossypol contents by near-infrared reflectance spectroscopy on NIR Systems model 5000 instrument (NIR Systems, Inc., Silver Spring, MD, USA) with routine analysis and calibration development carried out according to WINISI II manual (ISI FOSS NIR Systems/ TECTOR, Infra soft International, LLC.) (Qin *et al.*, 2010).

2.3. Determination of Cd content

For the quantification of Cd in cottonseeds, samples were dried to constant weight at 80 °C and ground into fine powder. They were then wet digested for 4-5 hours by adding a mixture of 5 ml HNO₃ and 0.5 ml H₂O₂. Each digested sample was transferred to a 50 ml flask for constant volume. Cd was quantified using an ICP-Mass Spectrograph (7500a, Agilent).

2.4. Observation of transmission electron microscope (TEM)

Seeds were fixed overnight in 2.5 % glutaraldehyde (v/v) in 0.1M PBS (Sodium Phosphate Buffer, pH 7.0) and washed three times with same PBS for 15 minutes each. The samples were post-fixed in 1 % OsO₄ (osmium (VIII) oxide) for 2 hours, then washed three times in 0.1M PBS (pH 7.0) with 15 minutes interval. They were then dehydrated by a graded series of ethanol (50 %, 60 %, 70 %, 80 %, 90 %, 95 %, and 100 %) for 30 minutes at each step, and finally transferred to absolute acetone for 40 minutes. The samples were then infiltrated and embedded in Spurr's resin overnight. After heating the specimens at 70 °C for 9 hours, the ultrathin sections (80 nm) were prepared and mounted on copper grids for viewing in

the transmission electron microscope (H-7650TEM) at an accelerating voltage of 80.0 kV. The analysis of Cd location was undertaken by energy dispersive X-ray analysis (EDAX GENESIS XM2) combined with TEM.

2.5. Statistical analysis

Data used for statistical analysis are the mean values of 10 plants in each treatment for two years. One-way ANOVA was performed using SAS v.9 software and Microsoft Office Excel 2010 for statistical significance at $p=0.05$. All the results were expressed as mean \pm SE for three replications, and a least significant difference (LSD) was calculated at 5% level of significance.

3. Results

3.1. Protein content under Cd stress

Protein is an important component of cottonseeds. The effect of Cd on protein content of the three cultivars was shown in Table 1. It could be observed that protein content was lower in 2011 than that in 2012. Among the three cultivars, TM-1 was the lowest one in protein content. Generally, cottonseed protein was increased with the increase of Cd levels with the highest at 400 μ M Cd. It was found that the average protein contents in cottonseeds were 7.42 %, 5.36 %, and 1.39 % higher at 400 μ M Cd than their controls for TM-1, ZD-90, and SGK3, respectively. Protein content was lower at 600 μ M Cd level than that at other Cd levels but still higher than that of their controls of the three cultivars. The biggest change in protein content among Cd treatment levels was found in TM-1 and the least one was SGK3.

3.2. Oil content under Cd stress

Oil content of three cotton cultivars under Cd stress was shown in Table 1. It had an opposite trend to protein contents among the three cultivars under Cd stress. Oil content was higher in 2011 than that in 2012. It was found that oil content was higher in cottonseeds of TM-1 than those of SGK3 and ZD-90 regardless

of Cd level. Oil content in TM-1 and ZD-90 decreased with increase in Cd level, and that in SGK3 was higher at any Cd levels than that of its control, although the differences were insignificantly.

3.3. Gossypol content under Cd stress

Gossypol is a specific cotton secondary metabolite consisted in almost every parts of cotton plant included cottonseeds, which is important for plant in resistance to many diseases and insects, although it is toxic to human beings and monogastric animals (Lukefahr et al., 1969). Gossypol contents were affected greatly by different Cd levels (Table 1). Inconsistent with protein and oil contents, gossypol contents in cottonseeds were differed in different experimental years and different cultivars.

Among the three cultivars, TM-1 was the highest one in gossypol content. With the treatment of Cd, gossypol content in cottonseeds of TM-1 was decreased, be the lowest at 400 μM Cd level. For other two cultivars, ZD-90 and SGK3, the gossypol contents in cottonseeds at 0 μM Cd level (CK) were 0.71% and 0.66%, respectively, much lower than that of TM-1. Interesting, at lower level of Cd treatment (200 μM Cd), the gossypol contents in cottonseeds of both cultivars was increased, although the increases were not significant. With the increase of the Cd level in the experiment, the gossypol content in cottonseeds of both cultivars was decreased. Again, the biggest change in gossypol content among Cd levels was found in TM-1, followed by ZD-90 and SGK3.

Table 1. Protein, oil, and gossypol contents in the cottonseeds of three cultivars treated with different Cd levels

Cd (μM)	Cultivars	Protein content (%)			Oil content (%)			Gossypol content (%)		
		2011	2012	Average	2011	2012	Average	2011	2012	Average
0	TM-1	32.17 \pm 1.58d	35.78 \pm 1.14c	33.98 \pm 2.01c	34.08 \pm 1.28a	27.58 \pm 1.80a	30.83 \pm 2.60a	0.88 \pm 0.03a	1.09 \pm 0.02a	0.98 \pm 0.02a
	ZD-90	38.76 \pm 2.35ab	42.14 \pm 1.41a	40.45 \pm 2.95a	27.42 \pm 1.73c	22.16 \pm 1.02c	24.79 \pm 2.42b	0.76 \pm 0.07b	0.67 \pm 0.08c	0.71 \pm 0.04c
	SGK3	40.36 \pm 1.08a	41.78 \pm 2.61a	41.07 \pm 1.10a	25.54 \pm 1.18cd	20.51 \pm 1.03d	23.03 \pm 1.93b	0.68 \pm 0.08c	0.65 \pm 0.07c	0.66 \pm 0.08c
	Average	37.1	39.9	38.5	29.01	23.42	26.22	0.77	0.80	0.78
200	TM-1	31.17 \pm 2.23d	38.00 \pm 2.15b	34.59 \pm 5.01bc	35.10 \pm 1.92a	24.45 \pm 4.43b	29.77 \pm 2.73a	0.88 \pm 0.07a	0.92 \pm 0.09a	0.90 \pm 0.04a
	ZD-90	37.99 \pm 2.09ab	43.79 \pm 1.13a	40.89 \pm 3.44a	27.98 \pm 1.32c	21.03 \pm 1.02cd	24.51 \pm 2.95b	0.80 \pm 0.01b	0.65 \pm 0.03c	0.72 \pm 0.08c
	SGK3	39.14 \pm 2.64a	41.71 \pm 1.49a	40.42 \pm 2.38a	27.06 \pm 2.61c	22.21 \pm 1.93bc	24.64 \pm 2.36b	0.71 \pm 0.05bc	0.69 \pm 0.05c	0.70 \pm 0.04c
	Average	36.1	41.17	38.63	30.05	22.56	26.31	0.80	0.75	0.77
400	TM-1	35.73 \pm 1.20c	37.28 \pm 1.99bc	36.50 \pm 1.06b	30.61 \pm 1.58b	27.41 \pm 1.67a	29.01 \pm 1.84a	0.70 \pm 0.05bc	0.84 \pm 0.08b	0.77 \pm 0.04b
	ZD-90	41.69 \pm 1.95a	43.54 \pm 2.56a	42.62 \pm 1.23a	23.67 \pm 2.09d	20.14 \pm 1.92d	21.91 \pm 2.41c	0.69 \pm 0.04bc	0.65 \pm 0.04c	0.67 \pm 0.04c
	SGK3	40.86 \pm 1.98a	42.42 \pm 1.39a	41.64 \pm 1.08a	25.64 \pm 1.94cd	22.06 \pm 1.56bc	23.85 \pm 2.08bc	0.66 \pm 0.01c	0.69 \pm 0.04c	0.67 \pm 0.03c
	Average	39.43	41.08	40.25	26.64	23.2	24.92	0.68	0.73	0.70
600	TM-1	32.93 \pm 1.17d	37.92 \pm 1.13bc	35.43 \pm 2.74b	32.46 \pm 1.26d	25.42 \pm 1.36b	28.94 \pm 2.87a	0.90 \pm 0.02a	0.78 \pm 0.03bc	0.84 \pm 0.07b
	ZD-90	42.00 \pm 1.14a	42.86 \pm 1.10a	42.43 \pm 0.49a	24.11 \pm 1.70cd	21.12 \pm 1.51bc	22.62 \pm 1.73c	0.63 \pm 0.01c	0.56 \pm 0.02c	0.60 \pm 0.04c
	SGK3	40.76 \pm 2.09a	41.26 \pm 2.13a	41.01 \pm 0.29a	26.55 \pm 2.09c	22.20 \pm 1.34bc	24.37 \pm 2.39b	0.57 \pm 0.01c	0.70 \pm 0.02c	0.63 \pm 0.07c
	Average	38.56	40.68	39.62	27.71	22.91	25.31	0.70	0.68	0.69

Values are the means of three replications \pm SE. Variants in each column possessing the same letter are not statistically significant at $p=0.05$.

3.4. Cd accumulation in cottonseeds

The mean value of Cd contents in cottonseeds of the three cultivars was depicted in Table 2. The result clearly demonstrated that the amount of Cd accumulated in cottonseeds increased with increasing of Cd level, and the Cd accumulation under Cd stress was twenty to thirty times more than that of their controls. There was a significant difference among the three cultivars at all different levels of Cd treatment. Without Cd treatment (CK), the Cd accumulation in cottonseeds was around $0.30 \mu\text{g}\cdot\text{g}^{-1}$, there were not different significantly among the three cultivars. With the increasing of the Cd level, Cd accumulation among

the cultivars was different greatly, especially at higher level of Cd treatment. SGK3 was the highest one in Cd accumulation in cottonseeds and TM-1 was the lowest one. At $200 \mu\text{M}$ Cd treatment, the Cd accumulation in cottonseeds of TM-1, ZD-90 and SGK3 were $1.97 \mu\text{g}\cdot\text{g}^{-1}$, $2.32 \mu\text{g}\cdot\text{g}^{-1}$, and $4.14 \mu\text{g}\cdot\text{g}^{-1}$, respectively, while at $600 \mu\text{M}$ Cd level, that were $4.29 \mu\text{g}\cdot\text{g}^{-1}$, $6.45 \mu\text{g}\cdot\text{g}^{-1}$, and $10.47 \mu\text{g}\cdot\text{g}^{-1}$, respectively. It reveals that, regarding Cd accumulation in cottonseeds of three different cultivars, there are greatly variation in Cd uptake and accumulation among the upland cotton genotypes, and it is possible to develop higher Cd up taking cultivars via germplasm screening and gene recombination among upland cotton germplasms.

Table 2. Cd accumulation in cottonseeds of three cotton cultivars treated by different Cd levels ($\mu\text{g}\cdot\text{g}^{-1}$)

Cultivars	Years	Cd treatments (μM)			
		0 (CK)	200	400	600
TM-1	2011	0.20±0.01d	1.99±0.13c	3.40±0.32b	4.55±0.34a
	2012	0.26±0.04d	1.95±0.03c	3.02±0.27b	4.04±0.36a
	Average	0.23±0.04d	1.97±0.03c	3.21±0.27b	4.29±0.36a
ZD-90	2011	0.05±0.04d	2.31±0.05c	5.17±0.07b	6.14±0.42a
	2012	0.55±0.35d	2.32±0.01c	4.58±0.42b	6.76±0.44a
	Average	0.30±0.35d	2.32±0.01c	4.87±0.42b	6.45±0.44a
SGK3	2011	0.14±0.05c	4.45±1.66b	6.37±1.49b	10.39±1.08a
	2012	0.58±0.31d	3.82±0.45c	6.25±0.09b	10.55±0.11a
	Average	0.36±0.31d	4.14±0.45c	6.31±0.09b	10.47±0.13a
Average		0.30	2.81	4.80	7.07

Values are the means of three replications ± SE. Variants in each line possessing the same letter are not statistically significant at $p=0.05$.

3.5. Ultrastructure of cottonseeds under Cd stress

The ultrastructures of the cottonseed cells in three cotton cultivars, SGK3, ZD-90, and TM-1, treated with

different Cd levels were shown in Figure 1 to Figure 4. The damage to the cottonseed cells in these cultivars became more and more prominent with the increase of Cd level as compared to their controls.

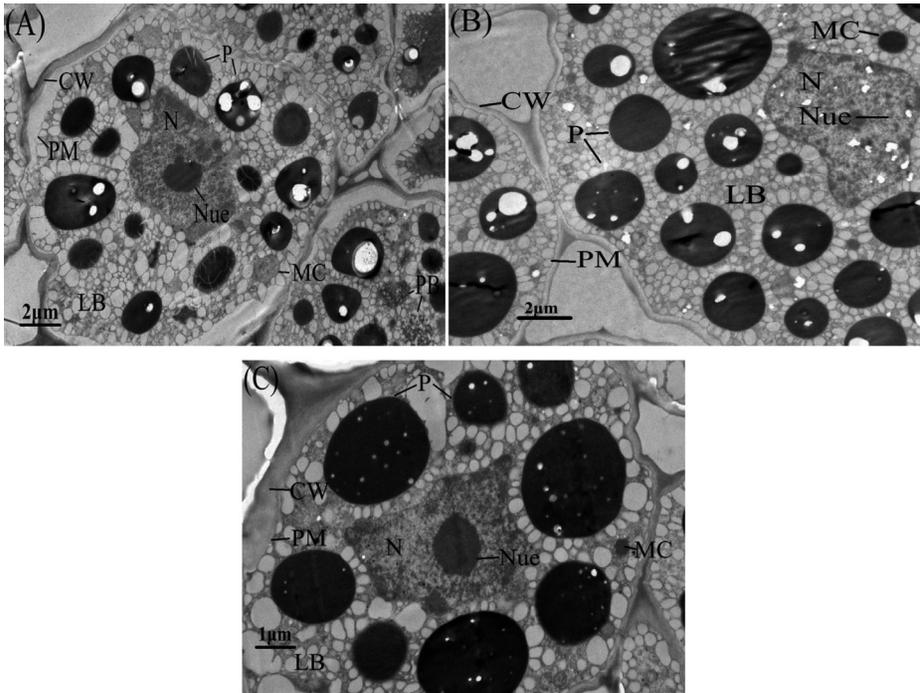


Figure 1. Ultrastructural observation of cottonseeds of the three cotton cultivars treated with 0 μM Cd. (A) SGK3; (B) ZD-90; (C) TM-1; CW: cell wall; PM: plasma membrane; P: protein; LB: lipid bodies; PB: protein bodies; N: nucleus; Nuc: nucleoli; MC: mitochondria.

Cottonseed cells in the controls of all cultivars had typical ultrastructures (Figure 1A-C). On the electron micrographs, dense cytoplasm, smooth, clean and continuous cell membrane and cell wall, centrally located well-shaped nucleus with one or two nucleoli and chromatin materials, round-shaped mitochondria, and well-shaped protein and protein bodies etc. could be observed clear enough. Moreover, the cells of the untreated cottonseeds possessed numerous and small lipid bodies, packed tightly as a single layer around proteins, or dispersed in organelle gaps. The changes of cottonseed cell ultrastructures began at 200 μM Cd level with obvious differences among the three cultivars (Figure 2A-D). The nucleus of the

cottonseeds was severely affected already in TM-1 at that Cd level, which was divided into a number of small nucleoli. Compared to controls, the shape of the nucleus was irregular. Mitochondria in cottonseed cells of SGK3 and ZD-90 became swollen, comparing with those of their control and TM-1. Furthermore, electron dense granules in the form of a ring were existed in ZD-90 and TM-1, but not in SGK3. Inside of those dense granules, there was a typical vacuole. Moreover, the cell wall was clean, the plasma membrane was almost adjacent to the cell walls, plenty of protein bodies surrounded by a single membrane were observed inside the cytoplasm, and lipid bodies were almost the same compared with those of the controls in three cultivars.

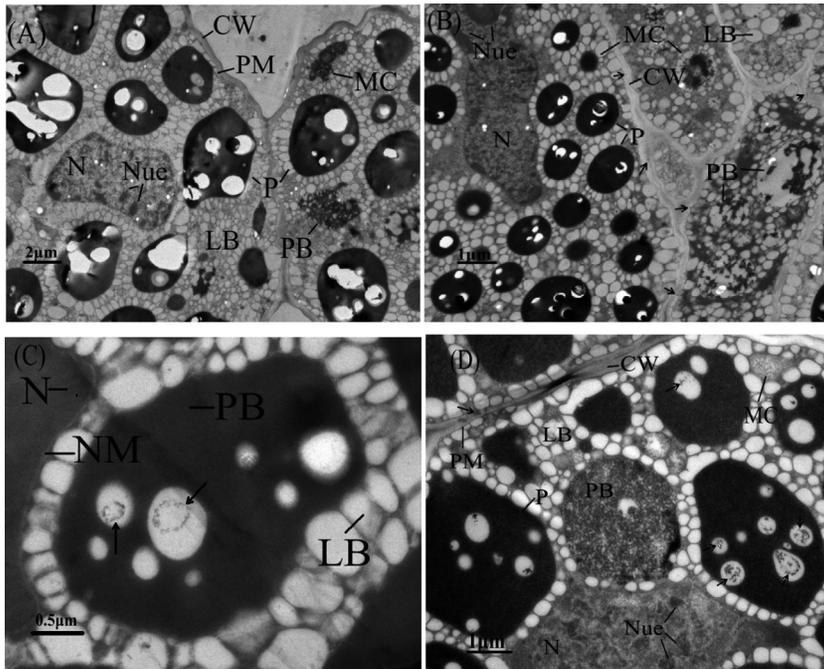


Figure 2. Ultrastructural observation of cottonseeds of the three cotton cultivars treated with 200 μM Cd. (A) SGK3; (B and C) ZD-90; (D) TM-1; CW: cell wall; PM: plasma membrane; NM: nuclear membrane; P: protein; LB: lipid bodies; PB: protein bodies; N: nucleus; Nuc: nucleoli; MC: mitochondria. Arrow (\rightarrow) indicates Cd deposition inside atypical vacuole and attached to the protein.

At 400 μM Cd level, the damages of the structure in cottonseed cells were more extensive (Figure 3A-G). The main alterations like plasmolysis, disintegration of nucleus, shrinkage of cytoplasm, and swell mitochondria could be observed in cottonseed cells of the three cotton cultivars. The cell walls increased in thickness and constriction, and many mucilage exudates were deposited on the cell walls. No protein bodies were observed. A number of cristae and vacuoles in mitochondria were visible in the cells of ZD-90. A few tiny lipid bodies around the protein and some collapse cells were noticed in TM-1. Moreover, a considerable amount of Cd was present in the forms of crystals and electron dense granules which were inside of atypical

vacuoles with protein or attached to cell walls. Severe damages in ultrastructural features were noticed at 600 μM Cd level (Figure 4A-F). The greatest changes occurred in the cottonseed cells in all three cultivars included detrimental plasmolytic shrinkage, disintegration of nucleus, shrinkage of cytoplasm, abnormal structures of organelle, thickening and constriction of cell wall, and cell collapse and disintegration etc. The cottonseed cells, as a whole, were seen to be out of shape and broken, and the tissues came loose and deformed. Also, accumulated Cd was found in the form of crystal and electron dense granules which were distributed in cytoplasm, intercellular space, or cell wall.

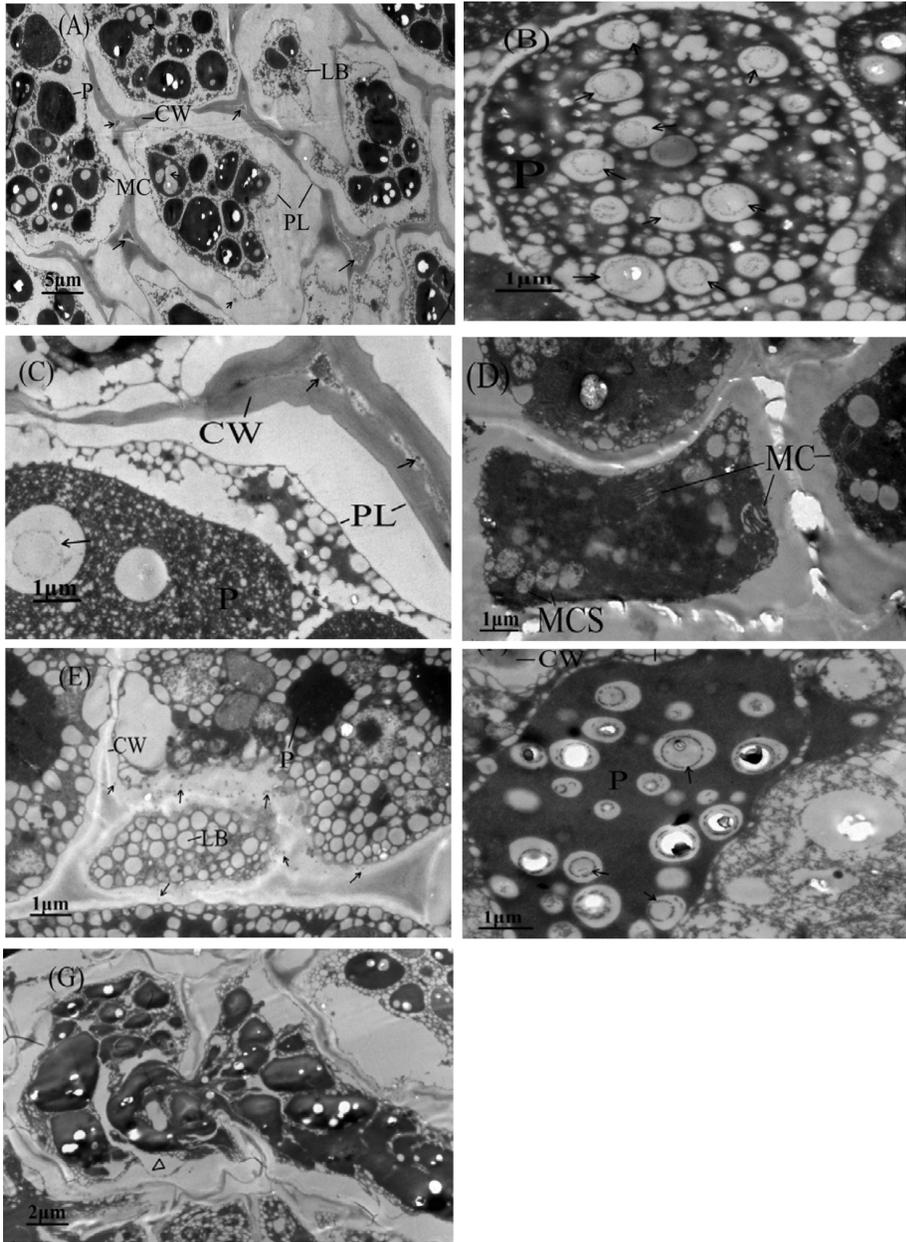


Figure 3. Ultrastructural analysis of seeds of the three cotton cultivars treated with 400 μM Cd. (A, B and C) SGK3; (D and E) ZD-90; (F and G) TM-1; CW, cell wall; PL, plasmolysis; P, protein; LB, lipid bodies; PB, protein bodies; MC, mitochondria; MCS, mitochondria swelling. Arrow (→) indicates Cd deposition inside atypical vacuole and attached to cell wall. Triangle (Δ) indicates cell collapse and abnormal structures of organelle.

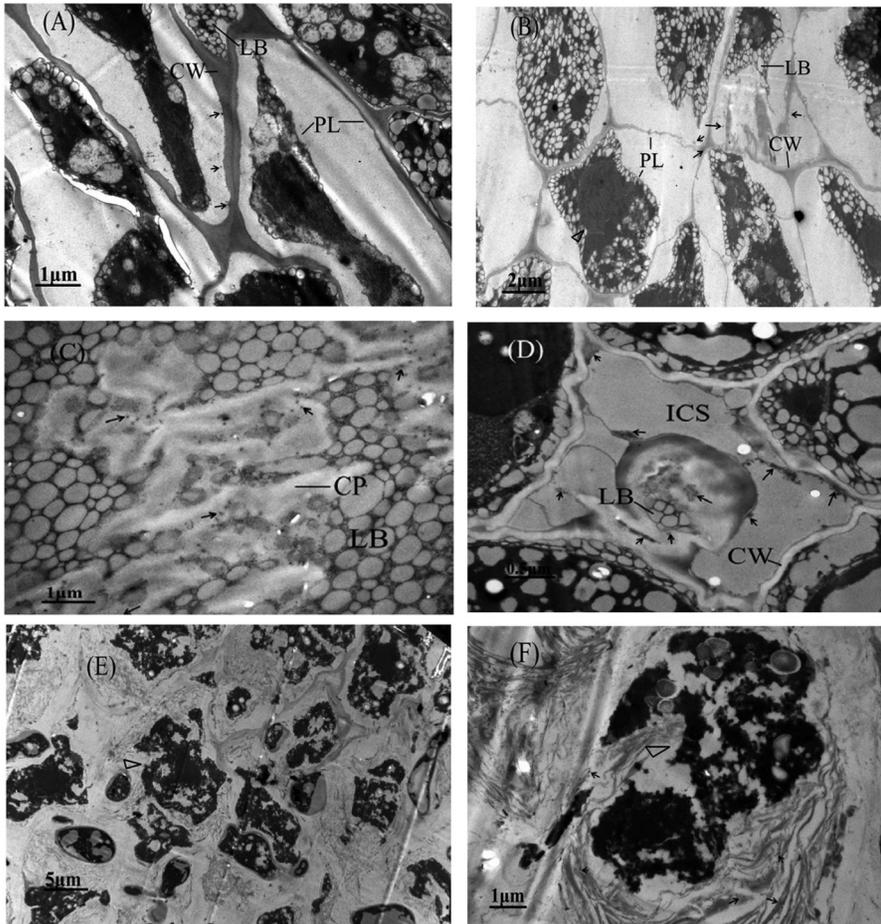


Figure 4. Ultrastructural analysis of seeds of the three cotton cultivars treated with 600 μM Cd. (A) SGK3; (B, C and D) ZD-90; (E and F) TM-1; CW, cell wall; PL, plasmolysis; LB, lipid bodies; CP, cytoplasm; ICS, intercellular space. Arrow (\rightarrow) indicates Cd deposition attached to cytoplasm and cell wall. Triangle (Δ) indicates cell collapse and disintegration.

Severe damages in ultrastructural features were noticed at 600 μM Cd level (Figure 4A-F). The greatest changes occurred in the cottonseed cells in all three cultivars included detrimental plasmolytic shrinkage, disintegration of nucleus, shrinkage of cytoplasm, abnormal structures of organelle, thickening and constriction of cell wall, and cell collapse and disintegration etc. The cottonseed cells, as a whole, were

seen to be out of shape and broken, and the tissues came loose and deformed. Also, accumulated Cd was found in the form of crystal and electron dense granules which were distributed in cytoplasm, intercellular space, or cell wall. The electron density of those granules was greater than that of other treatment levels. It can be concluded that the ultrastructural changes in cottonseed cells were significantly at this Cd level, and TM-1 was the

most severely damaged cultivar, followed by SGK3 and ZD-90.

3.6. Energy dispersive X-ray analysis (EDAX)

Distribution of Cd in cottonseeds was detected by EDAX, and the results were shown in Figure 5. The TEM images showed that there were some special ring structures, crystals, and electron dense granules in cottonseed cottonseed cells treated with different

Cd levels. The elemental microanalysis by EDAX was illustrated by the curves in Figure 5 (b) and Figure 5 (c), which pointed out Cd distribution in the cottonseed cells. Cd signal could be detected and its peaks was found in cottonseed cells treated with 400 μM Cd, suggesting that a certain amount of Cd existed in the form of rings, crystals, and electron dense granules. The signals of other elements shown in the figure may be caused by copper grids in the machine.

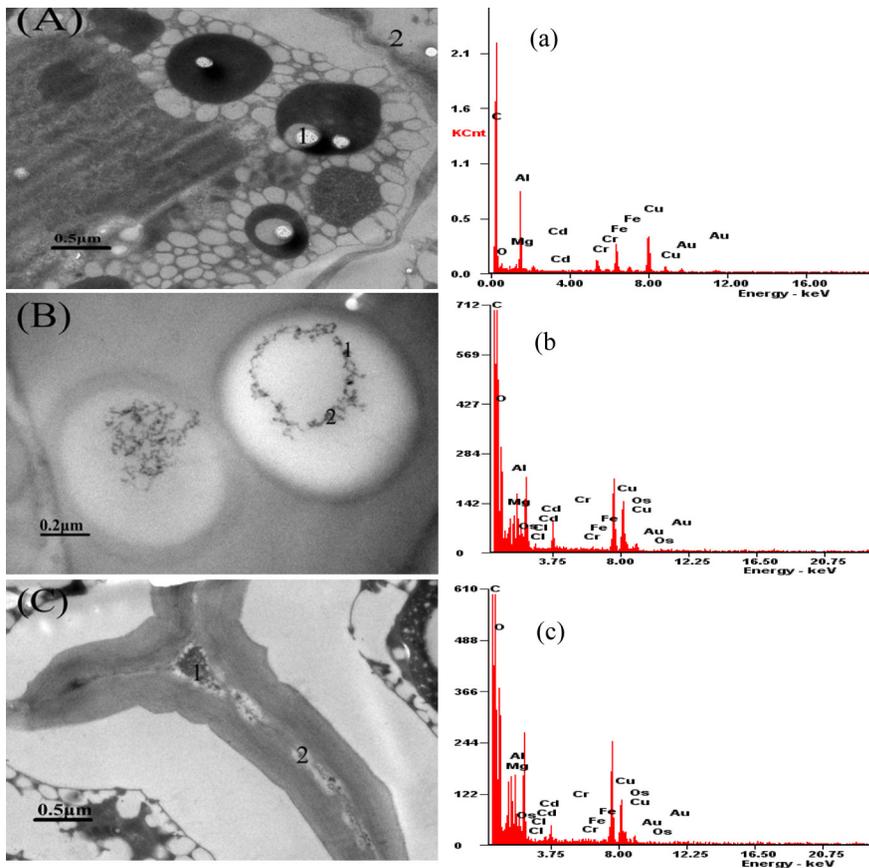


Figure 5. The microanalysis of Cd in seeds of cotton cultivar SGK3 under control and 400 μM Cd treatment levels by the transmission electron microscope (TEM) and the energy dispersive X-ray analysis (EDAX). In the left images of TEM (A, B, and C), the numbers were the sites of EDAX, and the right images (a, b, and c) were the results of EDAX. (A) was the TEM of the control; (B) and (C) were the TEMs of 400 μM Cd treatments. (a) was the EDAX of control; (b) and (c) were the EDAX of 400 μM Cd treatments.

4. Discussion

Cd is a non-essential nutrient element for plants, but it is one of the most deleterious heavy metals in contaminated soils. In our previous study, Cd stress lead to the changes in growth and development of cotton plants (Daud, *et al.*, 2009a; Daud, *et al.*, 2009b), caused serious reducing in cotton yield and fiber quality (Li, *et al.*, 2012). Our present results showed that the protein content increased at lower Cd levels and decreased at higher Cd levels. It could be inferred that the proteins or peptides in cottonseeds could be induced by lower level of Cd treatment. Changes of oil content in cottonseeds were inconsistent with that in corn grain which was increased at lower Cd levels and decreased at higher Cd levels (He and Luo, 1991). This might be due to the poor maturity of cottonseed caused by inhibiting plant growth and decreasing photosynthetic capacity under Cd stress (Li, *et al.*, 2012). While the gossypol contents in cottonseeds were decreased under Cd stress although lower level Cd increased its contents in some cultivars. The reason of the decrease in gossypol contents was not clear up to now. It may relate to changes in structures of pigment glands or gossypol synthesis and metabolism. Because it was involved in a special signal transduction pathway as one of the secondary metabolites itself or in other pathways affected by gossypol synthesis, the functional mechanism of gossypol under Cd stress is so complex and still needs to be deeply investigated.

It has long been recognized that metal accumulation capacity varies greatly among the plant species or varieties, and it is affected by various soil conditions as well (Shi and Cai, 2009). Our present experiment design was closely correlated with Cd contaminated soils. Generally, roots are the primary sites through which Cd gains access into the plant. Consequently, Cd is readily taken up by the plant through root and transported to the other parts by xylem. In most cases, a large fraction of Cd is retained in roots and only comparatively small amounts are transported to the shoots and the seedlings (Daud, *et al.*, 2009a; Daud, *et al.*, 2009b). However, the relative large quantity

of Cd was accumulated in cottonseeds indicated that the cotton is one of the most powerful crop to carry off Cd from the contaminated soil. Furthermore, the tremendous difference in Cd accumulation in cottonseeds among the experimental materials illustrated that there are great variation in capacity of Cd carrying off from soil among the upland cotton germplasm. He and Luo (1991) extracted Cd-binding proteins in maize with a ratio of 1:3 (protein : Cd), and indicated that the formation of Cd-binding proteins could limit the behavior of Cd and abate the injury of Cd to plants. Another kind of substance induced for heavy metal detoxification is phytochelatin. phytochelatin is catalyzed by phytochelatin synthase, the chemical form is usually as (r-Glu-Cys)_nGly (n=2-11) (Jiang, *et al.*, 2007). It may imply that some special amino acids such as Cys were involved in detoxification of Cd by phytochelatin combining amounts of Cd to abate the toxicity of plants. Cottonseeds are rich in protein and amino acids, Cd binding proteins and phytochelatin may be the important mechanisms of Cd detoxification for cotton stressed with higher level of Cd.

For the three cultivars in our experiment, the standard upland line, TM-1, was the poorest one in Cd accumulation in cottonseeds, followed by ZD-90 and SGK3. ZD-90 and SGK3 are two transgenic cotton cultivars with herbicide tolerant gene and insect resistant gene, respectively. The higher Cd accumulation in transgenic cottonseeds maybe due to the influence on exogenous genes such as Bt and EPSPS-G6 genes which affects the expression and function of other genes. It also may be the forming of disulfide bonds (-S-S-) that is required to phytochelatin and metallothionein binding Cd, as a result of the expression of foreign genes.

The cell is the basic unit in the structure and function of a plant. Knowledge of the cell structure contributes to the understanding of its metabolic mechanisms. In the present study, the ultrastructural changes in cottonseed cells were found to be dose-dependent. With the elevated levels of Cd, the damage to the structures of cottonseed cells became serious. The

nucleus became irregular and the number of nucleoli increased with the increase in Cd concentration. Finally, the nucleus was distorted and disintegrated. It indicates that the most severe influence on the nucleus occurred at highest Cd treatment. Nucleus is the center where genetic organization occurs and plays an important role in controlling the action of the cell through selective expression by the genes (Jiang, *et al.*, 2007). Similarly, it can be concluded that the increased number of nucleoli under Cd stress are used for ribosome formation and mRNA synthesis, which ultimately enhance the production of new proteins responsible for Cd tolerance (Sresty and Rao, 1999).

Plasmolysis was also serious consequences of Cd stress at ultrastructural level of cottonseeds. In our present results, organelles and cytoplasm was surrounded by cell membrane which shrunken at 400 μM Cd level and even discontinuous broken at 600 μM Cd level. This situation has also been previously observed by Daud *et al.* (2009a, 2009b) in cotton seedling. Changes were also observed in the cell wall under Cd stress including cell wall thickening and constriction which confirmed the findings of Schützendübel *et al.* (2001), who observed alterations in cell wall thickening with the involvement of Cd.

Furthermore, the presence of Cd in the form of the crystals and electron dense granules was found in this experiment. However, at 200 μM Cd level, SGK3 had no Cd deposition while the Cd accumulation in SGK3 was much higher than that in other two cultivars. It was shown that some macromolecules might be the binding sites of Cd and the intercellular spaces might be the places elevating of Cd toxicity. The present study supports the findings of other research work in maize (Lozano-Rodriguez, *et al.*, 1997) and *Juncus effusus* L. (Najeeb, *et al.*, 2011) in which increased Cd accumulation near the cell wall played an important role in Cd tolerance by preventing the circulation of free Cd ions in the cytosol. Rauser and Ackerley (1987) also found Cd in the cytoplasm of maize and *Agrostis* roots, which were similar to our results. It is indicated that the cytoplasm is one of the sites of Cd

accumulation in seeds. A large amount of Cd existed in the cytoplasm of ZD-90 at 400 μM Cd level indicated that the Cd bound by cell walls were saturation and excess Cd had entered into the cytoplasm, according to the study of Anap and Kalina (2007). Moreover, the rings inside the atypical vacuoles with the proteins were other forms of Cd that associated to Cd detoxification by binding proteins and phytochelatin.

5. Conclusions

The toxic effect of Cd on cottonseed compositions and ultrastructure was seriously, which varied significantly among the genotypes, SGK3 was the least affected among the three cultivars. The two transgenic cotton cultivars, ZD-90 and SGK3, showed higher tolerance to Cd according to the changes in components and cell ultrastructure of cottonseeds. SGK3 had a greater capacity in Cd accumulation, followed by ZD-90 and TM-1. In cottonseeds, Cd existed in the form of rings, crystals, and electron dense granules. They distributed in the intercellular space, cytoplasm, and the cell wall. The results indicated that cotton is a suitable crop for cleaning Cd contaminated soils by phytoremediation.

Acknowledgments

The project was financially supported by the National Basic Research Program (2010CB126006), National Natural Science Foundation of China (31371667), and the National High Technology Research and Development Program of China (2011AA10A102).

Reference

- Anap, C.M., Kalina, A.S. 2007. *Solanum nigrum* grown in contamination soil: Effect of arbuscular mycorrhizal fungi on zinc accumulation and histolocalisation. *Environ. Pollut.* 145, 691-699.

- Cao, Y., Huang, R.D., Jiang, W.C., Cao, Z.Q. 2005. Effect of heavy metal lead and cadmium on grain quality of maize. *J Shenyang Agric Univ.* 36, 218-220.
- Daud, M.K., Variath M.T., Ali, S., Najeeb, U., Jamil M., Hayat, Y., Dawoo, M., Khand M.I., Zaffar, M., Cheem, S.A., Tong, X.H., Zhu, S.J. 2009a. Cadmium-induced ultramorphological and physiological changes in leaves of two transgenic cotton cultivars and their wild relative. *J. Hazard. Mater.* 161, 463-473.
- Daud, M.K., Sun, Y.Q., Dawood, M., Hayat, Y., Variath, M.T., Wu, Y.X., Raziuddin, Mishkat, U., Salahuddin, Najeeb, U., Zhu, S.J. 2009b. Cadmium-induced functional and ultrastructural alterations in roots of two transgenic cotton cultivars. *J. Hazard. Mater.* 168, 614-625.
- Gu, J.G., Zhou, Q.X. 2002. Cleaning up through phytoremediation: a review of Cd contaminated soils (in Chinese with English abstract). *Ecol. Sci.* 21, 352-366.
- Guo, T.C., Xia, L.K., Zhu, Y.J., Kang, G.Z., Qin, X.L., Zhang, C.L. 2006. Preliminary study on effects of copper, cadmium stress on starch content and pasting property of winter wheat grain. *J Triticeae Crops.* 26, 107-110.
- He, D., Luo, J. 1991. Purifying of metal-binding proteins from roots of maize by HPLC anti-phase cartridges. *Chinese Sci.* 21, 490-496.
- Huang, D.F., Xi, L.L., Wang, Z.Q., Liu, L.J., Yang, J.C. 2008. Effects of irrigation regimes during grain filling on grain quality and the concentration and distribution of cadmium in different organs of rice. *Acta Agron sin.* 34, 456-464.
- Isaure, M.P., Fayard, B., Sarret, G., Pairis, S., Bourguignon, J. 2006. Localization and chemical forms of cadmium in plant samples by combining analytical electron microscopy and X-ray spectromicroscopy. *Spectrochim. Acta. Part B.* 61, 1242-1252.
- Jiang, H.M., Yang, J.C., Zhang, J.F. 2007. Effects of external phosphorus on the cell ultrastructure and the chlorophyll content of maize under cadmium and zinc stress. *Environ. Pollut.* 147, 750-756.
- Krämer, U. 2005. Phytoremediation: novel approaches to cleaning up polluted soils. *Curr. Opin. Biotechnol.* 16, 133-141.
- Küpper, H., Lombi, E., Zhao, F.J., McGrath, S.P. 2000. Cellular compartmentation of cadmium and zinc in relation to other elements in the hyperaccumulator *Arabidopsis halleri*. *Planta.* 212, 75-84.
- Li, L., Chen, J.H., He, Q.L., Daud, M.K., Zhu, S.J. 2012. Characterization of physiological traits, yield and fiber quality in three upland cotton cultivars grown under cadmium stress. *Aust. J. Crop Sci.* 6, 1527-1533.
- Liu, W.T., Zhang, Y.L., Chen, Z.M., Zhou, Q.X., Luo, H.Y. 2008. Cadmium and zinc absorption and distribution in various tree species in a mining area. *Chin. J. Appl. Ecol.* 19, 752-756.
- Lozano-Rodriguez, E., Hernandez, L.E., Bonay, P., Carpena Ruiz, R.O. 1997. Distribution of cadmium in shoot and root tissue of maize and pea plants: physiological disturbances. *J. exp. Bot.* 48, 123-128.
- Lukefahr, M.J., Shaver, N.T., Cruhm, D.E., Houghtaling, J.H. 1969. Sources and nature of resistance in *Gossypium hirsutum* to bollworm and tobacco budworm. *Proc. Beltwide Cotton Prod. Res. Conf.* p. 81-82.
- Ma, J.F., Ueno, D., Zhao, F.J., McGrath, S.P. 2005. Subcellular localisation of Cd and Zn in the leaves of a Cd-hyperaccumulating ecotype of *Thlaspi caerulescens*. *Planta.* 220, 731-736.
- Najeeb, U., Jilani, G., Ali, S., Sarwar, M., Xu, L., Zhou, W.J. 2011. Insights into cadmium induced physiological and ultra-structural disorders in

- Juncus effusus* L. and its remediation through exogenous citric acid. *J. Hazard. Mater.* 186, 565–574.
- Paiva, L.B., Oliveira, J.G., Azevedo, R.A., Ribeiro, D.R., Silva, M.G., Vitória, A.P. 2009. Eco-physiological responses of water hyacinth exposed to Cr³⁺ and Cr⁶⁺. *Environ. Exp. Bot.* 65, 403-409.
- Prasad, M.N.V 1995. Cadmium toxicity and tolerance in vascular plants. *Environ. Exp. Bot.* 35, 525-545.
- Qin, L., Shen, X.J., Chen, J.H., Zhu, S.J. 2010. Determination of protein and gossypol content in cotton kernel power with near infrared reflectance spectroscopy. *Spectrosc. Spect. Anal.* 30, 635-639.
- Rausser, W.E., Ackerley, C.A. 1987. Localization of cadmium in granules within differentiating and mature root cells. *Can. J. Bot.* 65, 643-646.
- Salt, D.E., Prince, R.P., Pickering, I.J., Raskin, I. 1995. Mechanisms of cadmium mobility and accumulation in Indian mustard. *Plant Physiol.* 109, 1427–1433.
- Satarug, S., Baker, J.R., Urbenjapol, S., Haswell-Elkins, M., Reilly, P.E.B., Williams, D.J., Moore, M.R. 2003. A global perspective on cadmium pollution and toxicity in non-occupationally exposed population. *Toxicol. Lett.* 137, 65-83.
- Schützendübel, A., Schwanz, P., Teichmann, T., Gross, K., Langenfeld-Heyser, R., Godbold, D.L., Polle, A. 2001. Cadmium induced changes in antioxidative systems, hydrogen peroxide content and differentiation in Scots pine roots. *Plant Physiol.* 127, 887-898.
- Sengalevitch, G. 1999. Problems of heavy metals contamination and using the agricultural lands in the region of KCM-AD-Plovdiv. *Prilozenie za ekologia kam bjuletin KCM.* 1, 10-14.
- Shi, G.R., Cai, Q.S. 2009. Cadmium tolerance and accumulation in eight potential energy crops. *Biotechnol Adv.* 27, 555-561.
- Sresty, T.V.S., Rao, K.V.M. 1999. Ultrastructural alterations in response to zinc and nickel stress in the root cells of pigeonpea. *Environ. Exp. Bot.* 41, 3-13.
- Vazquez, M.D., Barcelo, J., Poschenrieder, C., Madico, J., Hatton, P., Baker, A.J.M., Cope, G.H. 1992. Localization of zinc and cadmium in *Thlaspi caerulescens* (brassicaceae), a metallophyte that can hyperaccumulate both metals. *J. Plant Physiol.* 140, 350–355.
- Vitória, A.P., Rodriguez, A.P.M., Cunha, M., Lea, P.J., Azevedo, R.A. 2003. Structural changes in radish seedlings exposed to cadmium. *Biol. Plantarum.* 47, 561-568.
- Wagner, G.J. 1993. Accumulation of cadmium in crop plants and its consequences to human health. *Adv. Agron.* 51, 173-212.
- Wojcik, M., Vangronsveld, J., Tukiendorf, A. 2005. Cadmium tolerance in *Thlaspi caerulescens*-II. Localization of cadmium in *Thlaspi caerulescens*. *Environ. Exp. Bot.* 53, 163-171.
- Zhou, Q.X., Song, Y.F. 2004. Principles and Methods of Contaminated Soil Remediation. Science Press, Beijing.