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FUNCTIONAL AND ULTRASTRUCTURAL CHANGES OF THE PHOTOSYNTHETIC APPARATUS IN RESPONSE TO CD AND ZN IN *VICIA FABA* L. LEAVES

ABSTRACT:

Heavy metal as Cd can antagonise uptake and transport of other metal elements such as Mn, Zn, and Fe in leaves of *Vicia faba* L. concentrations of Cd or Zn up to 100 µM remarkably decreased chlorophyll content compared to control. The degradation in Chl. b under such stress was slower than that of Chl. a. and Cd had more inhibitory effect on both pigments than Zn even under low concentration (50 µM). The results cleared that Zn can strongly antagonise this toxic effect of Cd especially with high Zn concentrations and where full protection and restoration of chlorophyll levels was recorded. Measurements of Na, Mg, P, K, Ca, Cu, Al, Mn, and Zn bounded to the cell membrane, revealed a significant decrease of Ca, K and Mg in the presence of Cd or Zn, also the disappearance of Fe peak was observed at 100 µM Cd. This could be due to Cd ions interaction with the manganese cluster which is present in the evolving complex on the donor side of PSП. The effect of metal toxicity for Zn and or Cd, in this study, could be observed in many damages to the cell organelle ultrastructure. Changes in chloroplast ultrastructure and lipid composition of the thylakoid membranes alter the operability of the photosynthetic electron transport chain. The chloroplast treated with 100 µM Cd has a reduced number of intact grana, as well as reduced chloroplast size. Combined treatment of cadmium and zinc led to more changes in the shape of cellular components, such as, the shape of chloroplasts was almost spherical, the grana were completely damaged and a large part of chloroplast appeared with disturbed envelopes. Increasing Zn over Cd concentration treatment acquired beneficial effect as it can successfully antagonize the harmful effect of Cd.

KEY WORDS:

Cadmium, Zinc, ultrastructure, photosynthetic apparatus, *Vicia faba* L.

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INTRODUCTION:

The study of heavy metal pollution is of considerable importance and relevant to present scenario due to the increasing levels of pollution and its obvious impact on human health (Athar and Ahmad, 2002). Heavy metals at extremely micro concentrations affect different cellular components, thereby interfering with the normal metabolic function. The coexistence of essential and non-essential elements in the ecosystem leads to interactions that may be additive, antagonistic or synergistic (Siedlecka, 1995). Cadmium (Cd) is a non-essential heavy metal, which enter the environment mainly through Zn mining and smelting, industrial processes and the application of phosphate fertilizers (Waisberg *et al*., 2003; He *et al.,* 2013). It can be transferred to the food chain by plant uptake. The photosynthetic apparatus is one of the target sites of Cd action in plants. In fact, Cd can directly or indirectly interact with different components of the photosynthetic apparatus and can decrease electron transport efficiency, inhibit chlorophyll biosynthesis and reduce the photosynthetic carbon assimilation (Maksymiec, 2007). Cadmium reduces chlorophyll production by the inhibition of protochlorophyllide reductase. It can also interfere with the photosynthetic pigments by substituting Mg^{2+} ions with Cd^{2+} ions in chlorophyll molecules. These substituted molecules have much lower fluorescence quantum yields compared to magnesium chlorophylls. These two toxic effects reduce the production of chlorophyll and consequently photosynthesis, which can then lead to senescence and cell death.

Photosystem II and photosystem I, the light-harvesting units in the chloroplasts of plants that transfer light energy to chemical energy in the form of ATP and NADPH, are also affected by cadmium. Here the strong interaction of cadmium with iron leads to iron deficiency, as iron is required in both photosystems and their processes (Bandiera *et al*., 2016).

Cadmium is often associated with Zn as a contaminant up to 5% in the processed Zn-ores of Zn mines and smelters (Sterckeman *et al.*, 2000). Zinc accumulation at supra-optimal concentrations may delay or diminish the growth and root development and causes leaf chlorosis of Zn-exposed plants (Wang *et al*., 2009).

The study of the in vivo Chl fluorescence of green leaves provided basic information on the functioning of the photosynthetic apparatus and on the capacity and performance of the photosynthesis. Under optimum physiological conditions the major part of light absorbed by the photosynthetic pigments, chlorophylls and carotenoids is used for photosynthetic quantum conversion. This phenomenon consists of a complex reduction– oxidation chain along which electrons are transported through the photosynthetic apparatus from water to NADP via photosystem II (PSII) and photosystem I (PSI) (Delosme, 2003). Some amount of light absorption is de-excited by heat dissipation and a small proportion via Chl fluorescence, whose spectrum exhibits two maxima in the red (near 685–690 nm), and far-red region (near 730–740 nm) (Buschmann, 2007). However, under many stress conditions plants use less radiant energy for photosynthesis and have evolved numerous mechanisms that safely dissipate excess light energy to avoid photoinhibition and photooxidation with a concomitant increase in red and far-red Chl fluorescence (Baker and Rosenqvist, 2004). The inverse relationship between in vivo Chl fluorescence and photosynthetic activity can be used to study the potential photosynthetic activity of leaves and to detect stress effects on the green plants (Papageorgiou and Govindgee, 2004).

Zinc is known to have a stabilizing and protective effect on the biomembranes against Cd induced oxidative and peroxidative damage, loss of plasma membrane integrity (Cakmak, 2000) and also alteration of the permeability of the membrane with the help of enzymatic and non-enzymatic antioxidant.

The fact that Cd is a toxic heavy metal and Zn is an essential element makes this association interesting as it raises the possibilities that the toxic effect of Cd may be preventable by Zn. The co-existence of these two heavy metals in the ecosystem can lead to various synergistic and antagonistic interactions in their uptake and tissue content.

Nan *et al.* (2002) observed that no antagonistic Cd-Zn interaction at uptake level, while Hassan *et al.* (2005) showed that the increase in Zn level in the culture medium leads to a reduction in the Cd uptake and imitation in roots associated with an increase in its content in shoot of rice cultivar. Hart *et al.* (2002) showed that in both durum and bread wheat decreases in Cd uptake by roots with increasing Zn treatment is possibly due to a competition between Zn and Cd uptake. Thus, several studies have been conducted to investigate the Cd-Zn interaction at Cd and Zn uptake and accumulation sites in some plants species, indicating some kind of interaction between the two metals (Oliver *et al.,*1994; Cakmak *et al.,* 2000).

Thus, the present investigation has been undertaken to evaluate the antagonistic or synergestic effect of Zn and/ or Cd on photosynthetic apparatus, chlorophyll fluorescence and leaf ultra-structures of *Vicia faba* plant.

MATERIAL AND METHODS:

Plant materials and growth conditions:

Vicia faba L. cv. Nubaria1(obtained as a pure variety from the Agricultural Research Center, Ministry of Agriculture, Giza, Egypt) were seeded in 15 cm plastic pots and grown in a growth chamber at 25/20 c,75-80% relative humidity and 16/8-day night cycle. Light condition was at a photon flux density of 420 m mol photons m-2 s-1 at the top of plants provided by incandescent lamps. After seven days, all plants on alternate days were irrigated with half strength of Hogland solution. After an initial growth period of two weeks, plants were carefully taken and placed in sponge support collars in jars, Cadmium chloride or Zn sulphate was added at concentrations of 50 or 100 µ M.

The different treatments were: control, 50,100,150, and 200 µ M Zn, 50, 100 µ M Cd and the combinations 100 + 50, 100 + 100, 100 + 150, and 100 + 200 µ M Cd+ Zn.

These doses were chosen appropriately to expose the plants to from moderate to high levels of Cd or Zn ions. After 6 days of growth in these treatments, plants were harvested and leaves samples were removed washed and blotted gently to dry and used for biochemical analyses.

Measurement of leaf area:

Leaf area was determined using a moving belt electronic planimeter (Delta. T Devices burwell, UK).

Measurements of Photosynthetic Pigments and Chlorophyll Fluorescence:

Photosynthetic pigments, chlorophyll *a*, chlorophyll *b* and carotenoids were extracted and determined from expanded young leaves according to Inskeep and Bloom (1985). *In*

Vivo chlorophyll fluorescence measurements were monitored in fully-expanded and young leaves according to Branquinho *et al.* (1997) using a PAM 101 Chlorophyll Fluorometer (Watz, Effelrich, Germany). Variable fluorescence (F_v) was calculated as the difference between F_m and F_0 (Moustakas *et al*., 1993). Also, the maximum quantum yields of PSII (F_v / F_m) was calculated according to Maxwell and Johnson (2000).

Ultra-structure of tissues and cells:

The fragments of *Vicia faba* L. Nubaria 1 leaves from control and treated samples were fixed in a mixture of 2% formaldehyde and 2.5% glutaraldehyde at pH 7.4 in cacodylate buffer for two h and then thoroughly washed in the same buffer, and then post fixed with 1.0% (w/v) osmium tetraoxide in the same buffer for 2 h at room temperature. Subsequently, the samples were transferred to re-distilled water and stained with a 0.5% aqueous solution of uranyl acetate. After passing through increasing concentrations of ethanol and embedded in Spurr's resin at 70°C (Spurr, 1969). Semi-thin sections (1-µm) and ultra-thin (90-nm) ones were observed with light microscope (Leitz, Wetzlar, Germany) and a transmission electron microscope (model Joel-JEM 100CX, Japan), respectively. Other samples after staining with 2% uranyl acetate solution and lead acetate solution (Venable and Coggeshall, 1965). Ultra-thin sections were cut on an ultramicrotome (Leica EM UC6, Germany) with a diamond knife and were mounted on copper grids with 300 square meshes.

X-Ray microanalysis:

The concentrations of Na, Mg, P, K, Ca, Mn, Fe, Zn, and Cu in chloroplastic membrane were determined by x-ray microanalysis according to Fritz (1989) and described by Godbold and Jentschke (1998).

Statistical analysis:

Two-way ANOVA and the LSD at p≤*0.01* was determined following the method of Sokal and Rohlf (1995).

RESULTS:

Data presented in table 1 showed decreases in leaf area of *Vicia faba* plant in response to Zn and / or Cd treatments and with a highest value after imposition of 100 µM Zn or Cd metals treatment. This highly significant depression in leaf area by 100 µM of both metal was approximately 44% and 60 % of the control respectively. In contrast, there is an improvement in leaf area of *Vicia faba* plant treated with 100 µM Cd + 150 µM Zn or 100 µM Cd + 200 µM Zn and it was 44% in case of later treatment in respect to 100 µM Cd only.

The reduction in leaf area was greater by all Cd treatments as compared with those of Zn. The levels of photosynthetic pigments content (Chl. a, Chl. b, and Carotenoids) decreased as the concentrations of Zn and Cd increased in the culture media (Table 1). Chl. a was more affected in compared to Chl. b or carotenoids. Thus, when *Vicia faba* plant were exposed to 100µM of Cd, the amount of Chl. a reached a lower value of 9.7 mg g^{-1} fresh weight that was evaluated by 46.82 % of control. Similarly, total chlorophylls (a + b) contents significantly decreased as compared to the control. The total chlorophyll content in the leaves at 100 µM cadmium or 100 µM zinc were lowered to 13.63 and 19.67 mg g^{-1} Fw, respectively compared to 27.43 mg g^{-1} Fw in control.

Table 1. The effects of Zn and Cd on leaf area (cm2), chlorophyll contents, chlorophyll a / b ratio and carotenoids in leaves of broad bean plants Nubaria 1. Values are means ± SD

Treatments	Leaf Area	Chl a	Chl b	Chl a+Ch b	Chla/Chb	C
Control	12.60[a] ± 1.60	18.24 ± 2.22	6.8 ± 0.87	27.43 ± 3.19	2.68 ± 0.37	5
50 µM Zn	9.80[b] ± 1.24	16.92 ± 2.06	6.41 ± 0.82	23.33 ± 2.71	2.64 ± 0.36	5
100 µM Zn	7.10[c] ± 0.90	14.22 ± 1.73	5.45 ± 0.70	19.67 ± 2.29	2.61 ± 0.36	5
50 µM Cd	7.40[c] ± 0.94	13.12 ± 1.60	5.1 ± 0.65	18.22 ± 2.12	2.57 ± 0.35	4
100 µM Cd	5.00cd ± 0.63	9.7 ± 1.18	3.93 ± 0.50	13.63 ± 1.58	2.47 ± 0.34	4
100 µM Cd + 50 µM Zn	4.86d ± 0.62	8.69 ± 1.06	3.41 ± 0.44	12.1 ± 1.41	2.54 ± 0.35	4
100 µM Cd + 100 µM Zn	4.40d ± 0.56	9.45 ± 1.15	3.86 ± 0.49	13.31 ± 1.55	2.44 ± 0.33	4
100 µM Cd + 150 µM Zn	6.90d ± 0.88	10.38 ± 1.27	4.01 ± 0.51	14.39 ± 1.67	2.58 ± 0.35	4
100 µM Cd + 200 µM Zn	7.20c ± 0.91	13.86 ± 1.69	5.21 ± 0.67	19.07 ± 2.22	2.66 ± 0.36	5

The content of Chl. a was more than that of Chl. b in the plant leaves at all treatments. Reduction in the total chlorophyll content was due to more effect on chlorophyll a than on chlorophyll b content. This was also confirmed by their ratio. Carotenoids showed a similar reduction pattern to that of the chlorophylls and a significant reduction of total carotenoids especially due to higher concentrations of Zn and Cd metals was recorded (Table. 1). At 100 µM Cd, carotenoids content was 25% of the control

value, while at 100 µM Zn it was 10% of the control.

In combined treatment, all concentrations of Zn caused antagonistic and synergistic effects on Cd-induced toxicity to total chlorophylls and carotenoids, but maximum antagonistic effect was noticed with 200 µM concentration of Zn + 100 µM Cd, where there was increase by 23% as compared to 100µM Cd treatment alone.

Data represented in table 2 showed that Cd affects the structure of photosynthetic units and the electron transport system, it can be seen that after Cd-treatment the values of Fo in bean leaves increased and those of the variable Fv, decreased corresponds to the established ultrastructural disorders in chloroplasts. The Fv/Fm ratios decreased in the Cd-treated broad bean plants but this decrease in the photosynthetic quantum efficiency is insignificant but the ratio Fv/Fm, did not change significantly when the concentration of Zn in the culture medium increased, remaining within 0.77 - 0.70, which is typical for the functionally healthy leaves.

Combined treatment of Zn concentrations with Cd caused antagonistic and synergistic effects on Cd-induced inhibition to the maximum quantum efficiency of PS2 (Fv/Fm). Maximum antagonistic effect was noticed with 200 µM concentration of Zn + 100 µM Cd, where the value of Fv/Fm increased by 10% as compared to Cd treatment alone and retained Fv/Fm back to the control value. All concentrations except for 100 µM Zn + 100 µM Cd, induced synergistic effects where the value of Fv/Fm decreased as compared to100 µM Cd treatment alone.

Table 2. The effects of Zn and / or Cd on chlorophyll fluorescence as values of (F_o), (Fm), (Fv), and Fv/Fm in leaves of broad bean Nubaria 1. Values are means ± SD.

Treatments	F_o	Fv	Fm	Fv/Fm
Control	228 ± 28.68	780 ± 93.98	1008±117.21	0.773 ± 0.11
50 µM Zn	231 ± 29.06	814 ± 98.07	1045±121.51	0.778 ± 0.11
100 µM Zn	313 ± 39.37	1026 ± 123.61	1339±155.70	0.766 ± 0.10
50µM Cd	198 ± 24.91	533 ± 64.22	731±85.00	0.729 ± 0.10
100 µM Cd	326 ± 41.01	1006 ± 121.20	1332±154.88	0.701± 0.10
100 µM Cd+50 µM Zn	291 ± 36.60	720 ± 86.75	1130±131.40	0.637 ± 0.09
100 µM Cd+100 µM Zn	296 ± 37.23	700 ± 84.34	1100±127.91	0.636 ± 0.09
100 µM Cd+150 µM Zn	323 ± 40.63	861 ± 103.73	1184±137.67	0.727 ± 0.10
100 µM Cd+200 µM Zn	279 ± 35.09	780 ± 93.98	1011±117.56	0.771 ± 0.11

The content of Na, Mg, P, K, Ca, Cu, Al, Mn, Fe, and Zn bounded ions to the chloroplastic membrane were determined by X-ray microanalysis (Figs 1 & 2). In the presence of 100 µM Cd or Zn and the marked decreases were of Na, Mg, P, K, Ca, Cu, Al, and Mn ions. Disappearance of Fe^{2+} peak was observed at 100µM of Cd or Zn whereas it appeared again at 100 µM Cd + 200 µM Zn.

Combined treatment by 100 µM Cd + 100 µM Zn caused synergistic effect of both metals revealing that leaf contents of P, S, Mg, Ca and K as seen by the marked decrease in the height of their peaks compared with the control. While, treatment by 100 µM Cd + 200 µM Zn led to a marked increase of leaf contents of P, S, Mg, Fe, Ca, and K in broad bean plants compared to the treatment with 100 µM Cd + 100 µM Zn.

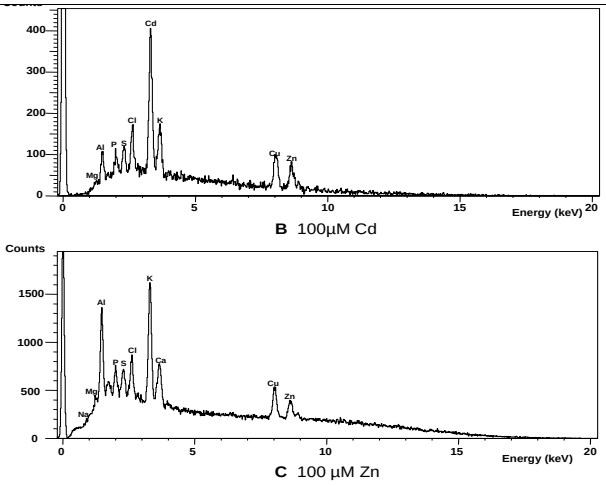
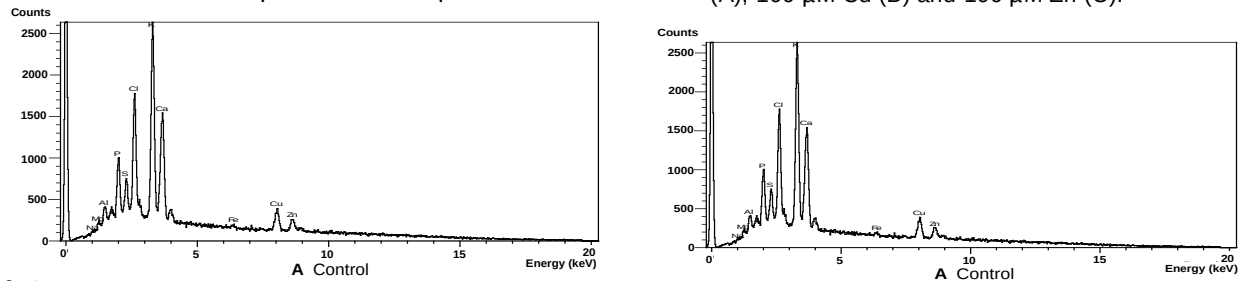


Fig. 1. X-ray microanalysis spectrum of broad bean leaf. The peaks belonging to individual elements in broad bean leaf are denoted by symbols; control (A), 100 µM Cd (B) and 100 µM Zn (C).



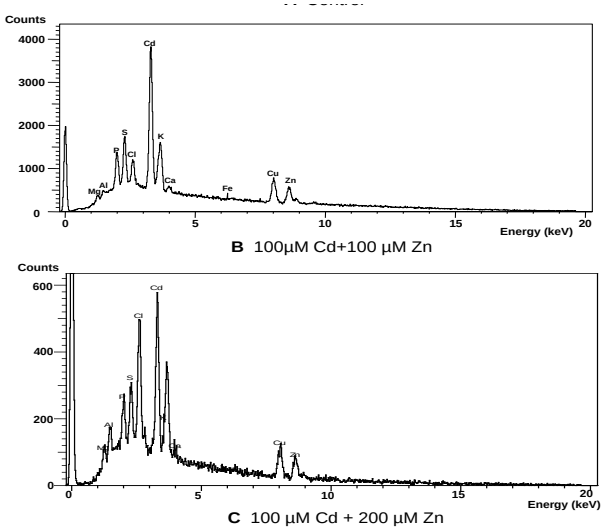


Fig. 2. X-ray microanalysis spectrum of broad bean leaf. The peaks belonging to individual elements in broad bean leaf are denoted by symbols; control A, 100 µM Cd+100 µM Zn B and 100 µM Cd + 200 µM Zn.

Transmission electron microscope (TEM) micrograph in figures 3 -7 showed that there was no observed structural distortion and subcellular arrangement of the leaf cell organelles was regular, integrated, intact and clear-cut in appearance in the control samples (Fig. 3A & B). Compact and smooth cell wall layers typical elliptical well developed chloroplasts containing several grana and the mitochondria showed a round shape in intracellular compartment (Fig. 3C & D).

Stromal-thylakoid layers had a distinct structure and were arranged in order (Fig. 3B). The grana lamella membrane and chloroplast membrane were distinctly legible (Fig. 3C & D). There was normal organelles distribution within the cell, the plasma membrane was stretched and parallel with the cell wall.

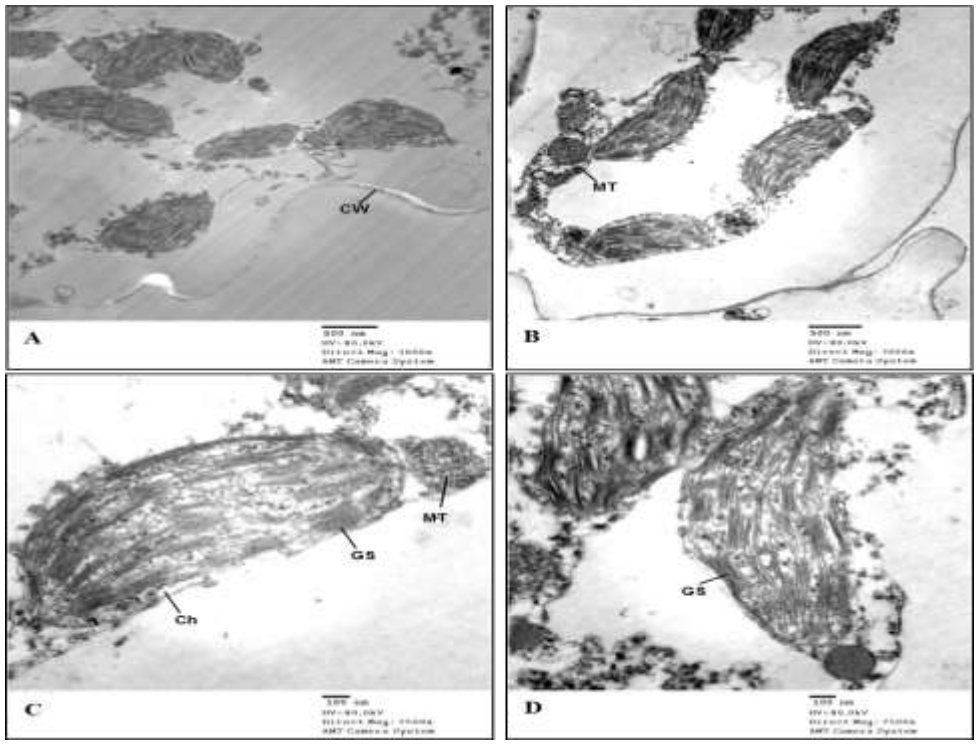


Fig. 3. Transmission electron micrographs of *Vicia faba* seedlings leaf ultrastructure of control. Ch: Chloroplast, CW: Cell wall, Gs: Granna stacks, Mt: Mitochondria. The magnification of panel A and B is 3000x; the magnification of panel C and D is 7500x.

The negative cadmium effect on all subcellular structure with chloroplast and nucleus was considerably more clearly expressed in Cd (100 µM) treated leaves (Fig. 4 A-D). The plasmolysis and the damage of cell membrane were obvious where it became away from the cell wall (Fig. 4A & D) and dark cadmium precipitant densely observed on the membrane and cell wall. Chloroplast migrated towards the plasma membrane and became aggregated together. Furthermore, it became swollen, smaller in size and their thylakoid system was dramatically affected, displaying membrane destruction and fewer layers of grana stacks (Fig. 4B). Thinning and partial tearing of the chloroplast envelope was observed (Fig. 4B & D).

In the chloroplast stroma (Fig. 4B), vesicular structures were observed, which were probably related to stromal thylakoid lysis. Starch grains were sporadically observed within some chloroplast (Fig. 4B). The nucleus became distorted and takes irregular shape with many condensed chromatids and stacked to the chloroplast and mitochondria. Also, the mitochondria were distorted and became small elliptical in shape aggregated beside nucleus, chloroplast and plasma membrane (Fig. 4A & D).

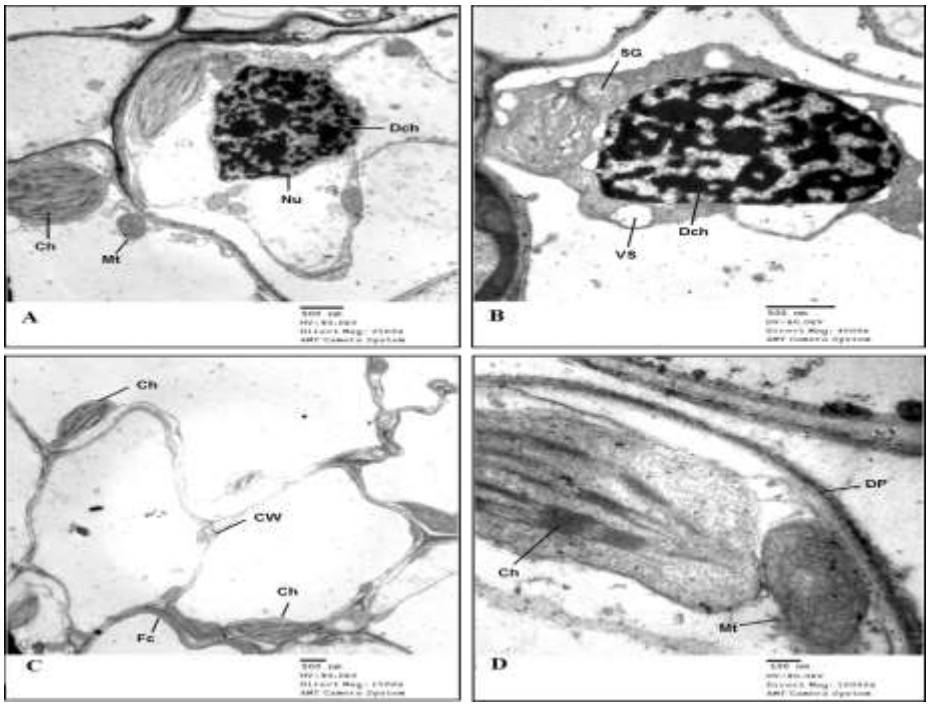


Fig. 4. Transmission electron micrographs of *Vicia faba* seedlings leaf ultrastructure treated with 100 µM Cd. Ch: Chloroplast, Dp: Dark precipitation, Fc: Folded cell membrane, Gs: Grana stacks, Mt: Mitochondria, Nu: Nucleus, Vs: vesicular structures, S.g: Starch grain, D.ch: dense chromatids. The magnification panel of A, B, C and D are 2500x, 4000x, 1500x and 10000 x approximately.

The subcellular structure of seedlings exposed to 100 µM Zn was distorted, and membrane structure was thicker, folded and exhibited distinct damnification (Fig. 5A). Also, dark precipitations of Zn appeared clearly alongside the plasma cell wall (Fig. 5A & B). Most of chloroplasts become more or less rounded or curved and not normally distributed as the control (Fig. 5C), some of them became more elongated (unfamiliar form) showing a lesser number of grana stacks compared to the control (Fig. 5A & B). Mitochondria were distorted and became elliptical in shape aggregated beside the nucleus and the cell wall (Fig. 5D). The nucleus performed elongated unusual form and may be tightly stacked to the plasma membrane and the chloroplast (Fig. 5C & D).

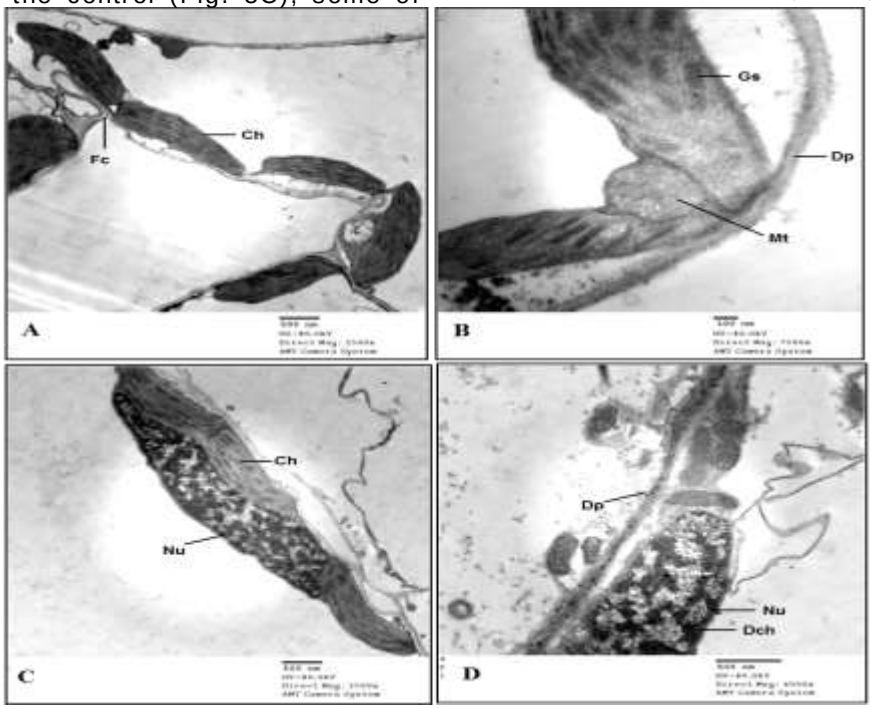


Fig. 5. Transmission electron micrographs of *Vicia faba* seedlings leaf ultrastructure treated with 100 µM Zn. Ch: Chloroplast, Cm: Cell membrane, Cw: Cell wall, Dc: Dense chromatids, Dp: Dark precipitation, Mt: Mitochondria, Nu: Nucleus, Fc: folded cell wall. The magnification panel of A, B, C, and D are 2500x, is 7500x, is 2500x, and is 4000 x approximately.

Figure 6 showed a drastic effect on the ultrastructure of leaf cells due to treatment

with 100 µM Cd and 100 µM Zn. The cells were more plasmolised smaller in size and all subcellular contents became packed and more or less aggregated at the middle of the cell specially chloroplasts and mitochondria (Fig. 6A & B). Further most proportion of chloroplasts (80%) became rounded (Fig. 6A & B). Chloroplast envelopes and the link between stromal thylakoids and grana were disrupted showing degradation of a large number of matrices. In addition, many starch grains appeared more obvious inside chloroplasts (Fig. 6C).

The plasma membrane was detached from the cell wall and was distorted (Fig. 6B). The cell wall became thicker displaying dense precipitations (DP) of heavy metals (Fig. 6C).

The mitochondria appeared rounded and highly distracted with dense chromatids. Nucleus exhibited unusual oval shape and attached to the chloroplasts (Fig. 6C & D).

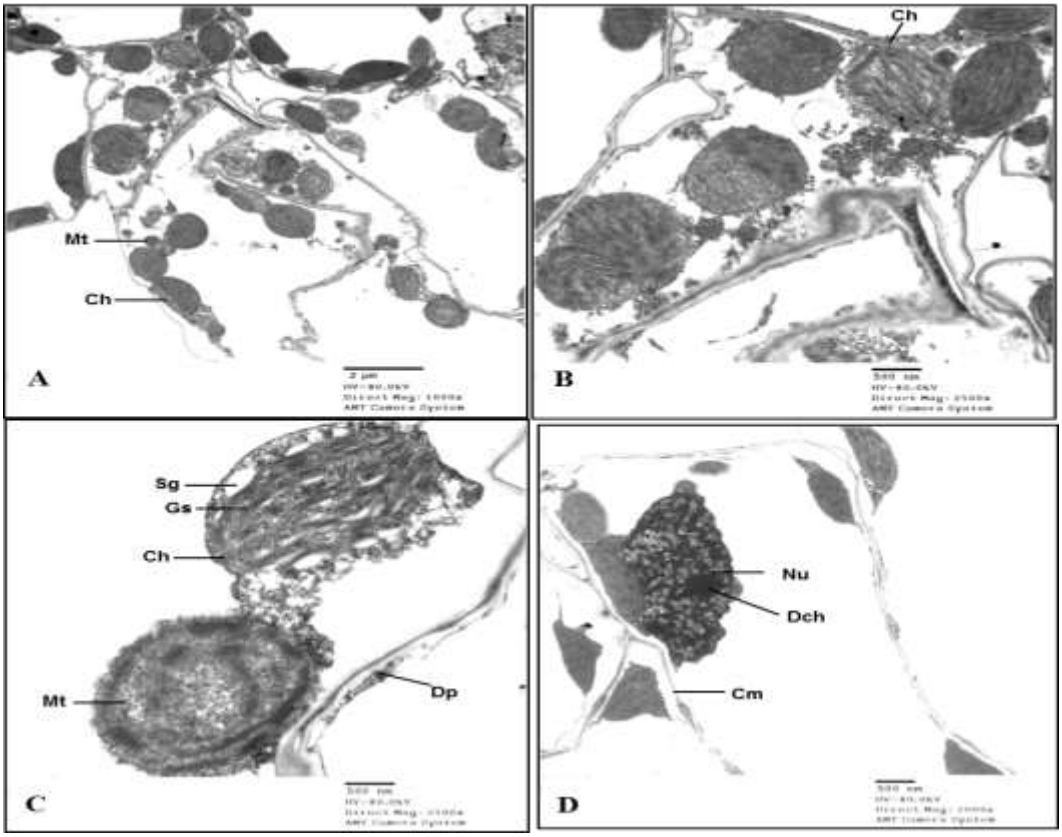


Fig. 6. Transmission electron micrographs of *Vicia faba* seedlings leaf ultrastructure treated with 100 µM Cd+100 µM Zn. Ch: Chloroplast, C.w: Cell wall, Dp: Dark precipitates, Gs: Grana stacks, Nu: Nucleus, Pm: Plasma membrane, Sg: Starch grains. The magnification panel of A, B, C, and Dare 1000x, 2500x, 2000x approximately.

Ultrastructure of plant cells treated with 100 µM Cd + 200 µM Zn showed a slight negative effect, compared to those treated with 100 µM Cd or 100 µM Zn; this may be due to the antagonistic effect between both of them. The chloroplast has an elliptical shape somewhat like the control but with less dense grana stacks and with more in numbers (Fig. 7A & B). Most of the mitochondria were rounded nevertheless little of them were oval (Fig. 7D). Unlike the previous treatments, the mitochondria were lying in a distance near to the chloroplasts and not stack with them (Fig. 7D), starch grains were also observed with fewer numbers but larger in size (Fig. 7A). The nucleus takes more or less a normal round shape compared to the control with less dense chromatids (Fig. 7C). Cell wall and plasma membrane were smooth showing a slight precipitation compared to the other treatments (Fig. 7B). Particularly at this combination, cytosolic vesicles around the chloroplasts were greatly detected (Fig. 7B, C, & D).

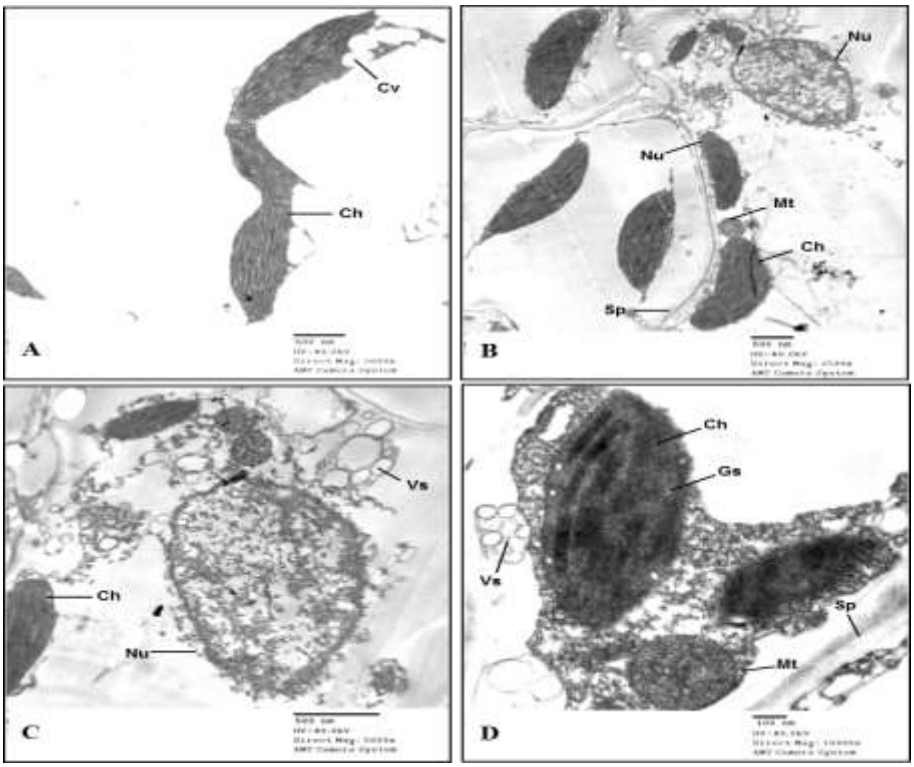


Fig. 7. Transmission electron micrographs of *Vicia faba* seedlings leaf ultrastructure treated with 100 µM Cd + 200 µM Zn. Ch: Chloroplast, C.v: Cytosolic vesicle, C.w: Cell wall, G.s: Grana stacks, M.t: Mitochondria, S.p: Slight precipitation. The magnification panel of A, B, C, and D are 3000x, 2500x, 5000x and 10000x approximately.

DISCUSSION:

Cadmium (Cd) is well known as a highly toxic environmental element due to its great toxicity and high mobility from soil to plants, it can be incorporated and accumulated by all organisms in large amounts and disturb physiological metabolisms in plants like transpiration, photosynthesis, respiration, and nitrogen assimilation (Wang *et al*., 2008). Zn is found to be involved in many cellular functions such as protein metabolism, gene expression, chromatin structure, photosynthetic carbon metabolism and indole acetic acid metabolism (Cakmak, 2000). The applied Cd in this study induced a significant decrease in the amount of photosynthetic pigments Chl.a, Chl.b, and carotenoids. This decline in chlorophyll and carotenoids has been proposed to be responsible for the reduction in photosynthesis and hence plant growth (Maksymiec, 2007). Also, Cd can induce iron deficiency (Marschner, 1983) which later caused inhibition of chlorophyll biosynthesis and several other reactions associated with photosynthesis (Siedlecka and BaszyńAski, 1993).

Heavy metal ions as Cd inhibit uptake and transportation of other metal elements such as Mn, Zn, and Fe by antagonistic effects and therefore the leaf lose the capacity of synthesis of pigments as also found by Das *et al*. (1997) and Gardea-Torresdey, *et al*. (2004). Furthermore, heavy metals may activate degrading pigment enzyme and accelerate the decomposition of pigment (Kumari *et al*., 2011). The reductions of chlorophyll content can be also due to the oxidation coming from an overproduction of ROS, which are generated by Cd toxicity (Laspina *et al*., 2005). Also, Cd inhibits PSI and PSII activity (Husaini and Rai, 1991). PSII is more sensitive to Cd than PSI and it is the primary site of action of Cd in photosynthetic electron flow in isolated spinach chloroplasts and *Nostoc linckia* (Yang *et al*. 1989; Husaini and Rai, 1991).

The concentration of Cd or Zinc up to100 µM induced a remarkable decrease in *Vicia faba* leaf chlorophyll content compared to control. The degradation rate of Chl. b under such stress was slower than that of Chl. a. As it is known, Chl.a is one of the most important center pigments in photosynthesis and therefore the decrease of Chl. a can inhibit the photosynthesis process greatly which was reflected on the plant leaf area. The treatment with Cd, had more inhibitory effect on pigments than Zn in broad bean leaves, even under low concentration of Cd (50 µM).

Alleviation of Cd toxicity by Zn was reported by Arunachalam *et al*. (1996). The current results cleared that Zn can strongly antagonize the toxic effects of Cd especially with high Zn concentrations as at 100 µM, there was full protection and restoration of the chlorophyll levels. Also, there was a remarkable increase in chlorophyll content by combining at 200 µM Zn compared to chlorophyll content of Cd treatment. This

showed that when the level of Zn become over that of Cd, it can probably maintain chlorophyll synthesis through sulphydryl group protection, a function primarily associated with Zn (Lebedev and Timko, 1998; Cakmak, 2000). In contrast, when low Zn concentrations were added (50 or 100 µM) combined with Cd (100 µM), severe reduction in chlorophyll contents was produced due to the synergistic interaction of Zn.

X-ray microanalysis for the determinations of Na, Mg, P, K, Ca, Cu, Al, Mn, Fe, and Zn bounded to the cell membrane, revealed a significant decrease of Ca, K, Mg in the presence of Cd or Zn, also the disappearance of Fe^{2+} peak was observed at100 µM Cd.

Lose of Ca, Mg, Fe and other elements due to the heavy metals treatment was similar to the senescence response suggesting that excess of Cd or Zn may lead to the progressive senescence of the leaves (Adams *et al*., 1990).

In the leaves of bean plant, Cd may displace Ca and Mg ions from exchange in sites and is strongly bound in the cell free space, as well as Cd ions may replace Mg ions in the chlorophyll molecule. Subsequently, Mg deficiency may be the reason of observed decrease in chlorophyll content and detected variable fluorescence ratio. Similarly, is calcium, a second messenger and an important element in the cell wall and in membrane structure/stability (White, 1998), however its deficiency may lead to serious cell damage. The recorded detrimental effect of Cd on Fe content was possibly due to ability of Fe^{3+} to be reduced to Fe^{2+} and then to be transported out the leaves (Agarwal *et al*.,1987). Also, Ouzounidou *et al.* (1997) reported that Fe, Na, K, Ca, and Mg content declined under Cd treatment. Nearly 90% of Fe in plants is localized in chloroplasts, where it is required for electron transport chain and synthesis of chlorophyll-determining proper course of photosynthesis and biomass production (Sagardoy *et al.,* 2010). However, the disappearance of iron under Cd treatment could be due to Cd ions interacts with the manganese cluster which is present in the oxygen evolving complex on the donor side of PSII, and accordingly the Mn ions are releases from manganese clusters into the interior of thylakoid membrane (Debus, 2000). It must be emphasized that Cd or Zn treatments decreased also, Ca levels affecting the sites of transport and Ca allocation in membranes and most probably affecting plastid membrane structure which could be the reason of differential changes occur within the chloroplast of *Vicia faba* leaves as also found by Liu *et al*. (2014).

Despite the strategies of *Vicia faba* to counteract the effect of metal toxicity, Zn and/or Cd, in the present study, caused many damages to the cell and organelle ultrastructure. The chloroplast disturbances observed were similar to those reported by He *et al.* (2011) who reported that heavy metals induced changes in chloroplast ultrastructure resemble those induced by senescence. Changes in chloroplast ultrastructure and lipid composition of the thylakoid membranes alter the operability of the photosynthetic electron-transport chain (Ouzounidou *et al*., 1997). This correlates with the progressive disorganization of chloroplasts and the poor development of thylakoid membranes and grana, and hence, a heavy metals-induced increase of chloroplast senescence (Vassilev *et al*., 2003). Starch accumulation in leaves of Cd-treated plants in this study could be attributed to heavy metal inhibition to plant growth.

Combined treatment between 100 µMCd + 100 µM Zn revealed that leaf concentrations of P, S, Fe, Mg, Ca and K in broad bean plants showed a marked decrease in intensities of the peaks compared with the control. While, Combined treatment between 100 µM Cd + 200 µM Zn revealed that leaf concentrations of P, S, Mg, Ca and K in broad bean plants showed a marked increase in intensities of the peaks compared with the control which may be due to the reduction in Cd content.

Cadmium (100 µM) had negatively influenced grana thylakoids. Pietrini *et al.* (2003) have found similar retardation of chloroplast development and severe disruption of grana thylakoids in soybean seedlings subjected to similar cadmium treatment. The chloroplasts observed in 100 µM Cd-treated have a reduced number of intact grana as well as reduced chloroplast size, which was also observed in *Sedum alfredii* treated with 400 mM Cd (Jin *et al*., 2008 & 2009) and *Pisum sativum* with 50 mM Cd (Sandalio *et al*., 2001). Vitória *et al*. (2003 & 2006) observed chloroplasts with increased size and distorted shapes due to $CdCl_2$. Baryla *et al*. (2001) found that Cd reduced number of chloroplasts per cell, and suggested that Cd interfere with chloroplast replication. Under cadmium stress, an overall changes occurred in the shape of leaf cells was reflected as folding of cellular envelope this make the diffusion path shorter (Cherif *et al.,* 2011).

Changes were established in the shape of the chloroplast ultrastructure, too where grana were highly disorganized and granal and stromal thylakoids were reduced in number. On the double-membrane envelope of some chloroplasts a small bright-grain deposition was established, which is probably related to the adsorption of Cadmium. It could be observed that the negative cadmium effect was not equally expressed in all chloroplasts,

this fact being also, reported by Barceló and Poschenrieder (1999). The disturbed organization of the chloroplast ultrastructure led to formation of structure-functional associations between the chloroplasts and mitochondria and an increase in the contact surface between the two organelles was observed.

Changes in the cellular organization at high Zn levels were evident and a clear alteration in the leaf cellular ultrastructure was detected in the plants grown at 100 µM Zn.

Accumulation of Zn precipitation along cell wall and chloroplast envelope was clear, that likely caused the observed alterations in the chloroplast morphology and the envelope desegregation. Thinning and partial tearing of the chloroplast envelope was also observed. The significant damage was in the mesophyll chloroplasts in the high concentration Zn-treated plants as was recorded by Ying *et al*. (2010).

Combined treatment of cadmium and zinc led to more changes in the shape of cells, as well as a dramatic change in the chloroplasts morphology, which showed few starch grains. The shape of chloroplasts was almost spherical, and the grana therein were completely damaged, also, a large part of the chloroplasts were with disturbed envelopes. This synergistic effect of Cd and Zn treatment on thylakoid was confirmed in *Vicia faba* plant as was previously reported in plants grown at high levels of heavy metals, such as Cd or Mn (McCarthy *et al*., 2008; Najeeb *et al*., 2009; Glińska *et al.,* 2016).

Increasing Zn over Cd concentration treatment (100 µM Cd + 200 µM Zn) acquired less negative effect and doubling concentration of zinc can successfully antagonize the harmful effect of Cd. The chloroplast has an elliptical shape like that of control but with less dense grana stacks. Cell wall was smooth showing a slight dark precipitation a large number of cytosolic vesicles compared to the other treatments.

The clearly detectable structures containing Zn around the chloroplasts and spread into the cytosol are similar to the so-called 'zincosomes' identified in yeast and mammalian cells grown in the presence of Zn (Haase and Beyersmann, 1999; Devirgiliis *et al*., 2004; Mohamed and Huaxin, 2015). Zincosomes are cytosolic vesicles that store Zn for metabolic needs. Also, the link between stromal thylakoids and grana was disturbed.

The presence of zincosomes in plant tissues is still controversial, although recent evidence of Zn-accumulating vesicles was detected in *Arabidopsis thaliana* seedlings (Kawachi *et al*., 2009). Based on current results, *Vicia faba* appears to possess similar structures. So, these zincosomes can therefore be interpreted as a strategy adopted by the plant to detoxify the cytosol from heavy metals (Azzarello *et al*., 2012; Bandiera *et al.,* 2016) and to prevent the translocation of Zn to the entire plant.

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التغييرات الوظيفية والتركيبية الدقيقة في جهاز البناء الضوئي في أوراق نبات الفول استجابة للتفاعل بين عنصري الكادميوم والزنك

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وجد أن عنصر الكاديوم وهو من العناصر المعدنية الثقيلة التي تمنع (تثبط) امتصاص وانتقال العناصر الأخرى مثل المنجنيز والزنك والحديد في أوراق نبات الفول. وباستخدام تركيز 100 µM من كل من الكادميوم على حدة قد أحدث نقصاً ملحوظاً في المحتوى الكلوروفيلي بالمقارنة بالكنترول. وأن النقص في محتوى كلوروفيل ب كان أقل منه في كلورفيل أ تحت ظروف هذا الإجهاد. بينما وجد أن التأثير المثبط للكادميوم تحت هذه الظروف كان أكثر من تأثير الزنك في أوراق نبات الفول حتى لو كان تركيز الكادميوم عند مستوى (50 µM). هذه النتائج توضح أن للزنك قوة كبيرة في تثبيط التأثير السام للكادميوم، خصوصاً إذا كان تركيز الزنك في المحلول المغذي أعلى بكثير من تركيز الكادميوم ولذلك فإنه يحمي تماماً محتوى الكلوروفيل. وفي وجود كل من الكادميوم أو الزنك. عند تعيين تركيزات عناصر الصوديوم والماغنسيوم والفوسفور والبوتاسيوم والكالسيوم والنحاس والألمونيوم والمنجنيز والزنك المصاحبة للجدار الخلوي وجد أنه قد حدث نقص معنوي في تركيز كل الكالسيوم والبوتاسيوم والماغنسيوم في وجود كل من الكادميوم أو الزنك مع اختفاء الحديد عند تركيز 100 µM كادميوم، وأن اختفاء الحديد في وجود الكادميوم قد يرجع إلى تداخل أيون الحديد مع تجمعات المنجنيز الموجودة على المركب المنطلق على الجانب المعطى في نظام PSII. وجد في هذه الدراسة أن الآثار السمية لكل من الزنك أو الكادميوم أو الاثنان معاً قد سبب تدمير التركيب الدقيق لعضيات الخلية. ونتيجة للتغير في التركيب الدقيق للبلاستيدة الخضراء ومكون الليبدات في أغشية الثيلاكويدات فقد تبدل عمل سلسلة انتقال الإلكترون في عملية البناء الضوئي. تحت تأثير المعاملة بتركيز 100 µM من الكادميوم وجد أن هناك اختزال لعدد البذيرات المترابطة مع نقص في حجم البلاستيدة. وعند المعاملة بالكادميوم كما والزنك معاً أدى ذلك الى تغييرات كبيرة في شكل مكونات الخلية، فمثلاً كان شكل الكلوروبلاست كروياً غالبا واختفت البذيرات تماماً وجزء كبير من الكلوروبلاست ظهر كأغلفة ممزقة. ولكن بزيادة تركيز الزنك عن الكادميوم نتج عنه تأثير إيجابي للتأثير السام للكادميوم مما يدل على أن الزنك ألغى بنجاح التأثير الضار للكادميوم.