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Cytogenetical and ultrastructural effects of copper on root meristem cells of *Allium sativum* L.

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ABSTRACT: Different copper concentrations, as well as different exposure times, were applied to investigate both cytogenetical and ultrastructural alterations in garlic (*Allium sativum* L.) meristem cells. Results showed that the mitotic index decreased progressively when either copper concentration or exposure time increased. C-mitosis, anaphase bridges, chromosome stickiness and broken nuclei were observed in the copper treated root tip cells. Some particulates containing the argyrophilic NOR-associated proteins were distributed in the nucleus of the root-tip cells and the amount of this particulate material progressively increased with increasing exposure time. Finally, the nucleolar material was extruded from the nucleus into the cytoplasm. Also, increased dictyosome vesicles in number, formation of cytoplasmic vesicles containing electron dense granules, altered mitochondrial shape, disruption of nuclear membranes, condensation of chromatin material, disintegration of organelles were observed. The mechanisms of detoxification and tolerance of copper are briefly discussed.

Introduction

Copper is an essential nutrient for plants; it plays an irreplaceable role in the function of a large number of enzymes catalyzing oxidative reactions in a variety of metabolic pathways (Lolkema and Vooijs, 1986; Marschner, 1995; Quartacci *et al*., 2000). Plants respond to both the deficiency and the excess of metals (Kukkola *et al.,* 2000). Various copper sources, including industrial and domestic wastes, agricultural practices, copper mine drainage, copper-based pesticides, and antifouling paints, have contributed to a progressive increase in copper concentrations in several environments (Andrade *et al*., 2004). High copper levels in soil can be phytotoxic, causing deleterious effects both morphologically and physiologically (Liu *et al*., 1995; Ke *et al*., 2007; Meng *et al*., 2007; Tanyolaç *et al*., 2007). Copper can lower the mitotic index, inhibit cell division and induce chromosomal aberrations (Liu *et al*., 1994; Jiang *et al*., 2001). Studies on the accumulation and subcellular localization of heavy metals in plants have been reported recently by energy dispersive X-ray spectroscopy (Kupper *et al*., 2000; Monni *et al*., 2002; Liu *et al*., 2007; Sahi *et al*., 2007), synchrotron radiation X-ray fluorescence spectroscopy (Shi *et al*., 2004) and electron energy loss spectroscopy (Liu and Kottke, 2004a; Rau *et al*. 2006). These techniques can provide essential information on subcellular localization and accurate elemental analysis of heavy metals. However, few investigations on toxic effects of Cu^{2+} on cell division, nucleolus, and on ultrastructural alterations in constructing anti-copper system in cells under copper stress have been reported.

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Allium sativum L. is a potentially useful plant to study the absorption and accumulation of heavy metals (Jiang and Liu, 2001; Liu and Kottke, 2003). In the current work we combined both cytogenetical and ultrastructural effects of the different copper concentrations on the root meristem cells of *A. sativum*, and to provide essential information on mechanisms of detoxification and tolerance.

Materials and Methods

Plant material, growth conditions and metal treatments

Healthy and equal-sized garlic cloves (*Allium sativum* L.) were chosen from bulbs showing no growth of either green leaves or roots. The experimental set up was similar to that of Fiskesjö (1988): 12 garlic cloves with the dry scales removed were used in each series, and were washed and rooted in tap water for 36 h. Afterwards, 10 of these rooted cloves were selected and directly placed in a container with either 10^{-5} M, 10^{-4} M or 10^{-3} M copper solution (provided as copper sulfate, $CuSO_4 \cdot 5H_2O$), respectively. Controls were grown on tap water. The test liquids were changed regularly every 24 h, seedlings were grown in a greenhouse equipped with supplementary 15-h light/9-h dark at 18-20ºC diurnal cycle.

Cytogenetical assay

Twenty root tips in each treatment group were washed with tap water and distilled water, and cut at 24, 48 and 72 h, respectively. They were fixed in ethanol + acetic acid (3:2) for 4 to 5 h and hydrolysed in 1 M hydrochloric acid + 95% ethanol + acetic acid (5:3:2) for 6 min at 60°C. For the observation of chromosomal morphology, 10 root tips were squashed in a carbol-fuchsin solution (Li, 1982) and for the observation of nucleolus changes, the others were squashed in 45% acetic acid, drying, and on day 2 staining with silver nitrate (Li *et al*., 1990; Liu and Jiang, 1991). Data for root length were analysed with standard statistical software (SigmaPlot 9.0).

Ultrastructure

For transmission electron microscopy observation, according to the results from cytogenetic investigation, seedlings treated at 10^{-4} M copper for 1 h, 2 h, 4 h, 8 h, 12 h, 24 h, 36 h, 48 h and 72 h.

The terminal 1–3 mm portion from each root in both the control and treated groups was fixed in a mixture of 2% formaldehyde and 2.5% glutaraldehyde in 0.2 M phosphate buffer (pH 7.2) for 2 h and, and then thoroughly washed in the same buffer, before post-fixation in 2% osmium tetroxide in the same buffer for 2 h.

TABLE 1.

Effect of $CuSO_4$ on cell division in the root tip cells of *A. sativum*

Time (h)	Concentration (M)	Mitotic index (‰)	Number of cells	Normal dividing cells (%)			Anomalous dividing cell (%)		
				metaphases	Anaphases	c-mitosis	Chromosome bridge	Chromosome stickiness	Rate of anomalous cells
24	Control	342	500	60.0	34.4	0	4.0	1.6	5.6
	10^{-5}	283	500	58.0	24.0	8.0	0.8	9.2	18.0
	10^{-4}	179	500	21.0	35.0	15.4	0.6	28.0	44.0
	10^{-3}	120	500	30.2	25.6	11.6	0.2	32.4	44.2
48	Control	406	500	56.2	40.4	0	3.0	0.4	3.4
	10^{-5}	131	500	49.0	21.0	9.4	0.6	20.0	30.0
	10^{-4}	127	500	36.6	18.0	7.4	1.0	37.0	45.4
	10^{-3}	64	500	32.6	8.6	18.2	0.4	40.2	58.8
72	Control	349	500	44.0	51.4	0	3.4	1.2	4.6
	10^{-5}	60	500	42.6	11.8	5.6	0	40.0	45.6
	10^{-4}	61	500	22.0	18.8	8.0	0.4	50.8	59.2
	10^{-3}	40	500	30.0	7.0	2.0	0	61.0	63.0

They were then dehydrated in an acetone series and embedded in Spurr's resin. For ultrastructural observations, ultrathin sections of 75 nm thickness were cut on an ultramicrotome (Leica EM UC6, Germany) with a diamond knife, and were mounted on copper grids with 300 square mesh. The sections were stained with 2% uranyl acetate for 50 min and lead citrate for 15 min. They were examined under a transmission electron microscope (JEM-1230, Joel Ltd., Tokyo, Japan).

Cytochemical tests

The Gomori-Swift reaction was used to detect cysteine-rich proteins. For such purpose, 100 nm thick sections of roots prepared as described above, were cut and mounted on gold grids.

Solution A containing 5 ml of 5% silver nitrate and 100 ml of 3% hexamethylenetetramine and solution B consisting of 10 ml 1∞ 44% boric acid and 100 ml 1∞ 9% borax were prepared. The final stain was obtained by mixing 25 ml of A, 5 ml of B and 25 ml of distilled water. The grids were floated in the silver methenamine solution for 90 min at 45ºC in the dark, and were then washed four times for 2 min. The grids were then floated on 10% sodium thiosulphate solution for 1 h at room temperature to dissolve metallic silver and rinsed in deionized water four times for 2 min. Finally, the sections were stained with uranyl acetate and lead citrate (Swift, 1968).

Controls were carried out to block S-H group and S-S group by the reduction of disulfide bonds in benzylmercaptan followed by alkylation of S-H groups in iodacetate boric acid. The procedures were described by Swift (1969) and Liu and Kottke (2004b).

Results

Cytogenetical investigation

Observations of roots treated with different levels of Cu^{2+} revealed structural damage that were not observed in the root cells of control plants.

Mitotic index: The mitotic index reflects the frequency of cell division and it is regarded as an important parameter. The mitotic index decreased progressively as a function of increased copper concentration and exposure time (Table 1).

Chromosomal aberrations: C-mitosis was observed in the root tip cells of all treated groups after treatment with Cu^{2+} (Fig. 1a). Anaphase bridges involving one or more chromosomes (Fig. 1b-c) were found after the Cu treatment. Anaphase bridges exhibiting stickiness were observed only in the treatment

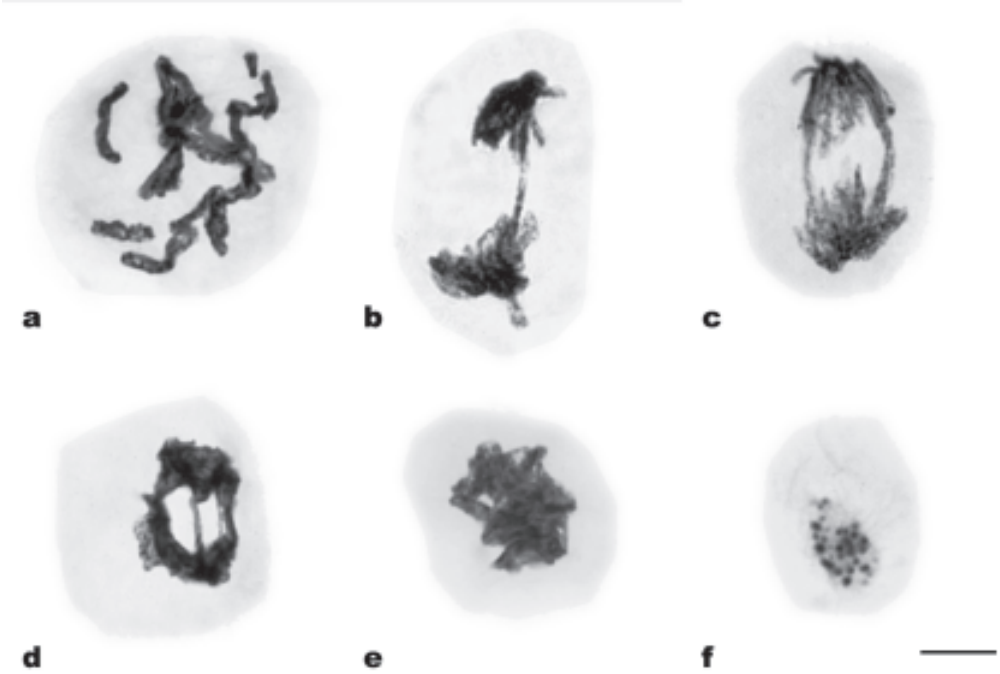

Figure1. The effects of Cu^{2+} on cell division in root tips of *Allium sativum*. a: C-metaphase (10^{-4} M Cu^{2+}, 24 h); b-d: Chromosome bridges (b, 10^{-4} M Cu^{2+}, 24 h; c, 10^{-3} M Cu^{2+}, 24 h; d, 10^{-3} M Cu^{2+}, 48 h); e: Chromosome stickiness (10^{-3} M Cu^{2+}, 48 h); f: Nucleus disintegration (10^{-3} M Cu^{2+}, 72 h). Scale = 10 μm.

with 10^{-3} M Cu (Fig. 1d). This type of toxic effect is most likely irreversible. The cells with chromosome stickiness were noted (Fig. 1e), and the frequency of cells progressively increased with increasing Cu^{2+} concentration. Some broken nuclei in the root tip cells treated with Cu (10^{-3} M) were observed (Fig. 1f).

Nucleolar organiser regions (NORs) are defined as nucleolar components containing a set of argyrophilic proteins, which are selectively stained by silver methods. After silver-staining, the NORs can be easily identified as black dots exclusively localized throughout the nucleolar area, and are called "AgNOR proteins" (Trerè, 2000). Normally, the diploid nucleus of *A. sativum* contains one or two nucleoli (Fig. 2a). The effects of copper on nucleoli varied with the different concentrations of copper sulfate solutions used. Two phenomena were observed after these treatments. Firstly, after 48 h treatment with 10^{-4} M copper, some particulates containing the argyrophilic NOR-associated proteins were observed together with the main nucleolus/nucleoli in the nucleus of some root-tip cells (Fig. 2b). The amount of the particulates increased progressively and nearly occupied the whole nucleus when the copper concentration increased to 10^{-3} M (Fig. 2c). Secondly, in concentrations of 10^{-3} M copper, the AgNOR proteins were extruded from the nucleus into the cytoplasm (Fig. 2d). The nucleolar proteins in the cytoplasm increase progressively (Fig. 2e) and aggregate into irregular shapes with longer duration of the treatment (Fig. 2f).

Ultrastructural investigation

A typical ultrastructure was exhibited in control cells. Plasma membrane was unfoled with a uniform shape in all parts. The numerous organelles were immersed in cytoplasm. Endoplasmic reticulum is mostly composed of parallel cisternae. Dictyosomes were also found in cytoplasm (Fig. 3a). The nucleus with well-stained nucleoplasm and distinct nucleolus was located in the center of cells, whereas a large vacuole or several vacuoles are distributed in root meristem cells.

Root tip cells exposed to 10^{-4} M copper solutions showed several ultrastructural alterations. Visible symptom of copper toxicity was noted after copper treatment for 1 h. The dictyosome vesicles markedly increased, which seems as a compact mass of vesicles in the cytoplasm (Fig. 3b). Endoplasmic reticulum with dilation of flattened cisternae appeared in cytoplasm after 2 h copper treatment, and these flattened cisternae were broken up into small closed vesicles (Fig. 3c), in which electron dense granules were occasionally observed in it when root cells exposed to 10^{-4} M copper for 4 h (Fig.

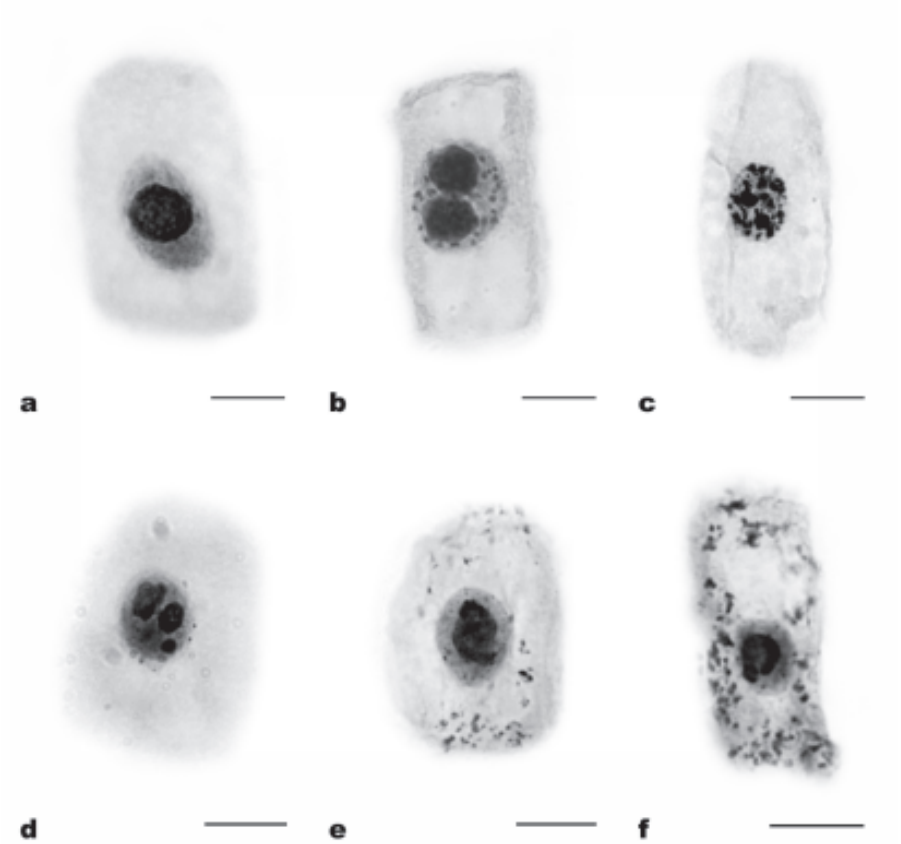

Figure 2. Effects of Cu^{2+} on nucleoli in root tip cells of *Allium sativum*. a: Control cell (48 h); b: Some particulate containing the argyrophilic NOR-associated proteins scattered in the nuclei (10^{-4} M Cu^{2+}, 48 h); c: More and more particles occupied the whole nucleus (10^{-3} M Cu^{2+}, 48 h); d-f: The particles scattered in the cytoplasm (d, 10^{-3} M Cu^{2+}, 48 h; e, 10^{-3} M Cu^{2+}, 48 h; f, 10^{-3} M Cu^{2+}, 72 h). Scale = 10 μm

3d). Any degenerative changes were not showed in the other organelles in relation to control cells. However, after 8 h copper treatment, larger vacuoles were rapidly formed by migrating to and fusing small vesicles derived from endoplasmic reticulum, and more electron dense granules in the vacuoles were accumulated (Fig. 4a). This tendency was more pronounced in the cells with prolonging copper treatment time. After 12 h copper treatment, abundance of parallel arrays of rough endoplasmic reticulum with regularly cisternae was also observed in cytoplasm (Fig. 4b). Due to an extension of cisternae and a loss of matrix density, alterations of mitochondria shapes were gradually showed in figure 4c. Electron-dense granules were taken into cells by means of invaginations of the plasma membrane. The invaginations form small vesicles that are pinched off the plasma membrane and carried, with their enclosed the material, into the cytoplasm, which appeared after 24 h treatment (Fig. 4c). The most significant ultrastructural changes were noted after 36-72 h of root cells treatment, revealing disruption of nuclear membranes, a highly condensed chromatin material and disintegration of organelles, which led to death of some copper treated cells after 72 h.

Cytochemical test

Cytochemical test were conducted to discover Cu^{2+} localization in root cells using the Gomori-Swift reaction which is highly sensitive and allows the detection of cysteine-rich proteins. In control root cells metallic silver grains as a result of positive reaction were not found (Fig. 4d). Swift reaction showed the presence of cysteine-rich proteins in root cells exposed to 10^{-4} M copper. Traced amounts of silver grains were, at early stage, noted in the cell walls of root cells at copper treat-

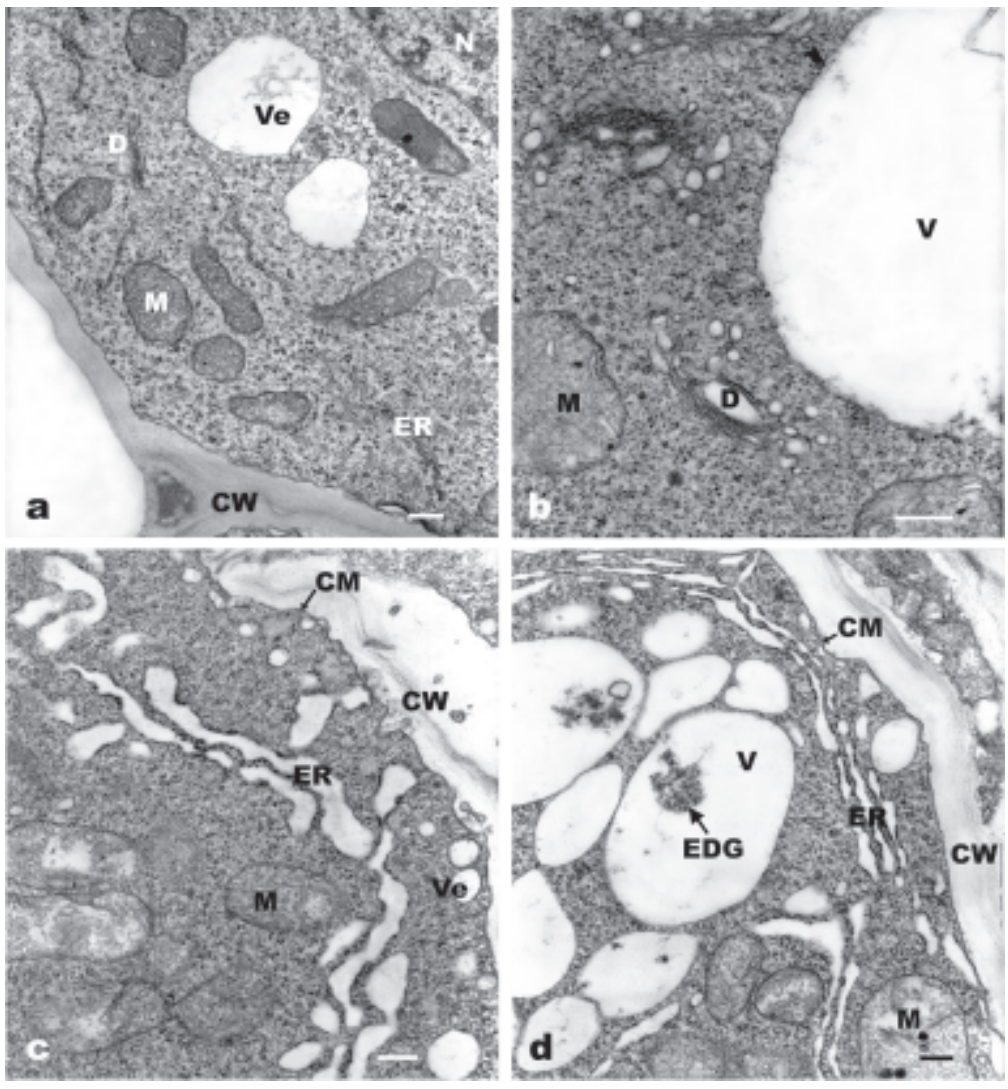

Figure 3. TEM micrographs showing toxic effects of Cu on ultrastructure of the root meristematic cells of *Allium sativum*. **a:** Control cells showing well developed root tip cells. **b-d:** The ultrastructural changes of root meristematic cells treated with 10^{-4} M Cu from 1 h - 72 h. **b:** Dictysome vesicles obviously increased in the root meristematic cells exposed to 10^{-4} M Cu for 1 h. **c:** Vesiculated ER was distributed in cytoplasm near cell wall (10^{-4} M Cu, 4 h). **d**: The electron dense granules were accumulated in vacuoles (10^{-4} M Cu, 4 h). C = cytoplasm, CM = cytoplasm membrane, CW = cell wall, D = dictyosome, ER = endoplasmic reticulum, EDG = electron dense granules, M = mitochondria, N = nucleus, V = vacuole, Ve = vesicle. Bar = 0.25 μm.

ment for 2 h, and then they appeared in cytoplasm and small vesicles near the cell walls (Fig. 4e). In vesicles and cytoplasm, the number of cysteine-rich protein increased as a consequence of excessive copper-exposure time from 4 h to 72 h, whereas, metallic silver grains in cell walls do not increased remarkably (Fig. 4f).

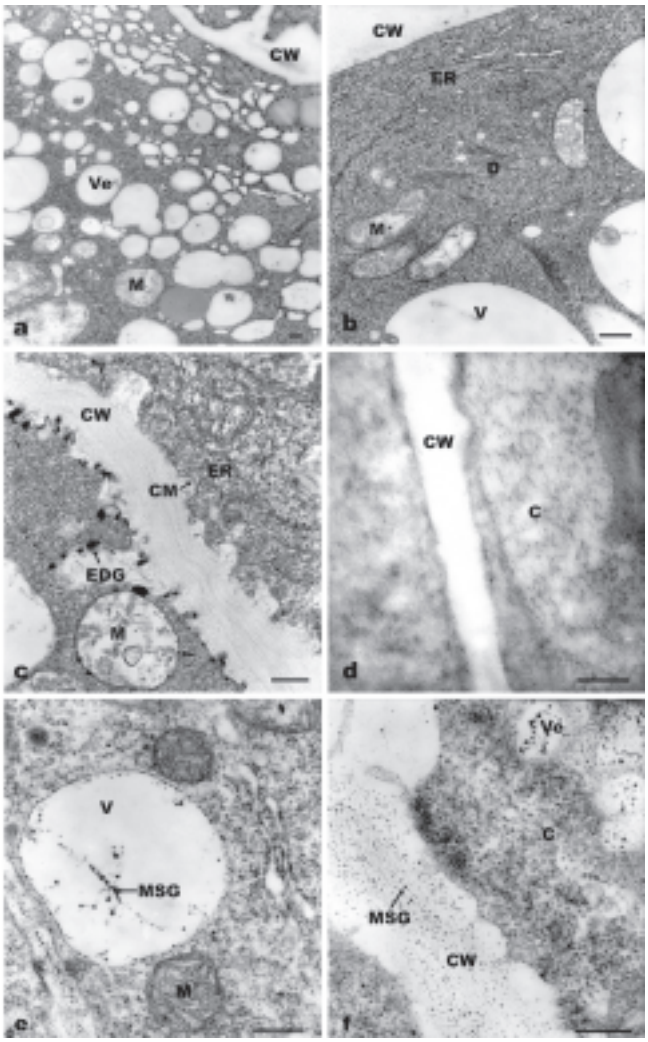

Figure 4. TEM micrographs showing toxic effects of Cu on ultrastructure of the root meristematic cells of *Allium sativum.* **a**: The vacuole increased by fusing small vesicles from ER and dictysomes in cytoplasm. **b:** Abundance of parallel arrays of rough ER with regularly cisternae exhibited in cytoplasm (10^{-4} M Cu, 12 h). **c:** Endocytosis of plasma membranes appeared. Arrow shows reduction of mitochondria. (10^{-4} M Cu, 24 h). d-f: Cytochemical test of the root meristematic cells of *A. strvum*. **d:** Without showing Gomori-Swift reaction in the cell of control. **e:** Metallic silver grains with Gomori-Swift positive reaction localized in the cytoplasm and vesicles (10^{-4} M Cu, 4 h). **f:** Showing metallic silver grains distribution in cell wall, in cytoplasm as well as vesicles (10^{-4} M Cu, 8 h). CM = cytoplasm membrane, CW = cell wall, D = dictyosme, ER = endoplasmic reticulum, EDG = electron dense granules, M = mitochondria, MSG = metallic silver granules, MT = microtubule, N = nucleus, V = vacuole, Ve = vesicle. Bar = 0.25 μm.

Discussion

The results in the present investigation indicated that copper affects *Allium sativum* at concentrations from 10^{-5} to 10^{-3} M. These are in agreement with the findings of Liu *et al*. (1994) for the effect of Cu^{2+} on tip cells of *A. cepa*. However, this results were with a few differences when comparison with the findings of Jiang *et al*. (2001) for the effect of Cu^{2+} on *Zea mays*. For instance, 1) there is not so many nucleolar particles scattered in the nuclei and so much nucleolar material released from nuclei into cytoplasm in *Z. mays*; 2) Copper toxicity on the nucleoli in root tip cells of *A. sativum* is stronger than those of *Z. mays*, which shows that *A. sativum* and *A. cepa* is more sensitive to copper compared with *Z. mays*.

Some reports revealed that copper caused the inhibition of root elongation by metal interference with cell division, including inducement of chromosomal aberrations and abnormal mitosis (Agarwal *et al*., 1987; Kahle, 1993; Jiang *et al*., 2001). Quzounidou (1994) stated that copper accumulation influences the tissue distribution of Ca, Mg, Fe and K. Jensén and Adalsteinsson (1989) also indicated that copper ions tend to displace Ca^{2+} ions from exchange sites and are strongly bound in root-free space. It is well known that the nucleolus is the metabolic center of RNA. The integrity of the nucleolus depends on the existence of Ca^{2+} (Wang, 1988). Because of the low level of free Ca^{2+} in the cells, calmodulin does not activate Ca-ATPase (Xu, 1985), which leads to failure in regulation of calcium concentration and disturbance or inhibition of various cellular metabolic processes.

The results from the present investigation revealed that excess copper caused adverse effects on garlic meristem cells. The plasma membrane and main organelles participated the establishment of anti-heavy metal toxic systems in the cells. The response to Cu^{2+} toxicity occurs in 1 h after copper treatment, resulting in an enlargement of dictyosomes. Usually, cisternae of endoplasmic reticulum are arranged in parallel in control cells. However, vesiculated endoplasmic reticulum could be observed markedly in cytoplasm. The vesicles derived from dictysomes and endoplasmic reticulum were formed into bigger vacuoles by fusing from each other. The feature of vacuolar compartmentation is the most obvious ultrastructure evidence of cells when cells resist heavy metals toxicity, which is in agreement with the work reported by Ouzounidou *et al*. (1995), Sresty and Madhava Rao (1999). It is very interesting that at copper treatment for 12 h, a large amount of endoplas-

mic reticulum with regular cisternae again appeared in the cytoplasm, similar to that in the control cells. One possible explanation is that target of toxic copper is at the molecular level and effects are always reflected in the structure of the cell and its organelles. Once excess Cu^{2+} ions entered cytosol, the synthesis of new proteins of endoplasmic reticulum involved in heavy metal tolerance is stimulated. Then, the vesicles from endoplasmic reticulum carried the proteins, which bind copper by formation of stable metal-phytochelatin complexes. In this way, free metal ions in cytosol decreased. Besides, some vesicles from endoplasmic reticulum carried polysaccharide and they were transferred to the dictyosomes where they are modified prior to secretion (Raven *et al*., 1986). Secretory vescles derived from cisternae of dictyosomes migrate to the plasma membrane and repair the damaged plasma membrane and discharging their contents in the region of the wall. Plants have a range of potential mechanisms at the cellular level that might be involved in the detoxification and thus tolerance to heavy metal stress (Hall, 2002). The results from the present investigation suggest that endoplasmic reticulum and dictysomes may participate in heavy metal detoxification in cells.

In this context, it is interesting that cysteine-rich proteins were found localized both in the cytoplasm and in cell walls and vesicles of copper treated cells (Fig. 4f). Rau *et al*. (2006) indicated that electron energy loss spectra demonstrated copper chelation by SH-groups of glutathione in the cytoplasm and the copper-binding as phosphates in vacuoles. Copper accumulation was associated with a reduced sulfur level in the electron-dense-precipitates of the cytoplasm and an increasing phosphorus level in the vacuole. Morelli and Scarano (2004) reported that phytochelatin synthesis started in the marine diatom *P. tricornutum* after copper exposure for 15 min, and phytochelatin accumulation in the cell reached a maximum rate at the treatment for 7 h. Data from size exclusion chromatography analyzed that an amount close to 60% of the total intracellular copper was bound to phytochelatin at seventh hour of exposure.

The evidence reviewed in this investigation and formed works strongly suggest that 1. Cell walls, a first barrier against copper stress, can immobilize some copper ions, therefore prevent contact with the sensitive plasmalemma and cytoplasmic components. The results here exhibited that the presence of cysteine-rich proteins were identified in cell walls exposed to copper. 2. Active endocytosis of plasma membrane and active secetory of vesicles from dictyosomes and endoplasmic reticulum appear at low concentration of heavy metals, which reflects the morphological feature during detoxification and tolerance to heavy metals. 3. At first stage, electron dense granules containing copper distribute in the cytoplasm and vesicles (Liu and Kottke, 2004b). However, vacuoles finally are one of main storage sites of heavy metals. 4. With the increased level of heavy metal ions in cytosol, cell and its organelle were seriously injured and lost their functions of anti-heavy metal toxicity, leading to some cells death.

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