

Physiological and ultrastructural studies on calcium oxalate crystal formation in some plants

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Abstract: Results from light, scanning, and transmission electron microscopic examination of CaOx crystal formation in 2 plant species, namely *Corchorus olitorius* L. and *Malva parviflora* L., revealed the presence of crystalline deposits. These deposits were of prismatic type in the case of *C. olitorius* and of druse type in the case of *M. parviflora*. Ultrastructurally, some differences were observed between crystal idioblast and noncrystal cells. Moreover, the effect of heavy metals and different calcium concentrations on the growth of calcium oxalate crystals in leaves of the 2 plants was investigated. Different analytical techniques were used to determine the influence of exogenous cadmium (Cd), lead (Pb), copper (Cu), and zinc (Zn) on CaOx deposition and to detect the presence of these metals in CaOx crystals. We found a positive correlation between the calcium concentration in the nutrient medium and the production of calcium oxalate crystals in the leaves of hydroponically grown plants. On the other hand, addition of the heavy metals to the nutrient medium decreased the number of crystals. Our investigation suggests that CaOx crystals do not play a major role in heavy metal detoxification in these 2 plant species but do play an important role in bulk calcium regulation.

Key words: CaOx, crystals, calcium level, heavy metals

1. Introduction

The plant kingdom exhibits a varied assortment of patterned mineralised structures formed by cells, including deposits of calcium oxalate (CaOx), calcium carbonate, and silica (Arnott & Pautard, 1970). CaOx crystals are by far the most prevalent and widely distributed mineral deposits throughout the families of higher plants (Arnott & Pautard, 1970; Kuo-Huang et al., 2002; Nakata, 2003; Franceschi & Nakata, 2005; Kuo-Huang et al., 2007; Mukherjee & Nordenstam, 2010). Crystals have been observed in virtually all the tissues of plants. Certain algae (Pueschel, 2001), lichens (Erdman et al., 1977), and fungi (Arnott, 1995) are also capable of producing significant quantities of the different forms of oxalate, particularly CaOx. In fact, the ability of plants to produce these various forms of oxalate may be helpful to them in certain phase(s) of their life cycles. In contrast, CaOx crystal formation in animals is generally considered to be pathological and extracellular. A good example of this situation is represented by urinary calculi (stones), which are often partly or entirely composed of CaOx. Most crystals produced in plants can be classified into 1 of 5 categories based on their morphology: crystal sand, raphide, druse, styloid, and prismatic (Franceschi

& Horner, 1980; Katayama et al., 2007; He et al., 2012). In whatever tissue the crystals are found, they most often accumulate within the vacuoles of specialised cells called crystal idioblasts (Franceschi & Nakata, 2005). The number and location of crystal idioblasts within the plant body also vary among taxa. Extracellular crystals have also been reported in some cases (Kuo-Huang et al., 2002). In some cases one crystal is formed per vacuole, whereas in other cases many crystals are formed within a vacuole. In the latter case, each crystal still forms within a membrane-delineated space, usually termed the crystal chamber, forming de novo in the vacuole (Arnott & Pautard, 1970). The idioblasts display ultrastructural modifications related to crystal precipitation. Crystal idioblasts are commonly reported to exhibit characteristic features including an enlarged nucleus, specialised plastids, greater amounts of endoplasmic reticulum and Golgi complexes, elevated levels of rRNA, and unique vacuolar components (Arnott & Pautard, 1970; Franceschi & Horner, 1980; Kostman & Franceschi, 2000; Franceschi & Nakata, 2005). The abundant Golgi complexes in these idioblasts have also been found to be involved in transporting a calcium-binding crystal idioblast specific protein, matrix protein, to the vacuole (Kostman & Franceschi, 2000). Ascorbic

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acid is suggested to be the immediate precursor of oxalic acid used for crystal formation (Horner et al., 2000; Keates et al., 2000; Franceschi & Nakata, 2005). Crystal idioblasts contain the pathway for ascorbate synthesis (Kostman et al., 2001; Nakata, 2003), thus making them autonomous for oxalic acid production. The morphology and distribution of crystals are constant within a species. This indicates that their presence, morphology, and distribution in a species are under genetic control (Nakata & McConn, 2000; Ilarslan et al., 2001; Franceschi & Nakata, 2005).

Although their functional significance in plant development remains unclear, various functions have been attributed to them, including calcium regulation in plant cells (Franceschi, 1989; Kostman & Franceschi, 2000; Volk et al., 2002), protection against herbivory (Molano-Flores, 2001), detoxification of heavy metals or oxalic acid (Franceschi & Nakata, 2005), tissue strength, light gathering, and reflection (Franceschi & Horner, 1980; Kuo-Huang et al., 2007).

However, the most commonly proposed function of these crystals is that their formation appears to be part of a mechanism for removal of excess biologically active calcium when other mechanisms have become saturated (Borchert, 1985, 1986; Franceschi, 1989; Pennisi & McConnell, 2001; Volk et al., 2002). For example, crystals in the legume *Phaseolus vulgaris* (Jáuregui-Zúñiga et al., 2005) play an important role in regulating levels of bulk calcium. Through this mechanism, large amounts of excess calcium can be precipitated as a highly insoluble salt of oxalate that is no longer osmotically or physiologically active (Zindler-Frank et al., 2001). In this way, the low levels of calcium required within the cell cytosol can be maintained. The precipitation of CaOx is a reversible process, releasing calcium during periods when calcium is limiting to growth of the plant (Webb, 1999; Volk et al., 2002). The CaOx crystals of plant crystal idioblasts represent a relatively simple model system for biomineralisation. Therefore, it is anticipated that an understanding crystal formation in plants will provide important new insights into the general area of biomineralisation and will be useful not only to biologists interested in how nature manufactures mineralised materials, but also to materials scientists and engineers who are interested in either harnessing or mimicking natural processes to create novel and useful ceramic structural materials (Jáuregui-Zúñiga et al., 2003).

Calcium oxalate crystals could play an important role in heavy metal detoxification (Jáuregui-Zúñiga et al., 2005). Several studies have shown that plants utilise organic acids (e.g., citrate, malate, and/or oxalate) as mechanisms enabling them to tolerate various heavy metals (Franceschi & Schueren, 1986; Ryan et al., 2001; Mazen, 2004). Some plants utilise oxalate to detoxify hazardous metals, such as lead (Yang et al., 2000), aluminium (Ma et al., 2001),

strontium (Franceschi & Schueren, 1986; Zindler-Frank, 1991), and cadmium (Choi et al., 2001), when present in the environment.

The present study was conducted as a contribution to other research efforts made toward understanding the biological significance of this important mineralisation process in plants. Investigation of some features of this process in 2 new model plant species [*Corchorus olitorius* L. (Tiliaceae) and *Malva parviflora* L. (Malvaceae)]

was the aim of this study. These 2 plant species are grown in Egypt near roads with heavy traffic. Thus, heavy metals are accumulated in these plants. Moreover, these plants represent preferred foods for Egyptians. The features examined in this study were as follows:

1- Occurrence, distribution, and ultrastructural description of crystals formed and the leaf tissue cells in which these crystals were borne.

2- The dynamic relationships between calcium in the nutrient growth medium and the formation of CaOx crystals.

3- Evaluation of the suggestion that CaOx crystals may play a role in the detoxification of toxic heavy metals by working as sinks for these metals.

2. Materials and methods

2.1. Pretreatment plant growth

Seeds purchased from a local market were allowed to germinate seedlings to grow in soil mixture of sand/clay (2:1) for 8 weeks in a greenhouse at 37/27 °C for *C. olitorius* and 23/13 °C for *M. parviflora*. During this period the plants were watered every other day to maintain soil at the field capacity. Every week during the growth period, the seedlings were irrigated with 1/4 strength Hoagland solution (Arnon & Hoagland, 1940). The soil-growing plants were used as a stock source for transplants to be recultured for all experiments conducted in this study. Whenever needed for an experiment, some healthy plants from this stock were very carefully liberated from the soil in which they were growing, and the root systems kept almost intact were washed thoroughly with tap water before their transplantation to a liquid culture, where they were grown under various treatments with different factors.

2.2. Treatment with various Ca concentrations

Plants that were grown hydroponically on 1/10 strength Hoagland nutrient solution (Arnon and Hoagland, 1940) for 5 days prior to treatment with various calcium concentrations were transferred to fresh new liquid cultures. In these new cultures, plants were grown on Hoagland nutrient solution in which calcium concentration was varied depending on the plant species. The Table summarises the different calcium concentrations with which each plant species was treated. Calcium nitrate ($\text{Ca}(\text{NO}_3)_2$) was used as the source of calcium in

Table. Calcium concentrations with which the 2 experimental plant species were treated.

Calcium levels	<i>Corchorus olitorius</i>	<i>Malva parviflora</i>
0.0 mM	+	+
0.5 mM	+	-
1.0 mM	+	+
4.0 mM	+	-
5.0 mM	-	+
6.0 mM	+	-
7.0 mM	+	-
10.0 mM	-	+
15.0 mM	-	+
20.0 mM	-	+
25.0 mM	-	+

the nutrient solution. The nitrate contents of the low and deficient calcium solutions were adjusted using NaNO_3 (Wu et al., 2006). Cultures were aerated continuously with fish-aquarium air bubblers. Plants in these cultures were left to grow at 25 ± 2 °C in a growth chamber at irradiance of $\sim 40 \mu\text{mol}/(\text{m}^2 \text{ s})$ for 16 h each day provided by fluorescent tubes and incandescent lamps. Nutrient solutions including the mentioned calcium concentrations were replaced with fresh ones every other day. Plants were grown for 20 days (Franceschi & Schueren, 1986). During this period new leaves appeared. They were sampled for preparation of leaf clearings used in the determination of crystal counts and sizes, and preparation of leaf specimens needed for microscopic examination.

The relationship between crystal formation and calcium level was tested in 2 sets of experiments. In the first set of experiments, the crystal formation criteria were studied in the experimental plants that were exposed to increasing calcium level in their growth media after they had been previously starved of calcium to ensure minimum crystal formation. In the other set of experiments, these criteria were studied in the experimental plants that were exposed to progressively decreasing calcium level in their growth media after their previous growth for a long growth period in media with saturated calcium level ensuring maximum crystal formation.

2.3. Treatment with heavy metals

Plants that were grown hydroponically on 1/10 strength Hoagland nutrient solution (Arnon & Hoagland, 1940) for 5 days prior to treatment with heavy metals were transferred to fresh liquid cultures. In these new cultures, plants were grown on Hoagland nutrient solution including 6.0 mM

calcium nitrate in the case of *Corchorus olitorius* and 20.0 mM for *Malva parviflora*. In these media, different concentrations of cadmium (as cadmium nitrate), lead (as lead acetate), copper (as copper sulphate), and zinc (as zinc sulphate) at 0.0, 1.0, 5, 10, 15, 20, 25, 50, and 100 μM were added. Cultures were aerated continuously with fish-aquarium air bubblers. Plants in these cultures were left to grow at 25 ± 2 °C in a growth chamber at irradiance of $\sim 40 \mu\text{mol}/(\text{m}^2 \text{ s})$ for 16 h each day provided by fluorescent tubes and incandescent lamps.

Nutrient solutions including the mentioned heavy metals at the mentioned concentrations were replaced with fresh ones every other day. Plants were grown for 20 days. During this time period new leaves appeared. They were sampled for preparation of leaf clearings used in the determination of crystal counts and sizes, and for preparation of leaf specimens needed for microscopic examination and for heavy metal content in leaf tissues.

2.4. Microscopic examinations and tissue analysis

2.4.1. Preparation of leaf clearings for light-microscopic observation of crystals

Leaves from treated plants were soaked in 70% acetone for 12 h at 60 °C until all chlorophyll was extracted. They were then dehydrated with acetone, infiltrated with xylene, infiltrated with cover bond (American Scientific Products, McGraw Park, IL, USA), a xylene miscible resin, and permanent slides of whole leaves made.

2.5. Determination of crystal size and counts per unit area

Crystals were counted using an ocular micrometer in an area of 0.3 mm² of unstained cleared leaves. Similarly, crystal sizes were determined. A Nikon Optiphot-Pol microscope fitted with polarising filters permitting the crystals to be easily viewed was used in these examinations. Crystal size was determined by measuring length and width in the case of *C. olitorius* and by measuring crystal diameter in the case of *M. parviflora*.

2.6. Light microscopy

Pieces of leaves formed during exposure of plants to treatments with heavy metals were fixed in 3% glutaraldehyde and 2.5% paraformaldehyde in 50 mM PIPES buffer (pH 7.2) for 12 h at 4 °C. The specimens were dehydrated with ethanol series. They were then infiltrated with Spurr epoxy resin (Spurr, 1969). After that, 1.5- to 2-g sections were cut with a glass knife using an ultramicrotome, stained with Methylene Blue stain, and examined and photographed with a Nikon Optiphot-Pol microscope using polarising filters.

2.7. Transmission electron microscopy

Pieces of leaves formed during the experiment were fixed in 3% glutaraldehyde and 2.5% paraformaldehyde in 50 mM PIPES buffer (pH 7.2) for 12 h at 4 °C. The fixed material

was prepared for transmission electron microscopy by postfixation in 1% OsO₄ in 25 mM cacodylate buffer for 2 h at room temperature, dehydration through ethanol series, and embedding in Spurr epoxy resin. For ultrastructural studies, the sections were cut with a glass knife, poststained with uranyl acetate and lead citrate, and examined (Spurr, 1969).

2.8. Determination of heavy metals in plant tissue

Leaves formed during exposure of plants to treatments by heavy metals were thoroughly washed with distilled deionised water and oven-dried at 80 °C for 72 h. The dry material was then finely ground and the sample for heavy metal determination was treated according to Heckman et al. (1987). The powder (1 g) was dry-ashed at 500 °C for 12 h, dissolved in 4 mL of concentrated HNO₃, and evaporated to dryness. The residue was then redissolved in 10 mL of 3 M HCl, refluxed for 2 h, filtered, and diluted to 25 mL with 0.1 M HCl. The determination of heavy metals was carried out with a PerkinElmer 2380 atomic absorption meter (PerkinElmer, United States).

2.9. Statistical analysis

The present study compared the data of treated and untreated (control) plants, using the least significant difference at 0.05, 0.01, and 0.001 levels. The data were analysed using one-way ANOVA, with the Origin program.

3. Results

3.1. Crystal presence, distribution, description, and ultrastructural features of crystal-bearing cells (crystal idioblasts)

Results using light, scanning, and transmission electron microscopic examination of CaOx crystal formation in the 2 plants used as experimental models revealed the presence of intracellular crystalline deposits. These deposits were of prismatic type in the case of *C. oleritorius* (Figure 1) and of druse type in the case of *M. parviflora* (Figure 2).

Regarding the distribution of crystal idioblasts in leaf tissues, they were found located essentially around veins in *C. oleritorius* and *M. parviflora*. They were seen in most mesophyll cells and in some bundle cells themselves but not in the surrounding bundle sheath cells. In the 2 studied plants, the crystal idioblast cells were not different in size or shape from other noncrystal cells (Figures 3, 4). In the 2 plants studied, crystals were formed intracellularly in the vacuoles enveloped by a membrane. Only a single large crystal was seen per crystal idioblastic cell (Figures 5, 6).

Ultrastructurally, some differences were observed between crystal idioblasts and noncrystal cells. Obvious differences noticed characteristic to crystal idioblasts were enlarged nucleus, modified plastids with few grana, and absence of starch. The cytoplasm of crystal-containing cell looks dense and rich in organelles, membranes, and vesicles (Figures 5, 6).

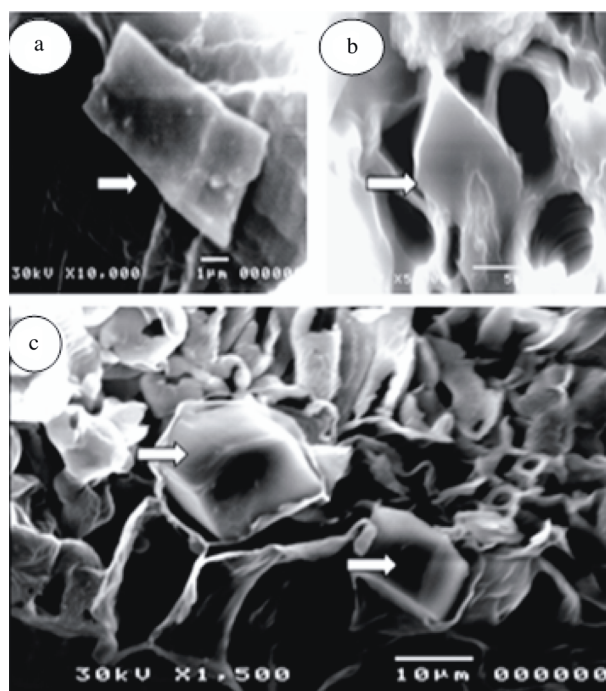


Figure 1. Scanning electron micrographs of calcium oxalate crystals in leaf cells of *Corchorus oleritorius*. (a, b, and c) Crystals (pointed to by arrows) are of the prismatic type and are borne intracellularly.

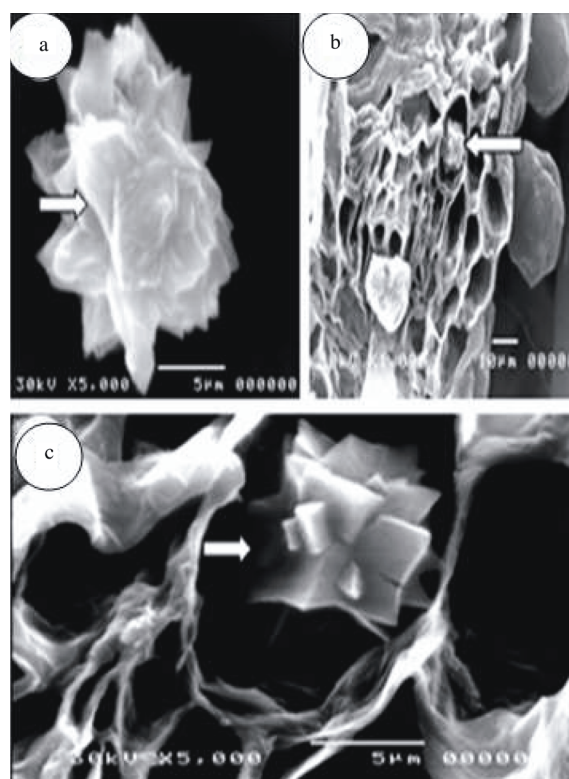


Figure 2. Scanning electron micrographs of CaOx crystals in leaf cells of *Malva parviflora*. (a, b, and c) Crystals (pointed to by arrows) are of the druse type and are borne intracellularly.

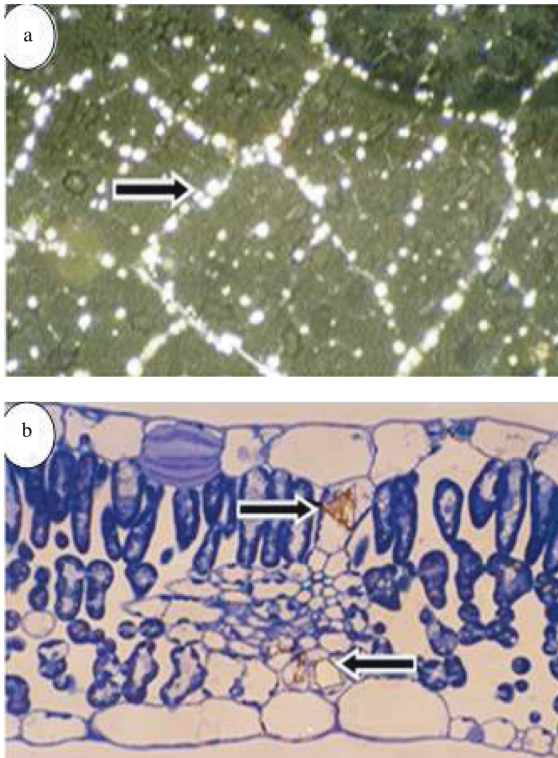


Figure 3. (a and b) Polariser and light micrographs showing CaOx crystals formed in leaf of *Corchorus olitorius*. (a) Enlarged portion of leaf clearing viewed by polarised light. Crystals (bright spots) are seen concentrated around a vein. Enlarged view of crystals (pointed to by arrows). Crystals are of the prismatic type. (b) Transmitted section by using light microscope shows distribution of crystals in the bundle sheath around the vein.

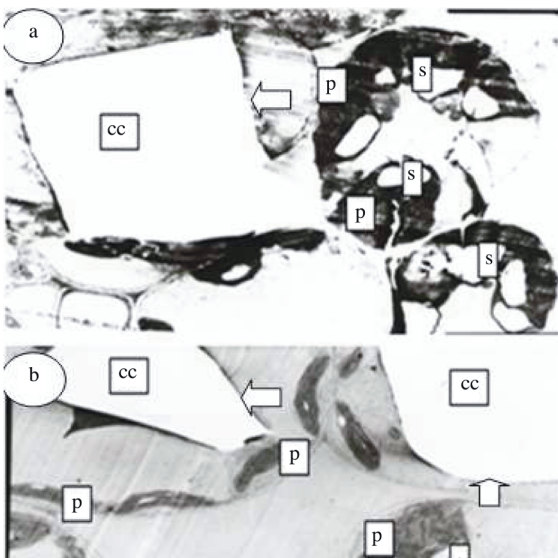


Figure 5. (a and b) Transmission electron micrograph of *Corchorus olitorius* leaf section showing the fine structure of the crystal bearing-cell. The white rectangle in the centre of the cell is the hole (crystal chamber) left after crystal drop during tissue preparation. Abbreviations: S = starch, P = plastid, C = crystal, CC = crystal chamber.

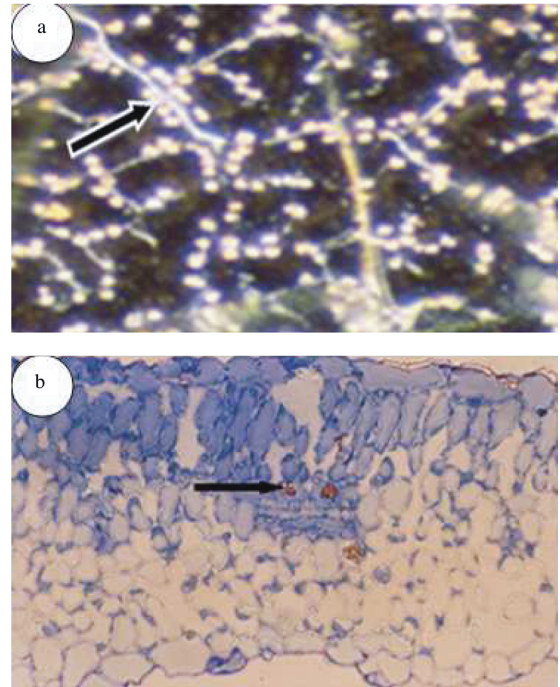


Figure 4. (a and b) Polariser and light micrographs showing CaOx crystals formed in leaf of *Malva parviflora*. (a) Enlarged portion of leaf clearing viewed by polarised light. Crystals (bright spots) are seen concentrated around a vein. Enlarged view of crystals (pointed to by arrows). Crystals are of the druse type. (b) Transmitted section by using light microscope shows distribution of crystals in the bundle sheath around the vein.

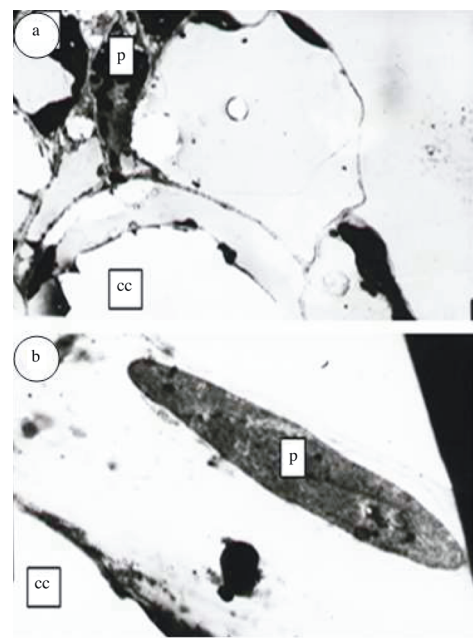


Figure 6. (a and b) Transmission electron micrograph of *Malva parviflora* leaf section showing the fine structure of the crystal bearing-cell. The white rectangle in the centre of the cell is the hole (crystal chamber) left after crystal drop during tissue preparation. Abbreviations: S = starch, P = plastid, C = crystal, CC = crystal chamber.

3.2. Dependence of crystal formation on calcium level

Two criteria were employed to assess the crystal formation process: crystal abundance (number of crystals per unit area of leaf tissue) and crystal size (length and width in the case of *C. olitorius* or crystal diameter in the case of *M. parviflora*). Generally, in all plants studied, crystal formation expressed by these criteria was found to highly significantly ($P < 0.001$) increase with the increase in calcium level in growth media and decrease with its decrease. With regard to calcium's effect on crystal size, the effects were highly significant ($P < 0.001$) in the case of *C. olitorius* but with nearly no effect on the crystal size in the case of *M. parviflora*. The inductive

effect of calcium on crystal formation was found to take place up to a certain calcium saturation level, after which no further effect or a negative effect took place. The saturated calcium effect was dependent on the plant species tested. For example, the saturated calcium levels were 6 mM in the case of *C. olitorius* and 20 mM in the case of *M. parviflora*. Any further increase in calcium concentrations in the growth medium beyond the mentioned saturation level led to a reduction in crystal formation in the case of *C. olitorius*. On the other hand, in the case of *M. parviflora*, the crystal formation process stayed unaffected by any further increase in calcium level (Figures 7–10).

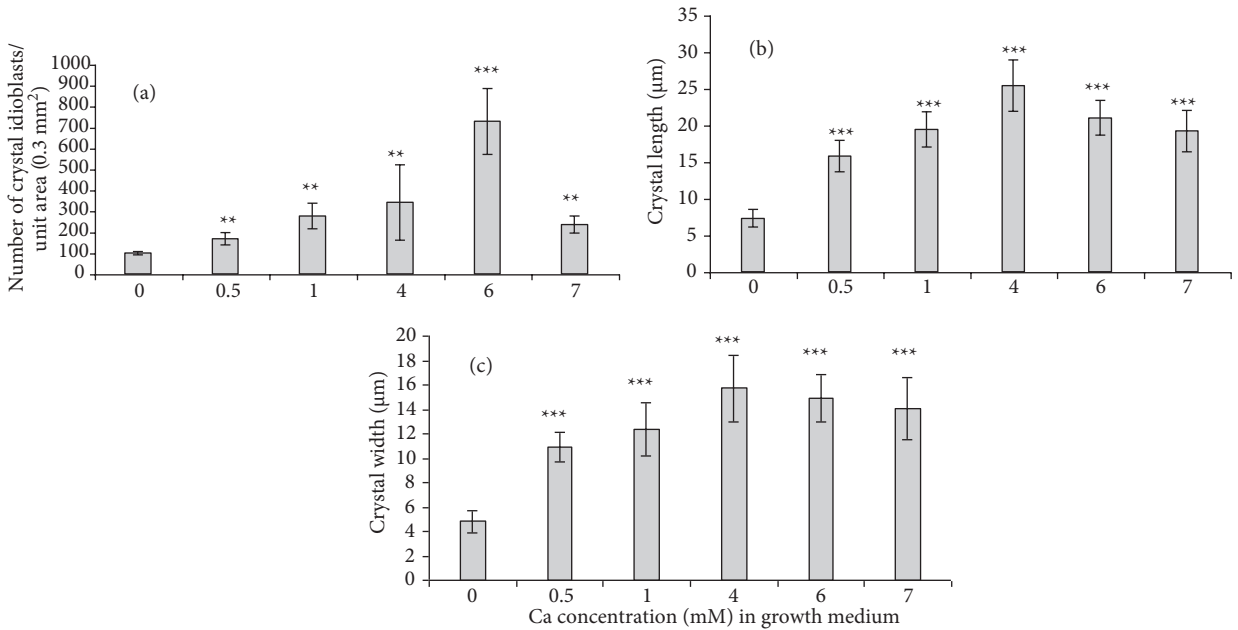


Figure 7. Effect of calcium concentration (0.0 (control), 0.5, 1.0, 4.0, 6.0, and 7.0 mM) in the growth media on the number of crystal idioblasts (a), crystal length (b), and crystal width (c) in leaf tissues of *C. olitorius*. In this experiment, plants were grown on the same mentioned calcium concentrations during the whole period of the experiment. Data are the means \pm SD of 10 repetitions. Asterisks indicate significant differences between the treatments and the control: *significant at $P < 0.05$; **significant at $P < 0.01$; ***significant at $P < 0.001$.

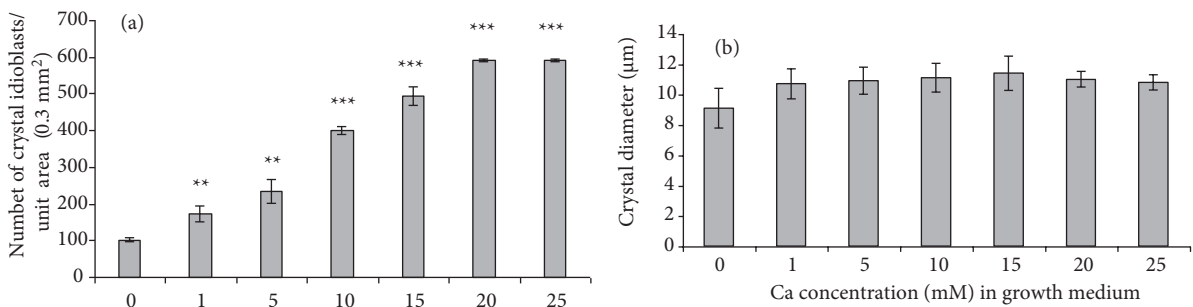


Figure 8. Effect of calcium concentration (0.0 (control), 1.0, 5.0, 10.0, 15.0, 20.0, and 25.0 mM) in the growth media on the number of crystal idioblasts (a) and crystal diameter (b) in leaf tissues of *M. parviflora*. In this experiment, plants were grown on the same mentioned calcium concentrations during the whole period of the experiment. Data are the means \pm SD of 10 repetitions. Asterisks indicate significant differences between the treatments and the control: *significant at $P < 0.05$; **significant at $P < 0.01$; ***significant at $P < 0.001$.

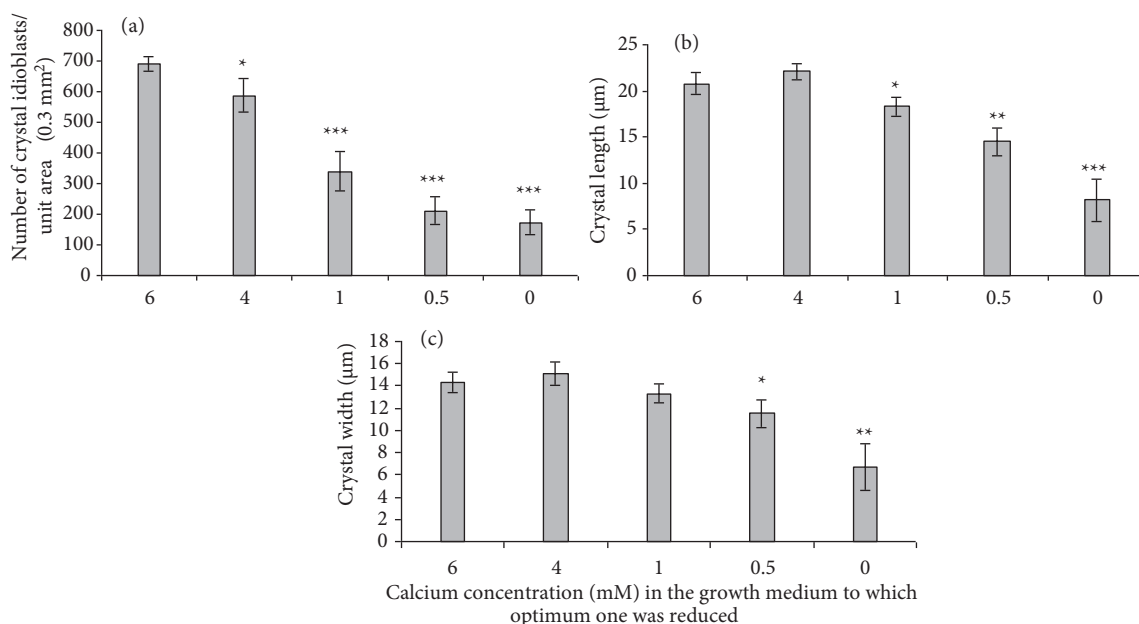


Figure 9. Effect of calcium decrease from the optimum concentration (6 mM) to lower concentrations (4, 1, 0.5, and 0 mM) in the growth medium on the number of crystal idioblasts (a), crystal length (b), and crystal width (c) in leaf tissues of *C. olitorius*. In this experiment, the same plants that were grown on the media with optimum calcium concentration (6 mM) were transferred to new media with lower calcium concentration. Data are the means \pm SD of 10 repetitions. Asterisks indicate significant differences between the treatments and the control: *significant at $P < 0.05$; **significant at $P < 0.01$; ***significant at $P < 0.001$.

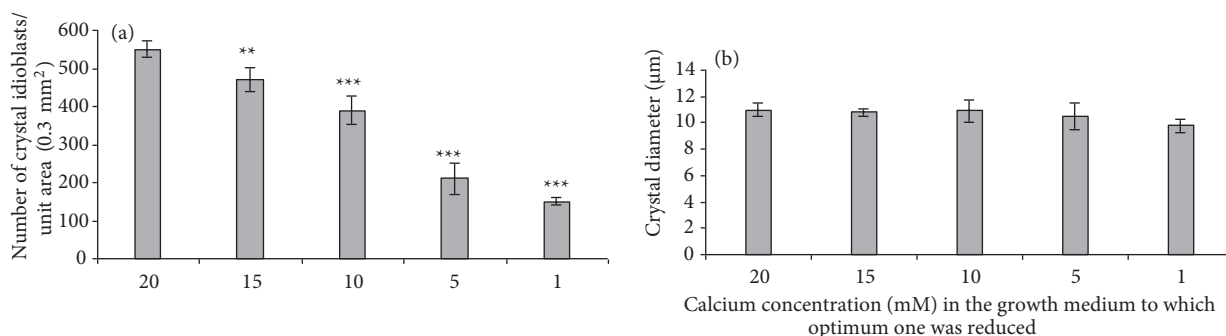


Figure 10. Effect of calcium decrease from the optimum concentration (20 mM) to lower concentrations (15, 10, 5, and 1 mM) in the growth medium on the number of crystal idioblasts (a) and crystal diameter (b) in leaf tissues of *M. parviflora*. In this experiment, the same plants that were grown on the media with optimum calcium concentration (20 mM) were transferred to new media with lower calcium concentration. Data are the means \pm SD of 10 repetitions. Asterisks indicate significant differences between the treatments and the control: *significant at $P < 0.05$; **significant at $P < 0.01$; ***significant at $P < 0.001$.

3.3. Heavy metal accumulation in plant leaf tissues

Results in this regard revealed that, overall, accumulation of Cd, Pb, Cu, and Zn in leaf tissues of the 2 plant species tested in this study significantly exceeded the levels of these metals present in untreated plants. The content of each of these metals in leaf tissues progressively increased with the gradual elevation in concentrations of these metals in growth medium. This increase was highly significant ($P < 0.001$) in the case of Cd and Pb in the 2 plants, while the increase in Cu and Zn was not significant in *M. parviflora*. Therefore, the levels of metal concentrations in the leaf tissues were dependent on plant species and type of metal (Figures 11, 12).

3.4. Effects of heavy metals on crystal abundance and size

In these experiments, plants were treated with various metal concentrations while growth media contained the optimum Ca concentrations suitable for crystal formation for each plant species. Generally speaking, the presence of the 4 heavy metals tested in this study led to reductions in crystal formation in leaf tissues of the 2 plant species used. The effect was obvious on crystal number as well as on crystal size. The extent of this effect was dependent on plant species, type of metal, and concentration. For example, while the effect of each of these metals was obvious on both crystal number and size in the case of *C. olitorius*, it was only

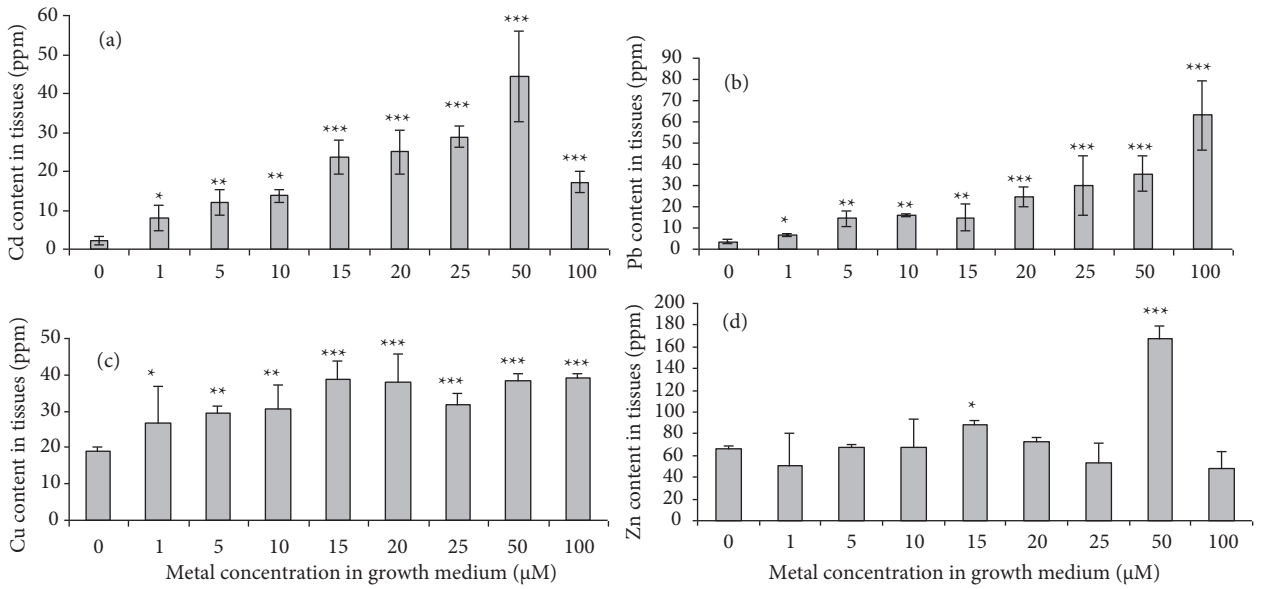


Figure 11. (a) Cd, (b) Pb, (c) Cu, and (d) Zn in the leaf tissues of *C. olitorius* plants after 15 days of exposure to different concentrations (0 (control), 1, 5, 10, 15, 20, 25, 50, and 100 μM) of each of these heavy metals in the growth media. Data are the means ± SD of 10 repetitions. Asterisks indicate significant differences between the treatments and the control: *significant at P < 0.05; **significant at P < 0.01; ***significant at P < 0.001.

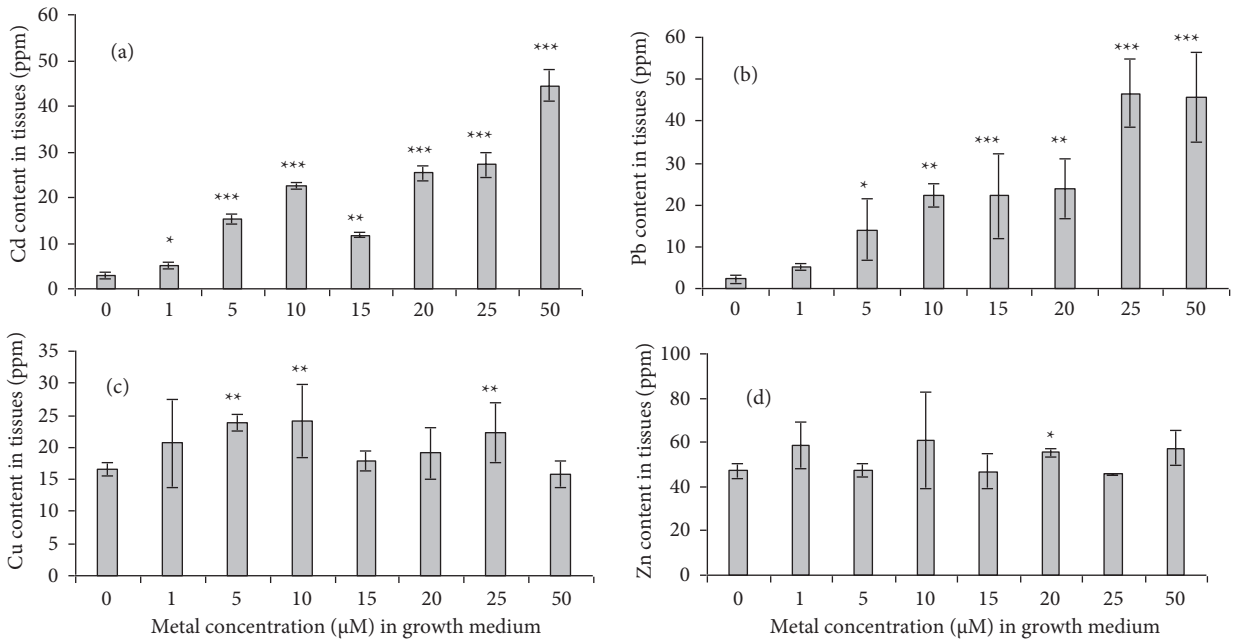


Figure 12. Contents of (a) Cd, (b) Pb, (c) Cu, and (d) Zn in the leaf tissues of *M. parviflora* plants after 15 days of exposure to different concentrations (0 (control), 1, 5, 10, 15, 20, 25, and 50 μM) of each of these heavy metals in the growth media. Data are the means ± SD of 10 repetitions. Asterisks indicate significant differences between the treatments and the control: *significant at P < 0.05; **significant at P < 0.01; ***significant at P < 0.001.

evident on crystal number in *M. parviflora*, while there was no effect on crystal size in this plant. In the case of Cd and Pb, the reduction was highly significant (P < 0.001) in crystal number with gradual increases in the concentrations of both metals in both plants. However,

the reduction in crystal size was highly significant (P < 0.001) in all concentrations of 2 metals in *C. olitorius* but it was highly significant only in higher concentrations in *M. parviflora* (Figures 13–16). In the case of Cu, the reduction in crystal number was highly significant in

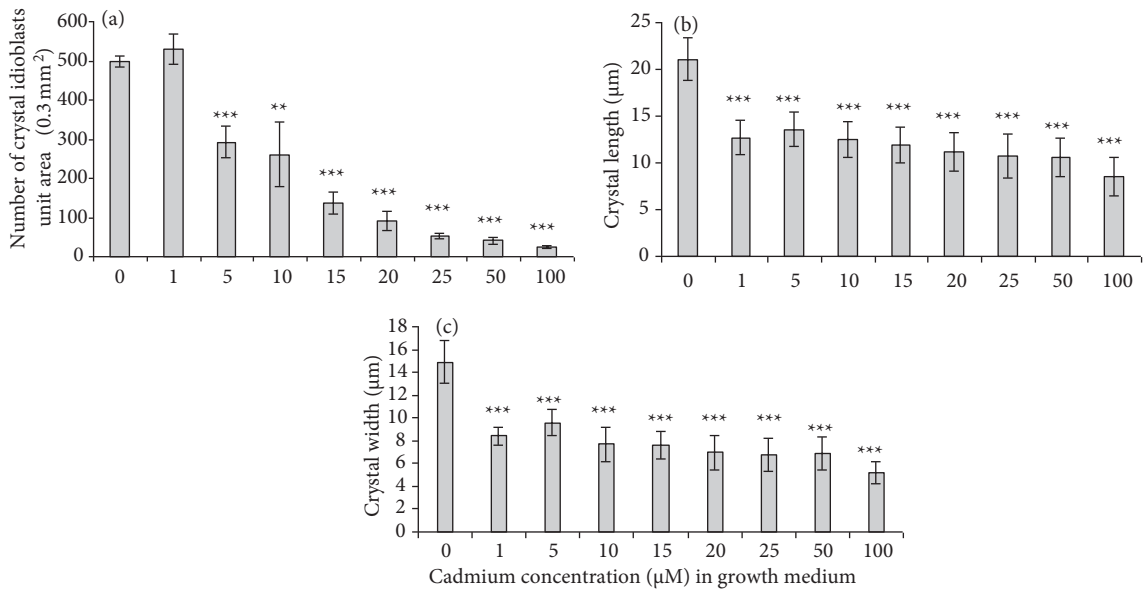


Figure 13. Effect of cadmium concentration (0.0 (control), 1.0, 5.0, 10.0, 15.0, 20.0, 25.0, 50.0, and 100 µM) in the growth media on the number of crystal idioblasts (a), crystal length (b) and crystal width (c) in leaf tissues of *C. olitorius*. Data are the means ± SD of 10 repetitions. Asterisks indicate significant differences between the treatments and the control: *significant at P < 0.05; **significant at P < 0.01; ***significant at P < 0.001.

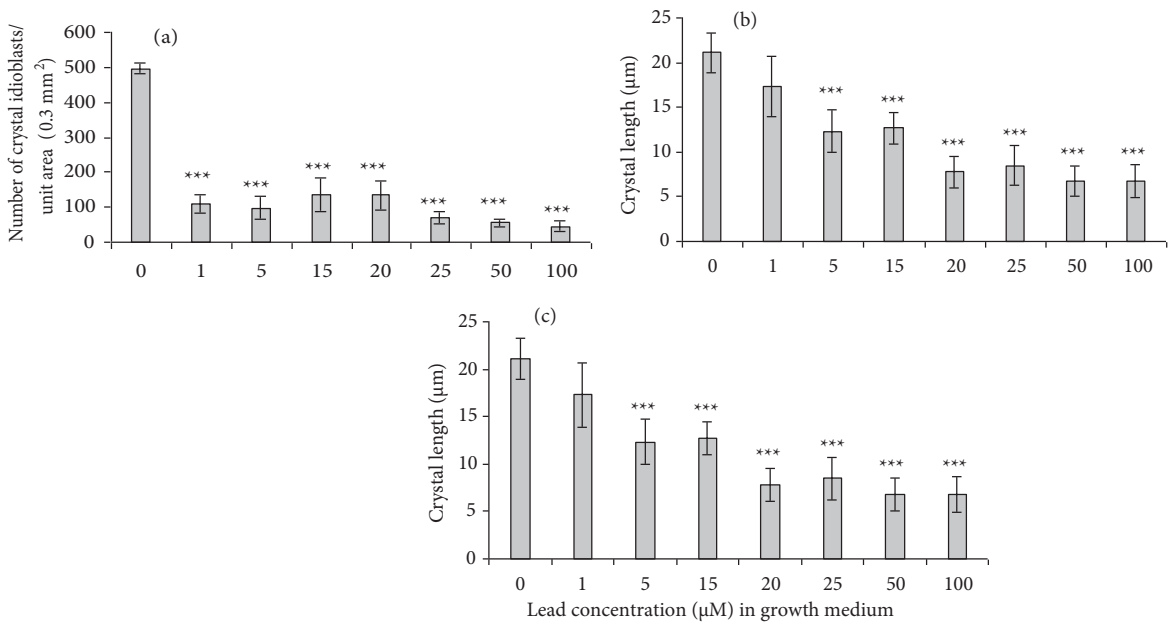


Figure 14. Effect of lead concentration (0.0 (control), 1.0, 5.0, 10.0, 15.0, 20.0, 25.0, 50.0, and 100 µM) in the growth media on the number of crystal idioblasts (a), crystal length (b), and crystal width (c) in leaf tissues of *C. olitorius*. Data are the means ± SD of 10 repetitions. Asterisks indicate significant differences between the treatments and the control: *significant at P < 0.05; **significant at P < 0.01; ***significant at P < 0.001.

both plants at all its concentrations except at the level of 20 mM concentration in *M. parviflora* as there was a significant (P < 0.01) increase in crystal number at this level. With regard to its effect on crystal size, the reduction in crystal size was highly significant in all its concentrations in *C. olitorius* but in *M. parviflora* there was no effect (Figures 17, 18). In the case of Zn, the

reduction in crystal number with a gradual increase in its concentration was highly significant in both plants but more obvious in *M. parviflora*. With regard to its effect on crystal size, there was a highly significant reduction in crystal size in both plants. However, the reduction in crystal size was significant only at 15 and 20 µM levels (P < 0.05) in *M. parviflora* (Figures 19, 20).

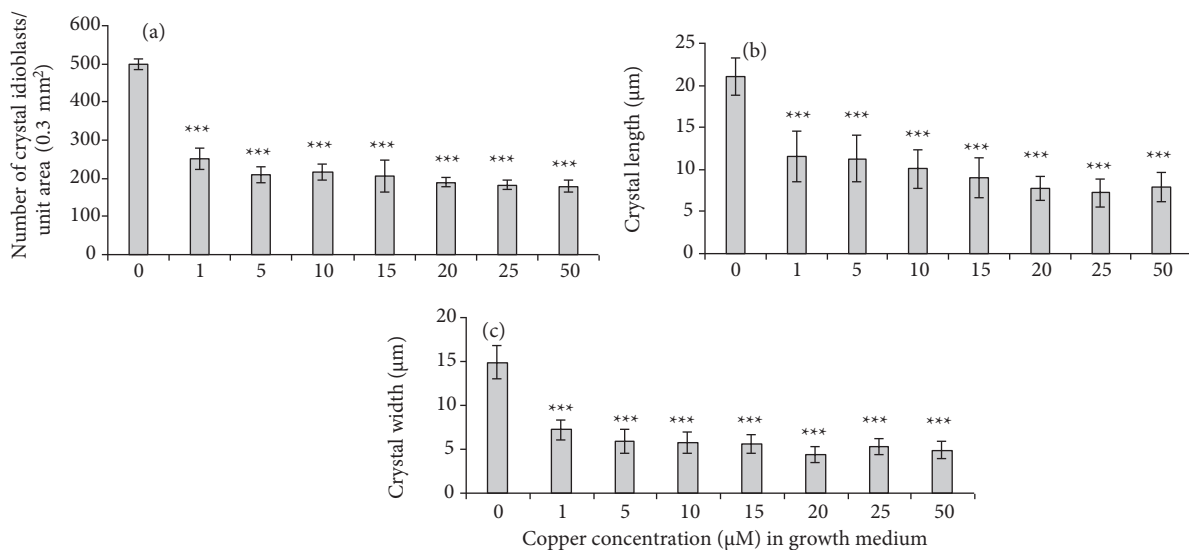


Figure 15. Effect of copper concentration (0.0 (control), 1.0, 5.0, 10.0, 15.0, 20.0, 25.0, 50.0, and 100 µM) in the growth media on the number of crystal idioblasts (a), crystal length (b), and crystal width (c) of leaf tissues of *C. olitorius*. Data are the means ± SD of 10 repetitions. Asterisks indicate significant differences between the treatments and the control: *significant at $P < 0.05$; **significant at $P < 0.01$; ***significant at $P < 0.001$.

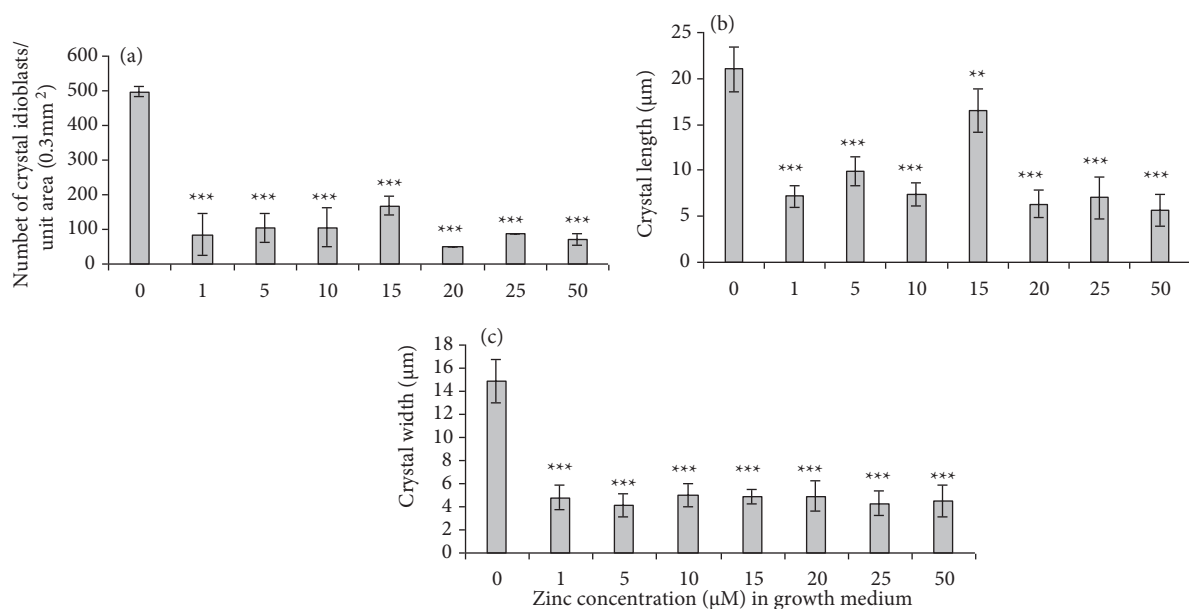


Figure 16. Effect of zinc concentration (0.0 (control), 1.0, 5.0, 10.0, 15.0, 20.0, 25.0, 50.0, and 100 µM) in the growth media on the number of crystal idioblasts (a), crystal length (b), and crystal width (c) in leaf tissues of *C. olitorius*. Data are the means ± SD of 10 repetitions. Asterisks indicate significant differences between the treatments and the control: *significant at $P < 0.05$; **significant at $P < 0.01$; ***significant at $P < 0.001$.

4. Discussion

Calcium oxalate (CaOx) crystals are found at all taxonomic levels in photosynthetic organisms, from small algae to higher plants (Franceschi & Nakata, 2005; Meric, 2009). The crystals are formed from endogenously synthesised oxalic acid and Ca taken from the environment, and they are produced and accumulated in species-specific morphologies.

Results using light, scanning, and transmission electron microscopic examination of CaOx crystal formation in the 2 plants revealed the presence of crystalline deposits. These deposits were of prismatic type in *C. olitorius* and of druse type in *M. parviflora* (Figures 1, 2). Regarding the distribution of crystal idioblasts in leaf tissues, they were located essentially around veins in *C. olitorius* (Figure 3) and *M. parviflora* (Figure 4). Thus, the constancy of crystal

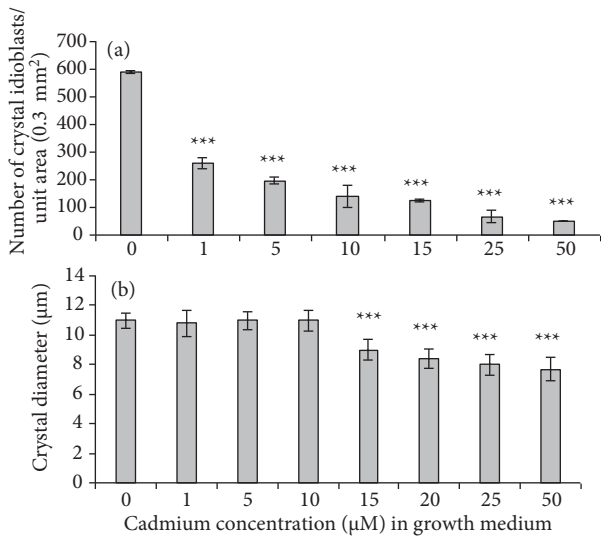


Figure 17. Effect of cadmium concentration (0.0 (control), 1.0, 5.0, 10.0, 15.0, 20.0, 25.0, and 50.0 μM) in the growth media on the number of crystal idioblasts (a) and crystal diameter (b) in leaf tissues of *M. parviflora*. Data are the means \pm SD of 10 repetitions. Asterisks indicate significant differences between the treatments and the control: *significant at $P < 0.05$; **significant at $P < 0.01$; ***significant at $P < 0.001$.

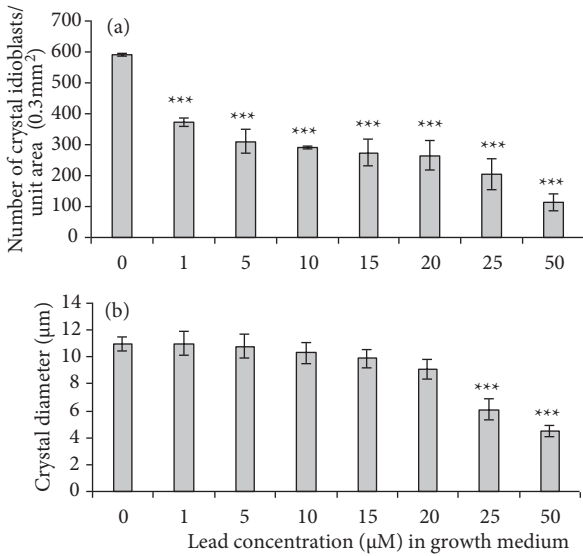


Figure 18. Effect of lead concentration (0.0 (control), 1.0, 5.0, 10.0, 15.0, 20.0, 25.0, and 50.0 μM) in the growth media on the number of crystal idioblasts (a) and crystal diameter (b) in leaf tissues of *M. parviflora*. Data are the means \pm SD of 10 repetitions. Asterisks indicate significant differences between the treatments and the control: *significant at $P < 0.05$; **significant at $P < 0.01$; ***significant at $P < 0.001$.

type and distribution may be considered a taxonomic character for classification of species (Franceschi & Nakata, 2005; He et al., 2012). The crystal pattern is also

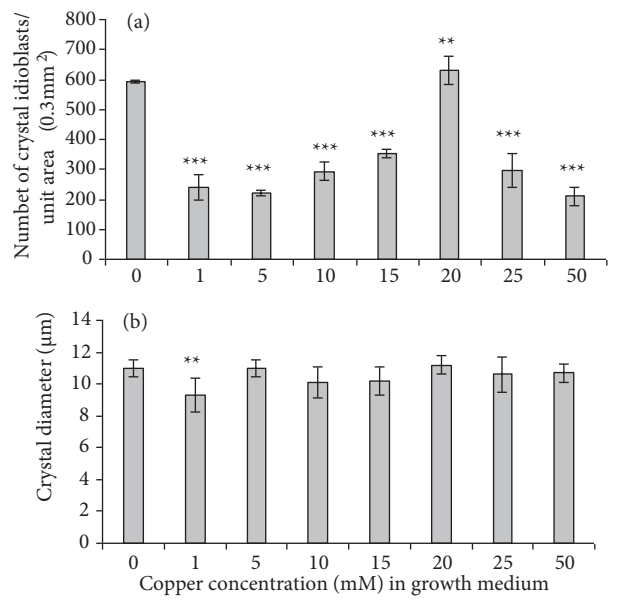


Figure 19. Effect of copper concentration (0.0 (control), 1.0, 5.0, 10.0, 15.0, 20.0, 25.0 and 50.0 μM) in the growth media on the number of crystal idioblasts (a) and crystal diameter (b) in leaf tissues of *M. parviflora*. Data are the means \pm SD of 10 repetitions. Asterisks indicate significant differences between the treatments and the control: *significant at $P < 0.05$; **significant at $P < 0.01$; ***significant at $P < 0.001$.

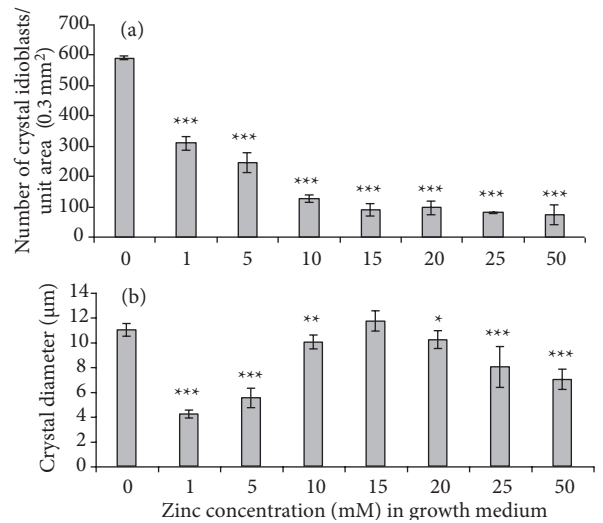


Figure 20. Effect of zinc concentration (0.0 (control), 1.0, 5.0, 10.0, 15.0, 20.0, 25.0, and 50.0 μM) in the growth media on the number of crystal idioblasts (a) and crystal diameter (b) in leaf tissues of *M. parviflora*. Data are the means \pm SD of 10 repetitions. Asterisks indicate significant differences between the treatments and the control: *significant at $P < 0.05$; **significant at $P < 0.01$; ***significant at $P < 0.001$.

often stable within a genus (Lersten & Horner, 2000). Ultrastructurally, some differences were observed between crystal idioblast and noncrystal cells (Figures 5, 6). An

enlargement of the nucleus and nucleolus is one of the first changes observed in developing crystal idioblasts (Horner & Whitmoyer, 1972; Rakovan et al., 1973). The cytoplasm of a young idioblast is often very dense and rich in organelles and vesicles (Rakovan et al., 1973). Plastids are often smaller than those in surrounding cells and have few grana (Horner & Whitmoyer, 1972). Invaginations of the plasmalemma, which produce structures analogous to the plasmalemmasomes, were described (Horner & Whitmoyer, 1972). The overall appearance of the young crystal idioblasts suggests a highly active cell. At maturity, most crystal idioblasts are probably still living cells. A thin layer of cytoplasm can usually still be seen around the periphery of the cell.

In the present study, there was a positive and dynamic relationship between crystal formation by the 2 studied plant species and calcium concentration in their growth media (Figures 7–10). This is in agreement with previous findings in other plants (Borchert, 1985, 1986; Juregui-Zuniga et al., 2005).

Studies in support of this hypothesis have shown, using a variety of plants, that the size and number of CaOx crystals are responsive to changes in the concentration of calcium in the plant environment (Webb, 1999). Several reports (Ilarslan et al., 2001; Pennisi & McConnell, 2001; Zindler-Frank et al., 2001; Monje & Baran, 2002; Morrow & Dute, 2002; Volk et al., 2002) support a role for CaOx formation in calcium regulation. Two of these reports examined whether crystals of different morphologies had varying sensitivities to fluctuations in calcium concentrations (Volk et al., 2002). Using the raphide and druse accumulating aquatic plant *Pistia stratiotes*, Volk et al. (2002) proposed that druse and raphide crystal formation serve different but related functions. The authors presented evidence indicating that raphide (needle-shaped) crystal formation may serve a dual function of calcium regulation and plant defence, while druse (globular) crystal formation is strictly involved in calcium regulation (Volk et al., 2002). Druse crystal formation was found to be dynamic and responsive to fluctuations in calcium levels. When calcium levels were

high, druse crystal size and number rapidly increased. When calcium was limited, druse crystal size and number decreased, presumably freeing up the calcium for utilisation by the plant. The disappearance of crystals in tissues under conditions of calcium deficiency and active growth has been reported in a number of plants (Arnott & Pautard, 1970; Webb, 1999; Mazen et al., 2003).

In our study, there was progressive accumulation of the heavy metals used in the leaf tissues of the plants studied (Figures 11, 12). This is in agreement with Bishehkolaei et al. (2011), who observed the accumulation of chromium in the cortical root cells of *Ocimum basilicum*. Moreover, addition of heavy metals to the nutrient medium decreased the number of crystals in the plants studied (Figures 13–20). This is not surprising because the toxicity of each of these metals interferes with the biological mechanisms mediating crystal formation. It is well known that heavy metals toxicate protein synthesis and function. This is in agreement with the study by Juregui-Zuniga et al. (2005), who reported that addition of heavy metals to the nutrient medium decreased the number of crystals in the leaves of *Phaseolus vulgaris*.

5. Conclusion

The data in this work indicated that ultrastructural analyses have demonstrated that this biomineralisation process is not a simple random physical–chemical precipitation of endogenously synthesised oxalic acid and environmentally derived Ca. Instead, crystals are formed in specific shapes and sizes. Furthermore, it was found that there was a positive and dynamic relationship between crystal formation by the 2 plant species studied and calcium concentration in their growth media, thus suggesting its biological role in calcium regulation. Moreover, addition of heavy metals to the nutrient medium decreased the number of crystals. Energy dispersive X-ray spectrometry did not detect the inclusion of heavy metals inside the CaOx crystals. Therefore, our study suggests that CaOx crystals do not play a major role in heavy metal detoxification in *C. olitorius* and *M. parviflora* but do play an important role in bulk calcium regulation.

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