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Original Pepar

Induction of biomarkers associated with cadmium detoxification in aquatic species

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Abstract

The aquatic species *Ceratophyllum demersum* and *Myriophyllum spicatum* were grown in different Cd concentrations (0, 25, 50, 75 mg/l) in a hydroponic system to analyze their detoxification capacity and the suitability of pigments and proline content to serve as biomarkers. Both studied species exhibited the same increasing pattern of Cd removal, when they treated with different Cd concentrations. *M. spicatum* exhibited a higher accumulation capacity than *C. demersum being* 1.5, 1.26 and 1.19 fold at the end of the experiment with the cadmium concentration of 25, 50 and 75 mg/l, respectively. Significant differences in pigment and proline contents between the treated and control samples indicated that Cd stress induced oxidative stress response in the studied species. Carotenoides and proline contents showed their partially increasing, especially during the short duration of Cd exposure. Chlorophylls a, -b exhibited the sever effects of Cd concentration on their contents. These responses reflected the suitability of the tested parameters to use them as biomarkers for heavy metal stress.

Keywords: Metals - Proline – Pigment – Phytoremediation – Hydrophytes

Introduction

Aquatic plants are well known for their potential to accumulate the heavy metals and they hold a prime position in food chain as primary producers, regulators of oxygen level. Aquatic plants play a significant role in biogeochemical cycling of toxic trace elements and are being increasingly considered for environmental phyto-management (Sinha *et al.,* 2002; Rai *et al.,* 2004; Mishra *et al.,* 2006). Aquatic macrophytes take up metals from the water, producing an internal concentration several folds greater than their surroundings. Many of the aquatic macrophytes are found to be the potential scavengers of heavy metals from water and wetlands (El-Khatib, 1991; El-Khatib and El-Sawaf, 1998; Ali and Soltan, 1999; Manal *et al.,* 2011). Aquatic plants can be used as indicators of low-level environmental contamination that might otherwise be difficult to detect (Mazej and Germ, 2009). In aquatic ecosystem, cadmium is an important widespread trace pollutant, having large solubility in water thus poses greater risk to aquatic ecosystem, with high toxicity to plants, animals and humans (Toppi and Gabbrielli, 1999). Cadmium is released into the environment by power stations, heating systems, metal-working industries, nickel-cadmium batteries and phosphate fertilizers (Mishra *et al.,* 2006), as well as from geochemical weathering of rocks. Despite its non-essentiality, cadmium is readily taken up by plants and can induce a number of physiological changes and phytotoxic symptoms including, chlorosis, growth inhibition, water imbalance, the inhibition of photosynthesis, phosphorus and nitrogen deficiency, reduced manganese transport and accelerated senescence (Mishra *et al.,* 2006; Ding *et al.,* 2007). It can enter chloroplasts and disturb chloroplast function by inhibiting the enzymatic activities of chlorophyll biosynthesis, pigment–protein complexes, O2-evolving reactions of photosystem II, electron flow around photosystem I and chloroplast structure (Siedlecka *et al.,*1997).Under Environmental stress such as heavy metal pollution, plants have shown proline accumulation (Ashraf and Foolad, 2007; Ahmad *et al.,* 2008). It has been often suggested that proline accumulation may contribute to osmotic adjustment at the cellular level and enzyme protection

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stabilizes the structure of macromolecules and organelles. Proline, as a compatible solute is demonstrated to play role in maintenance of (a) cellular osmoticum; (b) NADPH/NAD (P+) ratio; and (c) cytosolic pH, besides helping in detoxification of free radicals/toxic oxygen species (in particular singlet oxygen and hydroxyl radicals) (Alia and Saradhi, 1991; Alia *et al.,* 1995).

Aquatic plant species used in the present study are *Ceratophyllum demersum* L. (Coontail or hornwort) and *Myriophyllum spicatum* L. They are known to be a good accumulator of Cd (Gupta *et al,* 1996; Rai *et al.,* 1995; Arvind and Prasad, 2005; Mishra *et al.,* 2006), and could be used in ecological surveys as in-situ biomonitors of water quality due to its ability to concentrate pollutants in their tissues and reflect the environmental pollution (Nimptsch *et al.,* 2005). Accordingly, the aims of the present study was to:1)- compare the cadmium removal capacity of the two studied species; 2)- assess the using of pigment and proline contents of the two studied species as biomarkers reflecting the cadmium phytotoxicity.

Material and methods

Plant material, growth conditions and cadmium treatments

The studied species were *C. demersum* (family Ceratophyllaceae) and *M. spicatum* that taken from the main stream of the River Nile bank. *C.demersum* and *M. spicatum* (Haloragidaceae), and exposed to cadmium by growing the plants in hydroponic cultures in controlled environmental conditions. Before the experiment setup, whole plants of *Ceratophyllum* or *Myriophyllum* have thoroughly cleaned under running tap water to remove debris and other foreign particles, and then rinsed in bidistilled water. The plants transported to hydroponic culture and kept for 2 weeks in Hoagland nutrient solution to acclimatize, before adding the contaminants (Cowgill *et al.,* 1989). Healthy acclimatized plants (100 g fresh weight each) left to grow in nutrient solution in the holding tank assigned for the different treatments. They divided in four sets, The first one acts as a control, while in the others the hydroponic solution is enriched with Cd $(NO_3)2$ (Sigma, St. Louis, MO) up to 25,50, and 75 mg/l. Treated and control plants are sampled after 1, 3, 5 and 7 days for the experiment.

Cadmium analysis

Water samples of 15 ml for each, were collected from all compartments at 1, 3, 5, and 7 days intervals for cadmium analyses. Atomic absorption spectrophotometer (AAS) Model 210 VGP Buck Scientific used for Cd analyses. Cadmium concentration values expressed as mg / L (ppm).Plant samples (5 g each) were collected at the start, at 3 days, 5 days and at the end of the experiment (7-day) and analyzed for the presence of cadmium. Harvested plants washed in distilled water, and then dried in a convection oven for 24 h at 48 ^oC. After drying, the plants grounded to a fine powder. For analysis, dry plant material digested according to the wet digestion procedure involving concentrated nitric acid (Campbell and Plank, 1998). Ten ml concentrated nitric acid (HNO_3) and 0.5 ml hydrofluoric acid (HF) added to 0.5 g of dry plant sample in a closed Teflon vessel, designed for the purpose at a temperature of 130 ^oC for 24h. Digestion in solution continues until clear. The resultant liquid diluted up to 25 ml with distilled water then stored for analysis. Then, the Cd content was determined by the same Atomic absorption spectrophotometric method.

Photosynthetic pigments and proline content

The photosynthetic pigments, chlorophyll a (Chl a), chlorophyll b (Chl b) and carotenoids were determined according to (Lichtenthaler, 1987). The absorbance of pigment extract measured at wavelength of 452.5, 644 and 663 nm with spectrophotometer (Unico® 1200 spectrophotometer). The concentrations of pigments (*chl*.a, *chl*.b, and cartotenoids) calculated on a fresh weight basis and expressed as mg/g FW. (Bates *et al*., 1973). method used to determine proline content in the tissues of the studied species. Proline separated by addition of toluene and quantified by a spectrophotometer method using Unico® 1200 spectrophotometer, at 520nm. Proline content expressed as mg/g dry weight (DW).

Statistical analysis

Controls and treatments were performed in triplicate. Data were tested for statistical significance using the software of SPSS15.0 for Windows, followed by the MANOVA test for comparison of means of the tested parameters. The difference was considered significant at the $P < 0.01$ level. In addition, regression and correlation coefficients for the tested parameters were computed.

Results

Figure (1a) shows the increasing trend of Cd removal by the studied species with the increasing of time in all the experimental sets. Concerning *C. demersum*, the amount of Cd remains in the nutrient solution reached to 30.8%, 20.7% and 37.5% at the end of the experiment for initial Cd treatments of 25, 50 and 75 mg/l, respectively. Meanwhile, in the case of *M.* spicatum, the situation was 13.2%, 12.86% and 11.1% for initial Cd treatments of 25, 50 and 75 mg/l, respectively. Figure (1b) shows the removal efficiency of Cd by the studied species. It is noticeable, that *M. spicatum* attained the highest Cd removal efficiency in all the experimental sets, with all initial treatments 25, 50, 75 mg/l. It attained the highest removal efficiency 88.67 % at the highest treatment 75 mg/l by the end of the experiment. On the other side, the removal efficiency of *C. demersum* varied between 18.02% (1-day) and 79.14 % (7- day), when initial concentration of 50 mg/l Cd was used. Figure (1c) shows cadmium uptake *by C. demersum* and *M. spicatum*. At the experiment setup, the initial concentration of Cd in the tissues of both species was 0.017 mg/g DW for *C. demersum* and 0.016 mg/g DW for *M. spicatum.* Both species exhibited the same pattern accumulation, when they treated with different Cd concentratio the accumulation capacity of the two studied species exhibited significant differences (P<0.01) when different treatments were used. Higher accumulation values of 82.70 and 69.22 mg/g DW) were attained by *M. spicatum* and *C. demersum,* respectively, at 7 d, when Cd initial concentration of 75 mg/l was applied.

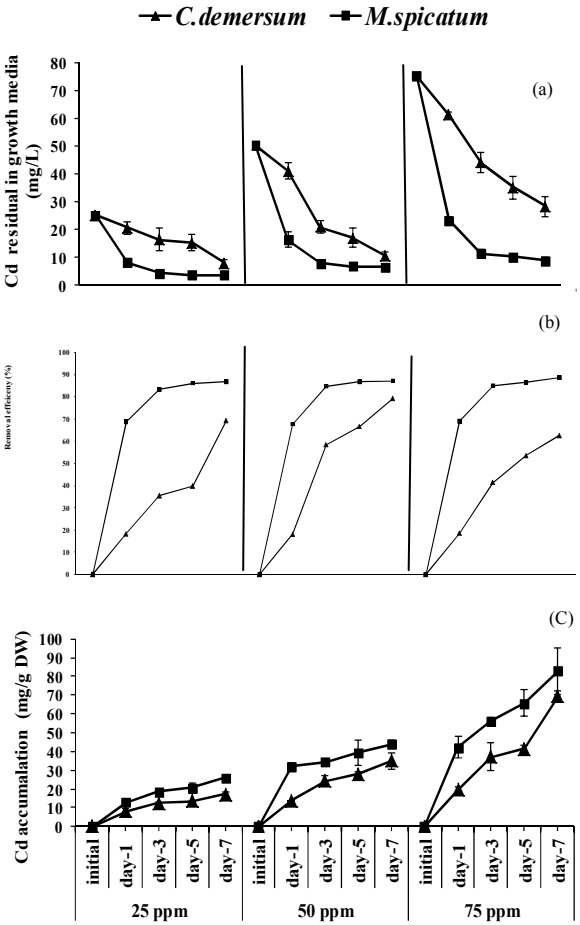

Figure. (1): Cadmium residual in the growth media (a), Removal efficiency of cadmium (b) and Cd accumulation by *C. demersum* and *M. spicatum* (c) with different Cd treatments during the experiment duration

Figure (2a-c) illustrates the changes in the photosynthetic pigments (chl a, chl b, and Carotenoid) in the leaves of the studied species due to cadmium detoxification. Chlorophyll concentrations in the studied species were drastically decreased with increasing Cd concentrations in the nutrient medium (Figure 2a-b). Compared to the control, the chlorophyll concentrations in the studied species were significantly different (P<0.01). Upon cadmium exposure, chlorophyll a and b content in the two studied species exhibited similar response with a gradual declining trend at all Cd concentrations. The maximum decline noticed after 7 d in the two tested species. Increase of Cd concentration in the nutrient media expressed significant effect (P< 0.01) on the production of chlorophylls in the studied species, where at higher Cd concentration (75 mg/l), chlorophyll a content reached its minimum values, being 36.18 mg/g FW (*C.*

demersum) and 34.6 mg/g FW (*M. spicatum*), at the end of experiment. The same is true for chlorophyll b, where minimum values of 27.05 mg/g FW (*C. demersum*) and 31.80 mg/g FW (*M. spicatum*) were recorded. Carotenoids contents of both two species showed contrast behavior to the chlorophyll content during the short duration of the experiment at the different medium concentration, except those of *C. dermersum* at 75 mg/l, where they showed a decreasing trend as those of chlorophylls (Fig 2c). After 7 d of the experiment setup, the carotenoids contents of the two species decreased to reach its minimum values of 14.40 mg/g FW (*C. demersum*) and 23.60 mg/g FW (*M. spicatum*). The two species exhibited maximum values of carotenoids contents at initial Cd concentration of 25 mg/l, after 3 d, being 76.90 mg/g FW (*C. demersum*) and 102.97 mg/g FW (*M. spicatum*).

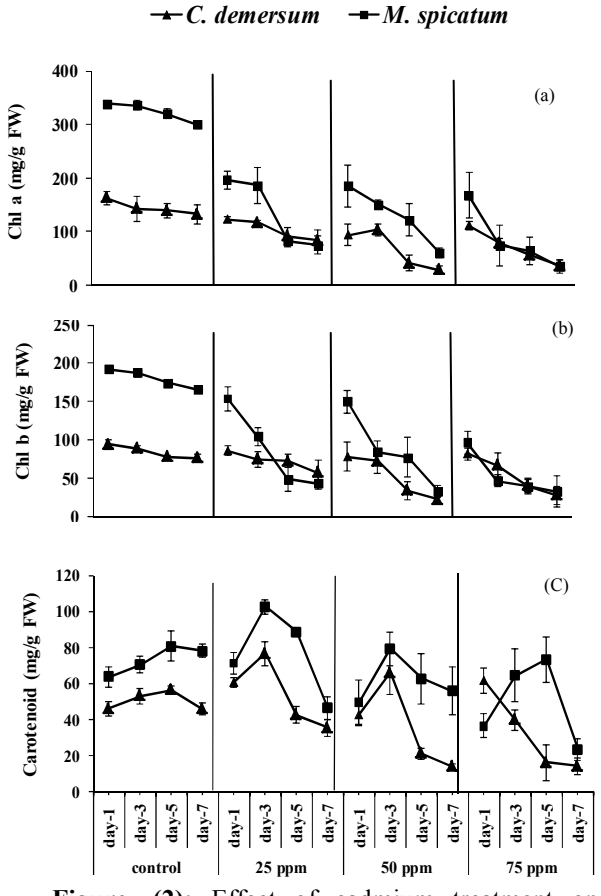

Figure. (2): Effect of cadmium treatment on photosynthetic pigments, chlorophyll a (a),chlorophyll b (b), and carotenoid (c) of *C.demersum* and *M.spicatum*.

The stress indicator amino acid proline content increased with the increase in the concentration of cadmium in the nutrient medium until initial Cd concentration of 50 mg/L (Figure 3). Compared to the control plants, the content of proline in the two studied species growing at 25 mg/L continued to increase significantly (P<0.01) until the end of experiment, where the proline content of *M. spicatum* was higher than those of *C. demersum.* At 50 mg/L, proline contents of the two studied species exhibited decline trend reaching its minimum values of 1.26 and 2.79 mg/g DW, after 7 d, in *C. demersum* and *M. spicatum,* respectively. At 75 mg/L, the two species exhibited their minimum values of 1.34 mg/g DW (*C. demersum*) and 3.11 mg/g DW (*M. spicatum*), at 7 d experiment.

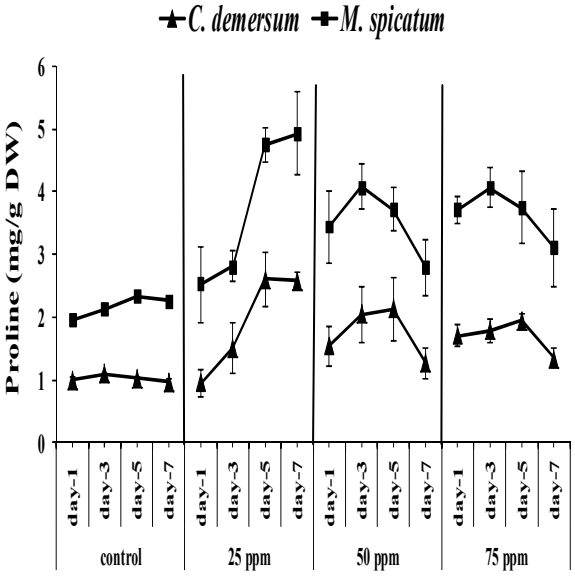

Figure. (3): Proline content by *C. demersum* and *M. spicatum* growing in growth media with different Cd treatments during the experiment duration.

Discussion

It is clear that the residual of Cd in growth media was greater in *C. demersum* and consequently, the highest removal of Cd recorded in *M. spicatum* under the three experiment treatments. The variation in the Cd concentrations indicated a highly significant difference (P< 0.001) between the two studied species. The regression coefficient (R^2=18.2 %) denoted the pronounced impact between the uptake and increasing Cd concentration in the medium, where the two studied species accumulated high amount of Cd in a dose dependent manner. Although, cadmium accumulation observed higher in *M. spicatum* than *C.*

demersum, a general increase in Cd accumulation in a given species occurs with an increasing Cd concentration in the nutrient media. The accumulation of Cd was the lowest at the 1 d treatment and the highest at the 7 d. All above-mentioned findings reveal that a mass balance should be given between cadmium accumulation in a species and cadmium removal from aqueous treatment solution. High significant difference in accumulation of Cd ($P< 0.01$) was recorded between two studied species. In their study, (Cardwell *et al.,* 2002). stated that the extent of metal accumulation within aquatic macrophytes is variable and depends on the type of plant species.

Generally, the two studied species exhibited high Cd accumulation capacity. This may be due to the two studied species are fully submerged, floating plants with narrow leaves and no root shoot partitioning. This feature provides large surface area for metal removal, which results in high accumulation. In comparison, *M. spicatum* expressed a higher accumulation capacity than *C. demersum* being 1.5, 1.26 and 1.19 fold on the end of the experiment at cadmium concentration of 25, 50 and 75 mg/L, respectively. Consequently, high concent-ration factor [metal content in plant tissue (82.71mg/g DW)/metal content in growth solution (75mg /L)] for *M. spicatum* reflects its high accumulation potential, which is an essential factor for phytoremediation (Andra *et al.,* 2010).

Cd stress causes many physiological changes in growing plants. Decrease in the chlorophylls is the primary bioindicator of Cd phytotoxicity. In comparison with those of control, chl a, and b content of the treated species showed decline trends, especially at higher Cd concentration, where after 7d, a drastic reduction in chls content was observed at cadmium concentration of 75 mg/L in the nutrient solution. In the present study, the effects of Cd treatments were significantly varied ($P <0.01$) from those of control. Both chlorophyll a and b contents in the two studied species exhibited similar response, with a gradual decline trend, at all exposure concentrations and durations. As reported in many studied (Macfarlane and Burchett, 2001; Sinha *et al.,* 2005; Mishra *et al.,* 2006; Srivastava *et al.,* 2006; Mishra and Tripathi, 2008). the content of photosynthetic pigments, including chl- Cd stress causes many physiological changes in growing plants. Decrease in the chlorophylls is the primary bioindicator of Cd phytotoxicity. In comparison with those of control, chl a, and b content of the treated species showed decline trends, especially at higher Cd concentration, where after 7d, a drastic reduction in chls content was observed at cadmium concentration of 75 mg/L in the nutrient solution. In the present study, the effects of Cd treatments were significantly varied ($P <0.01$) from those of control. Both chlorophyll a and b contents in the two studied species exhibited similar response, with a gradual decline trend, at all exposure concentrations and durations. As reported in many studied (Macfarlane and Burchett, 2001; Sinha *et al*., 2005; Mishra *et al*., 2006; Srivastava *et al*., 2006; Mishra and Tripathi, 2008), the content of photosynthetic pigments, including chl-a, b in their tested species reduced due to exposure to heavy metals including Cd. The mechanisms of this reduction documented as inhibition of enzymes involved in chlorophyll biosyn-thesis (Chandra and Kulshreshtha, 2004; Baryla *et al*., 2001; Benavides *et al*., 2005; Srivastava *et al*., 2006). impaired uptake of essential elements such as Mn and Fe (Somashekaraiah *et al*., 1992). and reduction in chloroplast density and size (Baryla *et al*., 2001; Benavides *et al*., 2005). In the present study, carotenoids content of both studied species showed contrast behavior to the chlorophyll content, during the short duration of the experiment. Carotenoids content of *C. demersum* and *M. spicatum* showed the highest values after 3 d at Cd concentration of 25 mg/L. While, the minimum value recorded by the end of the experiment at Cd concentration of 75 mg/L. The increase in the carotenoid level may be due to the ability of the plant to counteract the toxic effect of free radicals generated under stress (Toppi and Gabbrielli, 1999). The recorded decline in carotenoides contents with increase in metal concentrations reported as a common occurrence observed in several aquatic plants with other metals (Rai *et al.,* 1995; Sinha *et al.,* 2002). Generally, the computed negative correlation coefficient (-0.326) proved the inverse relationship between metal concen-tration and carotenoides content, especially

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Accumulations of Cd in the tissues of studied species were accompanied by concomitant induction in the levels of antioxidants (proline). Proline accumulation is not only regarded as an indicator of environmental stress but also considered as an important protective role against heavy metal stress (Sharma *et al.,* 1998). In the present study, the proline level increased by two and three-fold over that of control in *C. demersum* and *M. spicatum* in response to different concentrations of cadmium. These results are in agreement with those of Dinakar *et al*. (2008). who reported that Cd treated plant showed a significant increase in proline as compared to the control samples. Most heavy metals cause oxidative stress via generation of ROS (Dietz *et al*. 1999). It has been proposed that proline act as a free radical scavenger to protect plants away from damage by oxidative stress (Alia and Matysik. 2001). In addition, (Smain *et al*., 2009). reported that, cadmium, copper, and zinc induced an increase of proline contents in *L. gibba* and concluded that, the amplitude of proline changes was very important and could be a very good indicator of toxicity. It has been demonstrated that free proline could chelate with Cd ion in plants and form a nontoxic Cd proline complex (Sharma *et al.,* 1998). Accordingly, the increase in proline levels in the present study with increasing Cd concentration might consider as an indicator on a correlation between ROS generation (hydroxyl radicals mostly) and ROS scavenging by proline. This is stem up from the positive value (0.216) of correlation coefficient, which added an evident for this relationship.

Although a significant variation ($P< 0.01$) in accumulation value of proline was recorded between the two studied species, the two species often display the same pattern. The maximum proline accumulation was recorded at 25mg/l Cd (4.93 mg/g DW) in *M. spicatum* after 7 d. Generaly, *M. spicatum* had more proline content than *C. demersum*. This suggests that the hyperaccumulator, *M. spicatum* has stronger self-protection ability than *C. demersum*. The time–response curves displayed the same pattern with an increase followed by a decrease in proline contents, where, proline accumulation decreased after 3 d, at initial Cd concentration 50 and 75 mg/L. This is in agreement with the results of (Smain Megateli *et al*., 2009). in their study stated that the kinetics of the response showed a maximum proline accumulation after 4 days, which was followed by a decrease of the contents back to values close to those of the control after 7 d.

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تحفيز المؤشرات الحيوية المرتبطة بإزالة سمية عنصر الكادميوم فى النباتات المائية

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الملخص العربي

تم زراعة نوعين من النباتات المائية هما نبات السيراتوفيلم (*Ceratophyllum demersum*) و نبات الميروفيلم (*Myriophyllum spicatum*) عند تركيزات مختلفة من عنصر الكادميوم (صفر، ٢٥، ٥٠، ٧٥ ملجم/لتر) بنظام الزراعة المائية لاختبار قدرتهما على ازالة السمية الناتجة عن تواجد الكادميوم و كذلك ملائمة استخدام محتواهما من كل من الاصباغ و حمض البرولين كمؤشرات حيوية على التلوث. اظهر كلا النوعين نفس النمط المتزايد من ازالة الكادميوم من الوسط الغذائى، تحت تأثير المعالجات المختلفة. اظهر نبات الميروفيلم قدرة تراكم لعنصر الكادميوم اعلى من نبات السيراتوفيلم بواقع ١،٥، ١،٢٦ و كذلك ١،١٩ ضعف عند استخدام تركيز من الكادميوم ٢٥، ٥٠، و٧٥ ملجم/لتر، على التوالى. اظهرت نتائج الدراسة الاختلافات المعنوية بين محتوى النباتات المعالجة و نباتات الضابطة فى محتواهما من كل من الاصباغ و حمض البرولين موضحة استجابة الانواع تحت الدراسة الى حالة الاجهاد الناتجة عن معالجة النباتات بعنصر الكادميوم. اظهر محتوى نباتات الدراسة من الكاروتينات و حمض البرولين تزايدا جزئيا، بخاصة فى الفترة القصيرة من المعالجة، بينما اظهرمحتوى الكلوروفيل أ، ب الاثر الشديد لعنصر الكادميوم، مما يعكس مدى الملائمة لاستخدامها كمؤشرات حيوية للدلالة على حالة التلوث الناتجة عن العناصر الثقيلة.