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Effects of Copper on Accumulation, Antioxidant Activity and MDA Content in *Lemna minor*, *Lemna gibba* and *Spirodela polyrrhiza* (L.)

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ABSTRACT

The effects of different concentrations of copper on the accumulation, antioxidant enzyme activity, malondialdehyde (MDA), chlorophyll and carotenoid content of *Lemna minor*, *Lemna gibba* and *Spirodela polyrrhiza* L. were investigated. Duckweeds were treated with several concentrations of Cu (0, 10, 25, 50, and 100 μM Cu) for 4 days. According to results, Cu ions were accumulated mainly in *L. minor*, *L. gibba* and *S. polyrrhiza*. The MDA content and proline amount increased with increasing external Cu concentration in duckweeds. However the level of photosynthetic pigments decreased with increasing Cu concentration. Superoxide dismutase and catalase antioxidative enzyme activity increased significantly in plants exposed to 25-50 μM Cu compared to other treatments. These results show that *L. minor*, *L. gibba* and *S. polyrrhiza* can tolerate up to 25 μM Cu treatment.

Keywords: copper, duckweed, accumulation

Lemna minor, *Lemna gibba* and *Spirodela polyrrhiza* (L.)'da Akümülasyon, Antioksidan Enzim Aktivitesi ve MDA içeriğine Bakırın Etkileri

ÖZET

Lemna minor, *Lemna gibba* ve *Spirodela polyrrhiza* L. bitkilerinde akümülasyon, antioksidan enzim aktivitesi, MDA miktarı, klorofil ve karoteniod içeriği üzerine farklı konsantrasyonlardaki bakırın etkileri incelenmiştir. Su mercimeklerine dört gün boyunca farklı konsatrasyonlarda bakır uygulanmıştır. Elde edilen sonuçlara göre; bakır iyonları *L. minor*, *L. gibba* ve *S. polyrrhiza* oldukça fazla miktarda biriktiği belirlenmiştir. MDA içeriği ve prolin miktarı su mercimeklerinde artan dış bakır konsantrasyonunun artışıyla birlikte yükselmektedir. Fakat bununla birlikte fotosentez pigment derecesi artan bakır konsantrasyonuyla düşüş göstermiştir. Bitkilerin 25-50 μM Cu uygulaması diğer uygulamalarla karşılaştırıldığında süperoksit dismutaz ve katalaz enzim aktivitesi önemli bir oranda artış göstermiştir. Bu sonuçlar bize *L. minor*, *L. gibba* ve *S. polyrrhiza* bitkilerinin 25-50 μM Cu uygulamasını tolere edebileceğini ifade etmektedir.

Anahtar kelimeler: bakır, su mercimeği, akümülasyon

1. Introduction

Increase in environmental contamination caused by heavy metals is existing a important problem in the worldwide. Heavy metals are easily carried and amassed in aquatic ecosystems as dissolved and solid waste from domestic, industrial, and agricultural runoffs. They also accumulate in organisms. Thus, the accumulation of heavy metals in plants induces physiological and

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biochemical changes (Dhir et al., 2004), (Pavlíková et al., 2007). Modifications of the various organism was used greatly to evaluate the toxicity of these contaminants. Copper is an essential micronutrient for plants, as it is directly involved in numerous essential metabolic processes, including respiration, photosynthesis, protein synthesis, cell wall metabolism and lignification, ethylene sensing, and oxidative stress protection (Festa and Thiele, 2011). Indeed, these properties make copper ions indispensable for plant growth and development. However, copper can be toxic for plants when it is present at even slightly higher than optimal level (Martins and Mourato, 2006), (Reichman et al., 2006). High concentrations of Cu have a cytotoxic influence through the production of reactive oxygen species (ROS) containing hydroxyl radicals and hydrogen peroxide by Haber–Weiss reaction (Hegedus et al., 2001), (Mittler et al., 2004). To reduce functional and structural harm, the plants have improved different mechanisms against adverse effects (Teisseire and Guy, 2000). Amongst them, antioxidative enzymes and antioxidative compounds are quite efficient to scavenge ROS in plants. Aquatic plants could be used in phytoremediation (the exciting low-cost and eco-friendly technology) to reduce organic matter or remove metallic pollutants from water (Srivastava et al., 2006), (Eapen et al., 2007). To detect environmental pollution by aquatic plants as indicators is a credible and easy alternative to the customary sampling method (Zurayk et al., 2001). Due to Lemnacea potential accumulation capability of pollutants they admitted much attention by scientists (Zayed et al., 1998), (Qian et al., 1999). The genetic variety of diverse duckweed populations, the easy culture in laboratory situations and the fast herbal reproduction cycle are significant specifications that make the duckweed species pertinent models for ecotoxicological studies. Thus, duckweeds have been commonly used in toxicity tests of dissimilar chemicals and waste water (Pomati et al., 2004), (Razinger et al., 2007).

Previous several studies in duckweed reported that an extreme of copper obstructs enzyme activity, respiration, pigment synthesis and photosynthesis (Frankart et al., 2002), (Babu et al., 2003).

In this paper we studied (i) alterations in contents chlorophyll, Cu accumulation and the lipid peroxidation (MDA) of duckweed species, (ii) changes in the contents of antioxidant enzymes such as, catalase (CAT) and superoxide dismutase (SOD) in duckweed species. Hereby this can determine the concentration range which of duckweed species are appropriate for remediation of water body polluted by heavy metals.

2. Material and Methods

2.1. Plant Material, Growth Conditions and Metal Estimation

Fresh samples of *L. gibba*, *L. minor* and *S. polyrrhiza* (Lemnaceae) were collected from ponds in the Soysallı National Park, Kayseri, Turkey. Then plants were growned into plastic cups covered with containing

Hoagland's solution and grown for 7 d in the growth chamber (115 μmol m−2s−1 light with 16 h photoperiod, temperature 25 ± 2 °C) prior to the experiment (Aravind and Prasad, 2005). Heavy metal treatment was started on the 8th day and continued for four days, by applying Hoagland's solution containing 0, 10, 25, 50, and 100 μM Cu was prepared using copper sulfate. Seven daily plants, which were equal size as morphological (3 ± 0.2 mm caliber), were selected for the experiment. The total number for each species was 1200, for each treatment there were 1200/6 = 200 plants. The solutions were changed in every 24 h. The solution pH was maintained by titration to 5.8 ± 0.1 with NaOH or HCl solutions (0.1 M) when required. After 4 days, the plants were harvested and either used directly for analysis or frozen in liquid nitrogen followed by storage at -86 °C until further use.

Dried samples of plant were digested with 10 mL of concentrated HNO3, using a Millestone Start microwave digestion system (Demirezen Yılmaz and Uruc Parlak, 2011) . Determination of the Zinc contents in all samples was carried out by Perkin Elmer A Analist 400 (AAS). The samples were analysed in triplicate for Cu.

2.2. Determination of Pigment Concentration

Photosynthetic pigments of plants (100 mg) were extracted in 10mL of chilled acetone solution in the dark and after centrifugation at 4000×g for 10 min. The chlorophyll content was estimated by the method of Arnon (Arnon, 1949).

2.3. Estimation of lipid peroxidation

Lipid peroxidation was estimated by determining the malondialdehyde (MDA) content in according to Carreras and Pignata (Carreras and Pignata, 2001).

2.4. Antioxidative Enzymes

2.4.1. Enzyme Extraction

To acquire the enzyme extract 200–250 mg of fronds was homogenized in 2 ml of cold potassium phosphate buffer (0.1 M, pH 7.0). The homogenate was centrifuged at 15.000 g for 15 min at 4 °C (Hou et al., 2007). The supernatant was kept for enzyme determination.

2.4.2. Superoxide Dismutase

SOD activity was assayed by the method of Nishikimi and friends (Nishikimi et al., 1972). SOD activity was assayed by its skill to restrict the photochemical decrease of Nitro BlueTetrazolium (NBT). One unit (U) matches to the quantity of enzyme that inhibits diminution of NBT by 50% at 25±2 °C. A system free of enzyme presented as control.

2.4.3. Catalase

The CAT activity was assayed using a method adapted from Aebi (Aebi, 1984). The reaction mixture contained 50 mM of potassium phosphate buffer (pH 7), 15 mM H2O2 and a suitable aliquot of enzyme in the final volume of 1 ml. Decrease in the absorbance at 240 nm was measured.

2.5. Proline Analysis

Proline analysis was performed using the methodology described by Bates and friends (Bates et al., 1973). Concentrations of proline in the plant tissue were expressed on a FW basis.

2.6. Statistical Analysis

All values are expressed as mean(s) ± standard deviation (SD). Data was subjected to a one-way analysis of variance (ANOVA) to approve the changeability of data and validity of results. Turkey test was carried out to detect the important diverses among treatments. In the figures, the values are signed with different letters for the significance level ($P \leq 0.05$) as in proportion to the control.

3. Results and Discussion

3.1. Copper Content

Copper is vital in low concentrations for plant development and metabolism and plays key roles in the control of genes, oxygen transport, membrane structure and enzyme activities (Li and Xiong, 2004), (Rana, 2008). However, as copper is redundant, it turns out to be toxic and obstructs growth. According to the previous studies toxic metal concentrations mainly impede cell elongation and expansion (Srivastava et al., 2006), (Singh et al., 2012).In study, *L. minor*, *L. gibba* and S. *polyrrhiza* were grown in hydroponic culture in the presence of increasing Cu concentrations to assess their Cu uptake, growth and identify possible protection mechanisms. Growth of plants was not influenced by the presence of Cu in solution within an exposure time of 4 days for concentrations up to 100 µM (Table 1). Data showed that as the Cu concentrations of solution raised from 10 to 100 µM, the Cu concentration in plant tissues of both varieties also increased remarkably. S. polyrrhiza was chosen for further studies to get better Cu accumulation. The results presented here indicate that superfluous copper was toxic to duckweeds.

Table 1. Copper concentration in *L. minor, L. gibba* and *S. polyrrhiza* upon Cu exposure. Values represent mean ± S.E. (n = 3). Different letters indicate significant differences at $P < 0.05$.

Cu concentration [µM]	**Metal uptake by *L. minor* (mgkg $^{-1}$ DW)**	**Metal uptake by *L. gibba* (mgkg $^{-1}$ DW)**	**Metal uptake by *S. polyrrhiza* (mgkg $^{-1}$ DW)**
Control	0,12±0,00a	0,11±0,00^{a}	0,02±0,00^{a}
10	0,69±0,04a	9,93±0,07^{b}	15,38±0,36^{b}
25	5,44±0,32b	14,42±0,10^{c}	18,30±0,03^{b}
50	9,97±0,55c	15,53±0,68^{cd}	18,46±0,60^{b}
100	13,92±0,06d	17,03±0,56^{d}	18,91±0,87^{b}

3.2. Effects of Cu on Photosynthetic Pigments

After 7 days of treatment concentration of chlorophyll and carotenoid under Cu stress reduced importantly (Table 2). Saygideger and Dogan (2004), Rozentsvet and friends (2012), Parlak and Yilmaz have been reported alike pattern (Saygideger and Dogan, 2004), (Rozentsvet et al., 2012), (Uruc Parlak and Demirezen Yilmaz, 2012).

Table 2. The effect of Cu exposure on photosynthetic pigments contents in *L. minor, L. gibba* and *S. polyrrhiza*. Values represent mean ± S.E. (n = 3). Different letters indicate significant differences at $P < 0.05$.

***Concentration* [µM]**	***Total Chl content (mg g⁻¹ FW)***			***Carotenoid content (µmol g⁻¹ FW)***		
	L. minor	***L. gibba***	***S. polyrrhiza***	***L. minor***	***L. gibba***	***S. polyrrhiza***
Control	0,559±0,025 [a]	0,156±0,002[a]	0,348±0,007[a]	0,177±0,031 [a]	0,060±0,008 [a]	0,021±0,001 [d]
10	0,707±0,015 [ab]	0,187±0,001[ab]	0,348±0,002 [ab]	0,212±0,018 [a]	0,066±0,008 [a]	0,073±0,001 [a]
25	0,847±0,004 [b]	0,201±0,002[b]	0,475±0,001 [ab]	0,176±0,011[a]	0,059±0,013 [a]	0,104±0,001 [c]
50	0,669±0,008 [ab]	0,228±0,003[b]	0,402±0,002[b]	0,146±0,012[a]	0,053±0,002[a]	0,112±0,002 [c]
100	0,665±0,012[ab]	0,216±0,000[a]	0,265±0,001 [ab]	0,144±0,010 [a]	0,050±0,003 [a]	0,090±0,001 [b]

Upadhyay and friends (2014) gets this result, which carotenoid content decreased in *P. pectinatus* as compared to control while an increased carotenoid content under Cr and Pb treatment showed its scavenging potential in *P. crispus* (Upadhyay et al., 2014). The accumulation of heavy metals in plant tissues is supposed to lead to alteration in the essential physiological events in plants (Rout and Das, 2003). The degradation of chlorophyll pigments may finally decline photosynthetic efficiency in plants which might be one of the strong causes of reduction in growth of plant (Upadhyay and Panda, 2005).

3.3. Effect of Cu on Lipid Peroxidation Content

Lipid peroxidation is believed to be the best measure of harm induced by multiplying reactive oxygen species production (Matewally et al., 2005). Cu treatment prompted lipid peroxidation suggesting oxidative damage in plant. Because of high ROS production, which attacks membrane lipid leading to ion leakage and membrane deterioration, rate of lipid peroxidation in Cu-treated plants could be high. The effect was thoroughly connected to dose. The rise in

MDA content frankly associated with high ROS production and growth inhibition, showing that Cu-induced ROS production is the main reason of lipid peroxidation.

Our results displayed that with the increasing copper concentration, the MDA concentration increased (Fig. 1), which is similar to the effect of heavy metals on other higher plants (Singh et al., 2006), (Sinha and Saxena, 2006). MDA increased in different species-concentration dependent manner. The maximum increase in MDA content was observed in *L. minor* at 25 µM, as compared to control. In the experiments, MDA concentration increased directly with increased Cu levels in the solution. Present study confirms the results of previous studies on aquatic plants (Sinha et al., 2003), (Mishra et al., 2006).

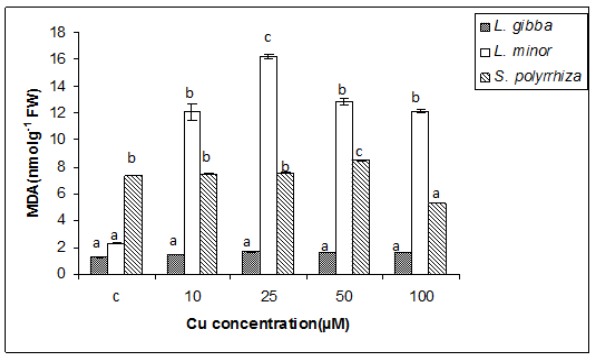

Figure 1. Effect of different concentrations of Cu on MDA content in *L. minor, L. gibba* and *S. polyrrhiza*. Values represent mean ± S.E. (n = 3). Different letters indicate significant differences at $P < 0.05$.

3.4. Response Of Antioxidant Enzymes to Cu Toxicity

As the plant is under environmental stresses, such as drought, metal exposure, and salt stres, the production of reactive oxygen species (ROS) rises, and so antioxidant enzymes are activated to scavenge more ROS and assist in detoxification (Uruc Parlak and Demirezen Yilmaz, 2012).

Superoxide radicals can be transformed rapidly to H2O2 by SOD (antoxidative enzyme defense system in plants). There have also been reports on alterations in the activities of antioxidant enzymes in response to metal stress (Rama Devi et al., 2003). Regulation of these enzymes is significant to keep the contents of superoxide and hydrogen peroxide under strict control. Superoxide dismutase, the first enzyme in the detoxifying process, has a substantial role in struggling oxidative stres in plants and turns superoxide radicals to H2O2 (Uruc Parlak and Demirezen Yilmaz, 2012), (Shalini and Dubey, 2003).

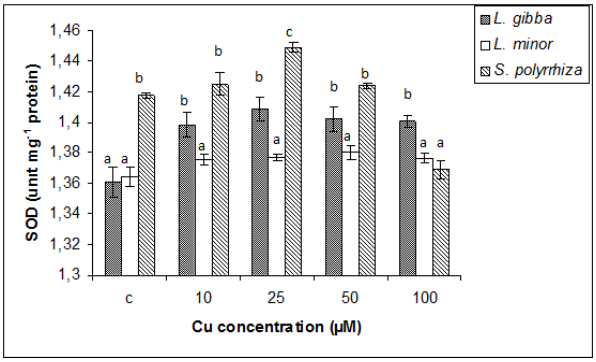

Figure 2. Effect of different concentrations of Cu on activities of SOD in *L. minor, L. gibba* and *S. polyrrhiza*. Values represent mean ± S.E. (n = 3). Different letters indicate significant differences at $P < 0.05$.

The increased SOD activity observed in the present study is consistent with studies in which other plant species treated with Cu (Upadhyay and Panda, 2009), (Xu et al.,

2011). In the experiment of SOD activity under lifted heavy metal stress was steadily stimulated with the increasing metal ion amount in medium (Fig. 2). Previous studies on various species informed such a raise in the activity of this enzyme after an exposure to Cu (Rama Devi and Prasad, 1998). Cu causes great inhibition of the activity of SOD enzyme. Similar results were reported in other sensitive varieties with lower SOD and higher Cu concentration in leaves of *Zea mays* (Tanyolac et al., 2007).

H2O2 is a preliminary to the highly-reactive hydroxyl radical and can be demolished by catalase (CAT) (Shalini and Dubey, 2003) . Although CAT activity multiplied with increasing copper levels in *L. gibba* and *S. polyrrhiza*, CAT activity decreased in *L. minor* (Fig. 3).

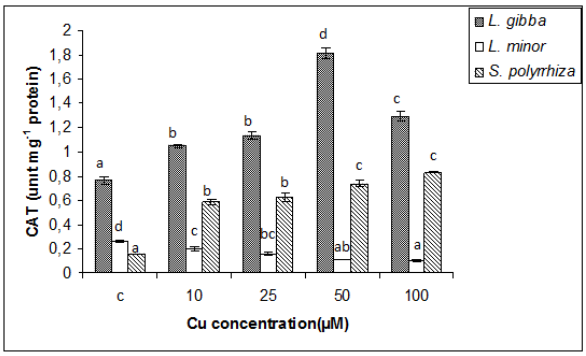

Figure 3. Cu farklı konsantrasyonlarının *L. minor L. gibba* ve *S. polyrrhiza'da* CAT aktivitesine etkisi. Ortalama değerler ± S. E. temsil (n = 3). Farklı harfler P <0.05 anlamlı farklılıklar göstermektedir. Effect of different concentrations of Cu on activities of CAT in *L. minor, L. gibba* and *S. polyrrhiza*. Values represent mean ± S.E. (*n* = 3). Different letters indicate significant differences at $P < 0.05$.

Nevertheless, the highest concentration of Cu (50 and 100 µM) demonstrated to be highly toxic in reducing the CAT activity. Our results showed that activities of CAT augmented in L. minor exposed to copper like the previous reports (Banu Doğanlar, 2013). Teisseire and Guy (2000) reported an increase of CAT activity in *L. minor* fronds subjected to a range of 0 to 0.64 mg/L of Cu (Teisseire and Guy, 2000). However, decreased CAT activity has also been reported in *L. minor* and *S. polyrrhiza* exposed to copper (Kanoun-Boule´ı et al., 2009). Their diversified results may be because of higher copper concentration (from 1.6 to 6.4 mg/L). CAT cleans the active oxygen species in plant cells. It attends in the main defensive system against accumulation and toxicity of hydrogen peroxide and can play the role in managing H2O2 grade in cells.

Increased SOD and CAT activities in *L. minor*, *L. gibba* and *S. polyrrhiza* show that these plants shave the enzymes capacity to adapt to Cu toxicity by developing an antioxidant defense system. Improvement of stress tolerance has been connected to an increase in the activity of antioxidant in many times.

3.5. Effect of Copper on Proline

To understand the contributions of non-enzymatic antioxidants in the *L. minor*, *L. gibba* and *S. polyrrhiza*, response to Cu toxicity, their proline contents were inspected. The amounts of proline in these plants grown under various concentrations of copper are shown in Table 3. The proline amount in control plants increased gradually with the duration of the experiment. Increased concentration of proline in plant

tissue specifies the protective role and tolerance against metals (Sinha and Saxena, 2006). In our study, Cu treatments increased the accumulation of proline. Increases in both proline and lipid peroxidation levels with increasing Cu concentration are determinative of a correlation between ROS generation and ROS scavenging by proline. Accumulation of proline in response to excess metal such as copper, cadmium, zinc, and nickel was defined in several plants (Matysik Alia et al., 2002). Present study confirms the results of previous studies on *P. pectinatus* and *P. crispus* plants (Upadhyay et al., 2014).

Table 3. Proline contents in *L. minor*, *L. gibba* and *S. polyrrhiza* upon Cu exposure. Values represent mean ± S.E. (n = 3). Different letters indicate significant differences at $P < 0.05$.

***Concentration* [µM]**	**Proline in *L. gibba* (µmol g $^{-1}$)**	**Proline in *L. minor* (µmol g $^{-1}$)**	**Proline in *S. polyrrhiza* (µmol g $^{-1}$)**
Control	0,030±0,012^{a}	0,010±0,001^{a}	0,014±0,000^{a}
10	0,038±0,005^{a}	0,019±0,002^{b}	0,023±0,008^{ab}
25	0,041±0,001^{a}	0,027±0,000^{c}	0,027±0,000^{ab}
50	0,044±0,000^{a}	0,031±0,001^{c}	0,036±0,000^{b}
100	0,050±0,000^{a}	0,042±0,000^{d}	0,055±0,000^{c}

4. Conclusion

After Cu exposure this showed that, the ROS accumulation was high and these enzymes have an important role in ROS detoxification. Considering these results, we powerfully propose that higher copper levels cause oxidative stress in *L. minor*, *L. gibba* and *S. polyrrhiza* cells and may cause membrane harm through production of ROS and interfere with chlorophyll metabolism. Hence, the data demonstrate that how *L. minor*, *L. gibba* and *S. polyrrhiza* can be used responds to its stressful environment. Among the antioxidative enzymes, SOD and CAT appear to play key roles in the plant's antioxidative defense mechanism under Cu toxicity. The ability of *L. minor*, *L. gibba* and *S. polyrrhiza* to both accumulate and tolerate moderate Cu level used in this study could be partly derived from ROS detoxification through an efficient antioxidant system. Interestingly, the use of *L. minor*, *L. gibba* and *S. polyrrhiza* could also be used for water stabilization in abandoned low-level Cu contaminated wetlands.

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