ISSN 1021-4437, Russian Journal of Plant Physiology, 2012, Vol. 59, No. 3, pp. 333–338. © Pleiades Publishing, Ltd., 2012.
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RESEARCH PAPERS

Antioxidant Activity in the Leaves of *Melilotus albus* and *Trifolium medium* from Man-Made Disturbed Habitats in the Middle Urals under the Influence of Copper

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Received April 1, 2011

Abstract—Physiological mechanisms of adaptation to copper-induced stress in two widespread legume plants, white sweet clover (*Melilotus albus* Merik.) and zigzag clover (*Trifolium medium* L.), growing in habitats differing in the man-made pollution. An antioxidant plant defense system was activated in response to 10 mM $CuSO_4$, which is a stress factor. Specific biochemical features related to adaptation to soil contamination with copper were observed in tested plant species. Superoxide dismutase was activated in response to stress in both species from various habitats. *M. albus* from the impact zone manifested the better capacity of proline accumulation as compared with plants from less polluted habitats. *T. medium* plants from the impact zone contained more active peroxidase. It was suggested that plants growing for a long time under stressful conditions manifest the greater tolerance to copper ions than plants, which did not experience stress or were subjected to the milder stress.

Keywords: Melilotus albus, Trifolium medium, heavy metals, copper, proline, superoxide dismutase, peroxidase, peroxidation of lipids.

DOI: 10.1134/S1021443712030065

INTRODUCTION

Anthropogenic influence is a mighty ecological factor affecting the plant kingdom. It frequently exceeds natural factors in the strength and diversity. Environment pollution with heavy metals is an extreme anthropogenic factor capable of plant organism damage.

Heavy metals are known to induce ROS generation and oxidative stress in the cells, protein denaturation, nucleic acid damage, and lipid peroxidation (POL). Both short-term and chronic action of heavy metals enhance POL. At present, it is known that POL activation at stress is not only the consequence of disturbed homeostasis but also a component of the adaptation process [1, 2].

Antioxidant enzymes and low-molecular antioxidants play an important role in the defense against the injurious action of free radicals and in plant adaptation to the action of man-made pollutants. Proline is a universal protector against stress in higher plants; it manifests diverse biological effects, obviously including antioxidant action [3]. A key enzyme protecting the cell against ROS is superoxide dismutase (SOD) converting superoxide anion-radical into hydrogen peroxide. According to available data [4–6], many factors elevating ROS concentration in the cell activate SOD. Peroxidase protects the cell against hydrogen peroxide produced as a result of superoxide reduction; peroxidase decomposes peroxide with the formation of molecular oxygen and water.

Copper is one of the main pollutants on the man-disturbed territories in the Middle Urals. At the low concentrations, copper is a micronutrient essential for plants. However, high copper concentrations are toxic; they activate free radical oxidation, resulting in the disturbance of the biological membrane structure and physiological and biochemical processes. It might be that plants inhabiting polluted territories have biochemical properties providing for their tolerance to technogenic factors, heavy metal in particular. It is known that legume plant tolerance to elevated concentrations of heavy metals in soil might be determined by the high content in their cells of specific proteins and other protecting compounds permitting partial neutralization of heavy metal toxicity [7–9]. This determined our choice of plant species for this study.

The objective of this work was to study separate components of the antioxidant defense system in the adaptation of wild plant species to copper excess in environment.

Abbreviations: POL—peroxidation of lipids; SOD—superoxide dismutase.

MATERIALS AND METHODS

Experiments were performed with perennial grasses from Fabaceae family: white sweet clover (*Melilotus albus* Medik.) and zigzag clover (*Trifolium medium* L.) [10], evrybiont species found in the gradient of pollution.

White sweet clover plants were taken from five habitats near Nizhny Tagil (Sverdlovsk oblast) differing in the degree of pollution. They are village Pokrovskoe, Ship Point, the III International village Rudnik, Alapaevsk branch-line, and Stroganov terrace. Zigzag clover grew only in three habitats: village Pokrovskoe, Ship Point, and Alapaevsk branch-line.

The total toxic load (S_i) on tested plots, as assessed by the content of heavy metals in soil, varied from 1.00 to 22.78 rel. units [11]. To calculate S_i in soil, the concentration of Cr, Mn, Fe, Co, Ni, Cu, Zn, Cd, and Pb were determined by the flame atomic absorption spectrometry with the AAS 300 spectrophotometer (Perkin Elmer, United States). The concentration of copper in plant leaves inhabiting natural biotops was measured by the same method after their ashing and subsequent extraction with 20% HNO_3 for 7 days.

Plant tolerance to copper in medium was assessed by POL severity, changes in the content of low-molecular antioxidant proline, and superoxide dismutase (SOD) and guaiacol peroxidase activities under normal and stressful conditions. Copper sulfate (10 mM) was chosen as a stressor.

Plant samples contained 10 plants of *M. albus* or *T. medium* harvested in each tested biotop in the period of mass flowering. Leaf samples were taken in the middle parts of shoots of plants at the middle-age stage of their development. A single typical leaf was taken from each plant, and 6 disks were cut with a borer. All leaf disks were firstly kept in the light in distilled water for 2 h to saturate them with water and preventing water deficit. Then half of the material (three disks) retained in distilled water (control), other three disks from each plant were transferred to the $CuSO_4$ solution for 2 h (experiment). Thus, experiments were performed in 10 replications (10 plants) with 6 recordings each. Water or $CuSO_4$ solution temperature was maintained at the level of 23° ± 1°C. The concentration of proline, activities of SOD and peroxidase, and POL intensity were determined in control and experimental plants.

The content of free proline in plants was determined by the method of Bates [12] modified by Kalinkina et al. [13] with the usage of the acid ninhydrin reagent. To this end, leaf sample (200 mg) was placed in the tube and poured with 10 mL of boiling distilled water; tubes were placed for 10 min in the boiling water bath. The prepared extract (1 mL), glacial acetic acid (1 mL), and ninhydrin reagent (1 mL) were introduced into the tube, which was placed in boiling water bath for 1 h and then cooled in ice. The color intensity was measured spectrophotometrically at 520 nm. The concentration of free proline was determined using the calibration curve.

POL intensity was determined after MDA accumulation with the usage of the reaction medium containing 0.25% thiobarbituric acid (TBA) in 10% TCA [14]. Plant material (300 mg of fresh leaves) was ground with the mortar and pestle in 1 ml of the reaction medium; the homogenate was placed in boiling water bath for 30 min. Then samples were cooled in cold water (10°C). The contents of samples was centrifuged at 10000 *g* for 10 min. Optical density (OD) was measured at 532 and 600 nm against the reaction medium. The MDA concentration was calculated after the formula:

$$\text{MDA (mmol/g fr wt)} = (OD_{532} - OD_{600})/(155 \times 0.3),$$

where 155/(mM cm) is the coefficient of TBA extinction, 0.3 is the weight of the plant material (g).

SOD activity was assayed by the method based on the measurement of inhibition of photochemical reduction of nitro blue tetrazolium [15]. Plant material was ground on ice in phosphate buffer (pH 7.8) with the addition of quartz sand. The homogenate was centrifuged at 15000 *g* for 20 min; SOD activity was assayed in the supernatant. The reaction medium prepared in 0.1 M phosphate buffer contained 12 mM L-methionine and 0.075 mM nitro blue tetrazolium. The reaction was started by the addition of 0.12 mM riboflavin. "Light control" and experimental sample were illuminated for 20 min, and "dark control" was kept in darkness. Reaction was run at 30°C and was stopped by placing tubes with experimental sample and "light control" in darkness. Optical density was measured at 560 nm against "dark control." SOD activity was calculated in arbitrary units after the formula:

$$\text{SOD activity} = \lg (D_c/D_0)/\lg 2 \times W,$$

where D_c is the optical density of light control sample, arb. units; D_0 is the optical density of experimental sample, arb. units; and W is the leaf fresh weight, g.

Peroxidase activity was assessed after the rate of guaiacol polymerization into tetraguaiacol, using slightly modified method of Chance and Maehly [14]. Plant material was ground on ice in 0.1 M phosphate buffer (pH 6.8–7.0) with the addition of quartz sand. The homogenate was centrifuged, and the supernatant was used as a source of enzyme. The reaction was started by the addition of 0.05 mL of the supernatant in the reaction medium containing 0.1 M phosphate buffer, 10 μM hydrogen peroxide, and 0.1% guaiacol. Reaction was run at 30°C. Peroxidase activity was assayed after the automatically recorded increase in the optical density during 2 min at 470 nm. In control sample, 0.05 mL of buffer was added instead of the supernatant. The data obtained were used for the calculation of enzyme activity. Peroxidase activity was calculated as μmoles of guaiacol/(g fr wt min) after the formula:

$$\text{Peroxidase activity} = (tg_0 - tg_c)V/26.6W,$$

Copper content in tested plot soils and in plants inhabiting them

Habitat	Zone	S_i, rel. units (after [11])	Copper content in soil, μg/g dry wt	Copper content in aboveground organs, μg/g dry wt (after [11]) M ± m
Village Pokrovskoe	background	1.00	12.26 ± 1.80	6.18 ± 0.46
Ship point	buffer	3.33	38.62 ± 0.59	9.72 ± 1.50
III International village Rudnik	buffer	6.19	101.57 ± 11.13	9.68 ± 1.28
Alapaevsk branch-line	buffer	8.36	78.64 ± 12.41	6.17 ± 0.56
Stroganov terrace	impact	22.78	951.49 ± 236.00	42.89 ± 10.50

where (tg_0 is the temporal change in the optical density of the experimental sample, tg_c is the temporal change in the optical density of the control sample, V is the total volume of the sample, 26.6 is the extinction coefficient of guaiacol, and W is the leaf fresh weight, g.

All spectrophotometric measurements were performed using a digital UV-VIS PD 303 UV spectrophotometer (APEL, Japan).

Statistical analysis of results was performed by routine methods of descriptive statistics with the determination of mean values (M), their errors (m), and standard deviations (S). Significance of differences was assessed using nonparametric criteria: U—criterion of Mann–Whitney and H—criterion of Kruskal–Wallis ($p \le 0.05$). Data were processed in Statistica 6.0 (StatSoft, 1984–2001). Bars in figures correspond to standard errors.

RESULTS

In correspondence with the total level of pollution in sites of sample harvesting, background, buffer, and impact zones were distinguished (table). It has been established in preliminary experiments that the main pollutant of these plots was copper [11]. The lowest copper content was characteristic of soil in the village Pokrovskoe (table). In the most heavily contaminated plot (Stroganov terrace), the concentration of copper exceeded by 78–130 times the background dose. At the same time, the content of copper in leaves of different plant species inhabiting tested areas varied to a lesser degree than in soil (table). Thus, in the leaves of white sweet clover from the buffer zones, the content of copper was 25.67 ± 3.97 μg/g dry wt and in leaves from impact zone, it was equal to 26.27 ± 4.15 μg/g dry wt.

To verify the suggestion that plants from polluted habitats are more tolerant to heavy metals than plants from the background zone, we subjected leaf fragments of these plants to short-term actin of 10 mM $CuSO_4$. Stress severity was assessed by POL intensity. In the leaves of *M. albus* and *T. medium* from all tested biotops, an increase in POL intensity as compared with control treatment was noted. In white sweet clover, this index was increased by 12–105% ($p < 0.05$) as compared with control, in zigzag clover – by 3–61% (Fig. 1). In plants from strongly contaminated plots, POL activation was less pronounced than in plants from relatively unpolluted biotops. Thus, the degree of stress-induced membrane damage was less in the leaves of plants from zones contaminated with copper than in the leaves from the background zone.

To understand the reasons of improved tolerance of plants from the impact and buffer zones to stressor action, activities of antioxidant enzymes, SOD and guaiacol peroxidase, and proline accumulation were determined. After 2-h treatment with Cu^{2+} of plants from the background zone, some decrease in proline concentration was noted: by 5–10% in *M. albus* and by 1–20% in *T. medium*. In plants from buffer and impact zones, the content of free proline increased under the influence of copper ions by 14–51% in *M. albus* and by 2–32% in *T. medium* ($p < 0.05$) (Fig. 2).

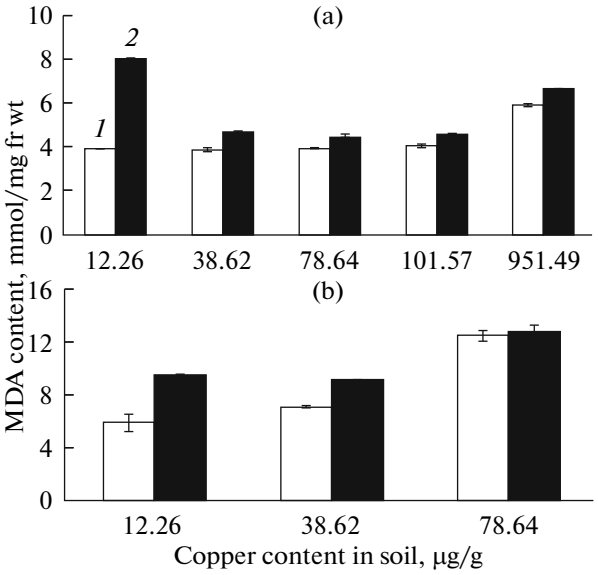

Fig. 1. POL activity in the leaves of *M. albus* (a) and *T. medium* (b) from habitats differing in man-made toxicity load under normal and stressful conditions.
(*1*) Control (H_2O); (*2*) experiment (10 mM $CuSO_4$).

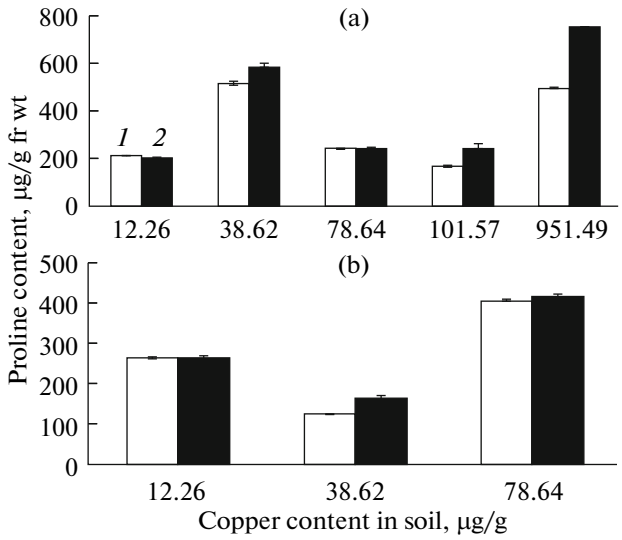

Fig. 2. Proline content in the leaves of *M. albus* (a) and *T. medium* (b) from habitats differing in man-made toxicity load under normal and stressful conditions.
(*1*) Control (H_2O); (*2*) experiment (10 mM $CuSO_4$).

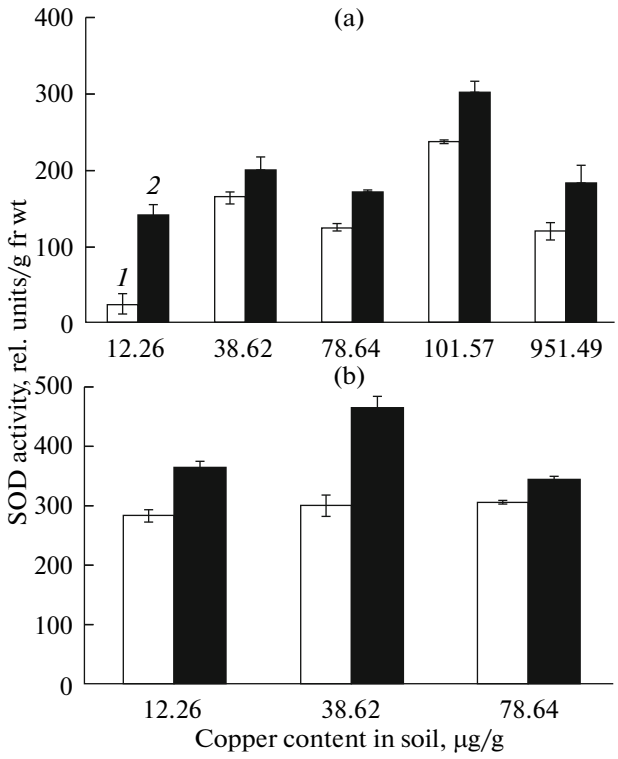

Fig. 3. Activity of superoxide dismutase in the leaves of *M. albus* (a) and *T. medium* (b) from habitats differing in man-made toxicity load under normal and stressful conditions.
(*1*) Control (H_2O); (*2*) experiment (10 mM $CuSO_4$).

SOD activity in the leaves of *M. albus* and *T. medium* from all tested plots was higher under stressful conditions than in control ($p < 0.05$) (Fig. 3).

In *T. medium* copper ion action on the leaves did not essentially change peroxidase activity, excepting plants from the buffer zone, where peroxidase activity decreased by 13% as compared with control (Fig. 4). In *M. albus* from the background zone and from one of the sites in the buffer zone (8.36 rel. units), peroxidase activity was decreased by 12 and 50%, respectively ($p < 0.05$). In other polluted territories, peroxidase activity was only slightly changed.

DISCUSSION

Such a transient metal as copper can catalyze generation of hydroxyl radicals from H_2O_2 and alcoxyl or peroxyl radicals from lipid peroxides, which creates the conditions of oxidative stress in the cells and induces POL [16, 17].

Observed accumulation of MDA in copper-treated plants from all zones (Fig. 1) indicates that copper induced oxidative stress, which resulted in membrane destruction and cell damage [18, 19]. POL enhancement differed in the degree in plants from habitats differing in the degree of pollution. In the leaves of both plant species (*M. albus* and *T. medium*) from impact and buffer zones, POL intensity changed less than in plants from the background zone. This can indicate the higher membrane stability and more efficient antioxidant system functioning under stress conditions in plants inhabiting on territories with copper excess in soil as compared with plants from uncontaminated plots.

The mechanisms of plant antioxidant defense under stress induced by heavy metals can include different components: low-molecular antioxidants, such as proline, ascorbate, anthocyanins, and others; and enzymes scavenging ROS (SOD, peroxidases, catalase) [6]. The balance between POL intensity and antioxidant system functioning is a necessary condition for the maintenance of normal cell life [20].

We demonstrated that components of antioxidant defense system functions in plant defense against copper-induced oxidative stress. However, the two tested legume species somewhat differed in the usage of different components during adaptation to plant treatment with copper ions. Thus, in *M. albus* plants from polluted habitats, the concentration of proline was increased more than by 1.5 times (Fig. 2); proline obviously was involved in ROS scavenging and prevented POL development under stress conditions. In *M. albus* plants from unpolluted or moderately polluted habitats, the content of proline changed insignificantly under the copper influence. It is likely that in these plants proline is not involved in the response to copper. In *T. medium* from background and impact zones, copper-induced stress did not essentially affect proline content, and in plants from the buffer zone, it increased slightly (1.2-fold).

Another important component of plant antioxidant system is the enzyme SOD. It might be supposed that the degree of plant tolerance to toxic copper concentrations depends on SOD activity as well because this enzyme scavenges superoxide-anion radicals appearing under stressful conditions.

Our experiments showed that basal SOD level (without copper treatment) in *M. albus* plants from the habitat uncontaminated with copper was much lower than in plants from buffer and impact zones, whereas SOD activities in clover taken from all tested biotops were similar. In experiments on plant treatment with copper ions, we observed changes in SOD activity in both *M. albus* and *T. medium* harvested in all tested habitats. It seems likely that SOD activation and thus a decrease in the superoxide anion-radical concentration are factors of rapid cell defense against oxidative stress when the system of proline biosynthesis is not still sufficiently active, and this is confirmed by research of other authors [5].

One more component of plant antioxidant system is peroxidase. This enzyme oxidizes hydrogen peroxide produced during SOD interaction with superoxide radical [20]. Since SOD activity and thus H_2O_2 content as a product of superoxide radical dismutation increased sharply in *M. albus* plants from background zone, activation of peroxidase is expected. However, peroxidase activity was not essentially changed and even decreased under copper action. It might be that stress-induced hydrogen peroxide excess is removed from the cell by another enzyme, catalase [6].

It is of importance that the basal level of peroxidase activity in *M. albus* was much higher (by 5–10 times) in plants from the background zone than from buffer and impact zones. Thus, in *M. albus* just the reaction of superoxide anion-radical dismutation catalyzed by SOD is a stress-induced reaction.

In contrast, in *T. medium* the basal level of peroxidase activity only slightly differed (not more than 1.5-fold) between plants from background and buffer habitats. In both plant species, stress primarily activated SOD. It might be that H_2O_2 accumulation in stressed cells is related to its signaling role [21, 22] providing for the rapid switching on plant defense mechanisms.

Thus, it is established that copper ions induce stress in plants, which is manifested in POL intensification. The POL intensity depends on the activity of antioxidant system, in particular, proline accumulation, the activation of SOD and guaiacol peroxidase. As judged from the increase in the proline content and SOD activity in response to copper action, *M. albus* plants from polluted biotops manifested a better capability of adaptation to chronic action of excessive amounts of heavy metals than plants from background and buffer zones. *T. medium* plants from polluted habitats had the higher basal level of peroxidase activity than plants from the background zone. It is likely that this allows them to survive under conditions of chronic excess of heavy metals in soil. Under laboratory conditions, stress induction by copper ions did not induce substantial changes in such components of the antioxidant system as proline and guaiacol peroxidase.

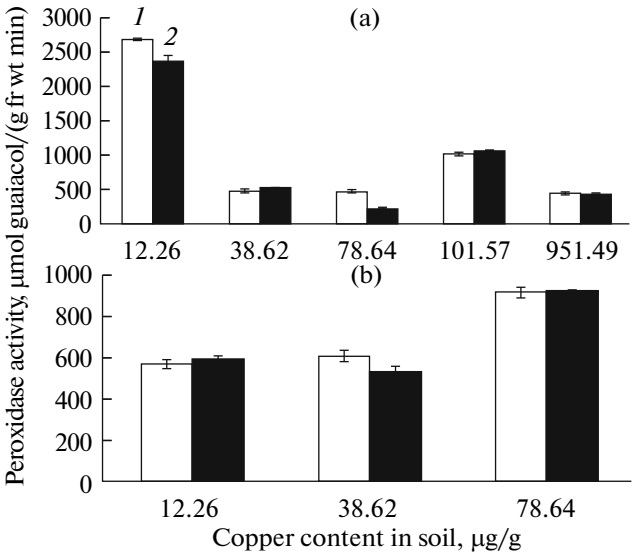

Fig. 4. Activity of peroxidase in the leaves of *M. albus* (a) and *T. medium* (b) from habitats differing in man-made toxicity load under normal and stressful conditions. (*1*) Control (H_2O); (*2*) experiment (10 mM $CuSO_4$).

Thus, plants of two legume species, *M. albus* and *T. medium*, possess the biochemical mechanisms of adaptation to copper excess in medium, which are manifested in SOD activation and, as a consequence, in reduced membrane injury, as evident from a decrease in POL intensity. Some specific features of adaptation to habitats contaminated with heavy metals, copper in particular, were also found in the two species. In the impact zone, *M. albus* plants could synthesize and accumulate more proline, whereas *T. medium* had more active peroxidase than plants from background zone. Thus, it might be that plants growing under long-term stress manifest better tolerance to copper ions than plants not experienced stress or subjected to weak stress.

ACKNOWLEDGMENTS

This work was supported by the Federal program *Scientific and Scientific-Pedagogical Personnel of Innovation Russia* (State contract nos. P2364, P1301, 14.740.11.1032.H), by the President of Russian Federation (MK-881.2010.4), and State Social-Pedagogical Academy of Nizhny Tagil (no. 1.1.08).

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