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Influence of excess copper on lettuce (*Lactuca sativa* L.) grown in soil and nutrient solution

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Abstract

Lettuce is as a world-wide popular leafy vegetable, commonly grown in urban and suburban gardens exposed to the potential risk of soil contamination by intensive and uncontrolled usage of copper-based pesticides. In this research, the physiological response of two lettuce cultivars to excess copper was investigated in controlled conditions, where copper was administered in nutrient solution as 0.5 mM Cu for two days and in an experiment performed outdoors till the lettuce heads formation, using two soils with high total copper content (Cu 120 and 180 mg kg^{-1} soil). The evaluated parameters were copper accumulation in leaves, lipid peroxidation level, leaf protein and proline content as well as total and specific peroxidase activity. The results indicate similar physiological response mechanisms to copper induced oxidative stress in lettuce grown in different experimental conditions, depending on the severity of stress and cultivar tolerance. Based on the established copper accumulation in lettuce leaves, observed antioxidative activity (GPOX) and increased proline concentration, it could be concluded that the exposure of lettuce plants to excess copper in nutrient medium resulted with altered plant metabolism due to oxidative stress. A short treatment with high copper concentration in nutrient solution resulted in significant difference between cultivars in lipid peroxidation level, showing higher tolerance of cultivar Triatlon that accumulated more copper but had lower TBARS content than cultivar Nadine. Significant correlative relationships between free proline accumulation and peroxidase activity in leaves under influence of copper stress imply possible metabolic connections of enzymatic and non-enzymatic antioxidative response pathways in lettuce.

Key words: Copper, guaiacol peroxidase, heavy metal accumulation, lettuce, lipid peroxidation, nutrient solution, oxidative stress, proline, protein content, soil pollution.

Introduction

Vegetables are vital to the human health and in particular provide the well-known trace elements [1]. Diets rich in fruits and vegetables delay the onset of many age-related diseases and contain a complex mixture of antioxidants but also pro-oxidants, including iron, copper, H_2O_2, haem, lipid peroxides and aldehydes [2]. Plants growing on contaminated soils will reflect elevated concentrations of heavy metals in the soils to varying extents, depending on the soil total concentrations, physico-chemical conditions (especially pH) and the genotype of the plant [3]. In general, the inhibition of plant growth and crop production by excess heavy metals such as Cu, Cd, Zn or Ni in contaminated soil was considered as a global agricultural problem [4]. Further investigations are still required to understand the specific role of these pollutants in disturbing the plant metabolism [5]. The major sources of soil contamination with heavy metals are human activities, such as the prolonged application of Cu-based fungicides that was considered as one of the usual causes of high levels of Cu in the soil [6]. Copper is an essential component of electron-transfer reactions, mediated by proteins such as superoxide dismutase, cytochrome-c oxidase and plastocyanin, but the reactivity of copper can lead to the generation of harmful reactive oxygen species [7]. The induction of peroxidases (POX or POD; EC 1.11.1.7) is a general response of higher plants to uptake of toxic amounts of metals [8, 9]. There are some other stress indicators such as proline accumulation, which was considered as important for future investigation on stress thresholds for productivity and product quality of cultivated plants [10]. Lipid peroxidation (TBARS) is also widely used as a biochemical marker for the free radical mediated injury in plant and animal tissues, and the quantification of lipid peroxidation could be a relevant bio-assay for Cu toxicity [11]. Measurement of antioxidants as stress markers will remain an essential aspect in assessing stress responses in plants, but specific aspects related to plant species and tissues must be considered [12].

Although the mineral nutrition of higher plants is of fundamental importance to agriculture and human health, many basic questions remain unanswered, particularly in relation to the accumulation of essential heavy metals [13]. The availability of metals to the organisms is largely determined not only by the external ambient conditions but also by the physicochemical characteristics of the microenvironment of the organism [14]. Therefore, the aim of this work was to analyze the physiological response of lettuce cultivars grown outdoors in the soil with high Cu content, in comparison with the response of the same cultivars to high Cu concentration in nutrient solution in controlled conditions (growth chamber). Lettuce was selected as a world-wide popular leafy vegetable, commonly grown in urban and suburban gardens that are exposed to the potential risk of soil contamination by intensive and uncontrolled usage of copper-based pesticides.

Material and Methods

The experiments with lettuce grown in nutrient solution and soil with excess copper: Seeds of two lettuce (*Lactuca sativa* L.) cultivars (iceberg lettuce Triatlon, butter head lettuce Nadine) were sown to plug plates filled with commercial substrate and grown for three weeks in glasshouse conditions, till the four to five leaf stages. The uniform plantlets were selected and uprooted from the substrate, washed thoroughly with running deionised water and planted on perforated polystyrene fasteners of glass pots containing modified nutrient solution for leafy vegetables[15]. The composition of the nutrient solution was as follows (mg L^{-1}): N (as NO_3), 404; P, 76; K, 202; Ca, 127; Mg, 123; Fe, 24; Mn, 2; Cu, 0.05; Zn, 0.22; B, 3; and Mo, 0.02. EC and pH were adjusted to 1.2 mS cm^{-1} and 6.5, respectively. The nutrient medium was renewed every 3 days and aerated for one hour each day. The pots were kept for three weeks in a growth chamber, in a completely randomized block design, with rotation of pots in a chamber at every change of nutrient solution. The temperature was maintained at 20^{o}C, with 70% relative humidity and 12-h photoperiod. Light was supplied by cool white fluorescent lamps providing the photosynthetic photon flux density of 120 µM m^2 s^{-1} at the leaf level. Subsequently, the plantlets were treated with 0.5 mM Cu (final concentration, applied as sulphate) in nutrient solution for 2 days, while the control plants remained on previously applied nutrient solution with Cu 0.05 mg L^{-1}.

In the experiment with lettuce grown in the soil, plantlets were transferred from the glasshouse and grown in plastic pots (10 L volume) filled with vineyard soil or field soil taken in the vicinity of the vineyard to ensure that both variants have the same soil type but different Cu level (Table 1). Lettuce plants were grown outdoors for 80 days (April 19 – June 31, 2007), with mean daily temperature of 19.2^{o}C and regular watering with tap water when necessary, until the formation of lettuce heads.

Both experiments were performed in split-split-plot design, with four replicates per treatment, and each replicate contained four plants.

Soil and plant analysis: The determination of soil pH was in 1:5 suspensions of soil using 1 M KCl solution and deionized water, respectively. Soil organic matter (humus content) was determined by sulfochromic oxidation [16]. P_2O_5 and K_2O content in the soils were determined from ammonium lactate-acetic acid extractions (AL)[17] and measured by VIS spectrophotometry and atomic absorption spectrophotometry (AAS), respectively. The total copper content in the soil was determined using aqua regia extraction [18], while it's exchangeable fraction was determined using EDTA-extraction [19] with some modifications. Briefly, 10 g of soil was extracted with 20 ml of 0.01 M Na_2-EDTA + 1 M CH_3COONH_4 for 30 min under stirring, prior to be filtered. The concentration of copper in soil and plant was measured by AAS and expressed in mg kg^{-1} soil or plant DW. Dry samples of lettuce leaves (1 g) were subtracted to wet acid digestion by means of 5 mL 4% $HClO_4$ in concentrated H_2SO_4, with addition of 10 mL H_2O_2 and heating at 380^{o}C for 30 min. Clear and cold solution was diluted with deionised water to 100 mL. Copper concentration was measured by AAS.

Determination of lipid peroxidation, peroxidase activity, protein and free proline: The most developed fresh lettuce leaves from each plant were powdered in liquid nitrogen. Lipid peroxidation was measured as the amount of thiobarbituric acid (TBA) reactive substances (TBARS) determined by the thiobarbituric acid reaction [20]. Total peroxidase activity (GPOXt) was determined using guaiacol as a substrate [21] by following the formation of tetraguaiacol at 470 nm, expressed as ΔA_{470} min^{-1} mg^{-1} FW. Peroxidase specific activity (GPOXs) was calculated taking into account the protein concentration in leaf tissue, estimated using bovine serum albumin (BSA) as a standard [22]. Free proline concentration in lettuce leaves (µM g^{-1} FW) was determined spectrophotometrically, using sulphosalicylic acid extraction, reaction with acid ninhydrin and toluene separation[23].

Table 1. The agrochemical properties of soil used in pot experiment with lettuce.

	Field soil	Vineyard soil
pH in H_2O	8.51	8.59
pH in 1 M KCl	7.72	7.65
AL -extractable P_2O_5 (mg kg^{-1} dry soil)	506.0	156.0
AL -extractable K_2O (mg kg^{-1} dry soil)	816.5	294.3
Organic matter content (%)	2.81	2.76
Cu total content - aqua regia-extractable (mg kg^{-1} dry soil)	120.0	180.0
Cu exchangeable – EDTA-extractable (mg kg^{-1} dry soil)	25.7	41.4

Statistical analysis: The results obtained from both experiments were evaluated statistically by analysis of variance, and least significant difference (LSD) was calculated when significant ($P<0.05$) F-ratio occurred. The relationships among tested stress response parameters and copper concentration in plant tissues were evaluated using single correlation and regression analyses with t-test application ($P<0.01$).

Results and Discussion

The response of lettuce cultivars on the copper excessive supply in nutrient solution or in the soil was evaluated by plant copper accumulation in leaves, lipid peroxidation level, leaf protein and proline content as well as total and specific peroxidase activity.

Ginnochio *et al.* reported that mean copper concentrations were higher in lettuce than those in tomato and onion plants as well as in vegetables grown on acidic soils [24]. The soil used in our experiment had relatively high pH what might influence the exchangeable fraction of copper in the soil (Table 1). Presumably, if the pH was lower, considering better availability of micronutrients and heavy metals at lower soil pH, the exchangeable fraction would be higher. Greater variations in soil pH and organic matter would be expected to increase the relative significance of pCu^{2+} over total copper, since pCu^{2+} integrates the impact of pH, organic matter and other soil characteristics on the fractionation and speciation of total copper [25]. Here, field soil taken in the vicinity of the vineyard showed unexpectedly high copper content (in total 120 mg kg^{-1} whereas 25.7 as EDTA-Cu; Table 1), what implies that copper-based fungicides applied through many years in the vineyard were transferred by the wind to the surrounding fields. The critical levels for many European countries are in the range of 20-60 mg kg^{-1} [26]. As reported by Brun *et al.* [19], the average Cu content observed in vineyard soils in the south of France ranged from 31 to 251 mg kg. On the contrary, an overview of transition metals in the soils of the sub-Mediterranean winegrowing region in Slovenia [27] showed only 7 mg kg^{-1}, but with significant increment with a vineyard's age.

The accumulation of copper in lettuce leaves was significantly higher in plants grown in vineyard soil (cv. Triatlon 18.4% and cv. Nadine 29.5% higher than in the plants grown on field soil, Table 2). Within particular soil, cultivar differences were not significant. However, Jiang [28] reported much lower copper concentrations in field grown lettuce (7.3 mg kg^{-1} leaf DM) and stated that copper and other micronutrient concentrations were in general agreement with textbook values. Relatively high copper concentration in lettuce grown on field soil in our research emphasises soil contamination, although there was no vineyard, at least in the last hundred years. The difference in leaf copper concentration between control and stressed plants in nutrient solution was much higher, they accumulated 41.1% (Nadine) and 83.3% (Triatlon) more copper than control plants in only two days at 0.5 mM Cu supply (Table 3).

As an indication of stress, an increase of the thiobarbituric reactive metabolites (TBArm) content that occurred immediately following copper application in *Phaseolus vulgaris* seedlings, was reported previously [29]. It was observed in the primary leaves from the first hour after copper uptake, but the effect on shoot growth was less pronounced. Our results indicate that the uptake and translocation of Cu to lettuce leaves in plants grown in soil did not cause severe oxidative stress and lipid peroxidation or it might have been prevented by the increased antioxidant activity. However, short exposure to high copper concentration in nutrient solution resulted with high difference between cultivars in lipid peroxidation level. Cultivar Nadine showed much higher TBARS level in leaves after 48 h in copper treatment, 35.8% higher than untreated plants, while in cv. Triatlon it was only 4.8% higher, indicating higher tolerance of cv. Triatlon to copper stress.

The leaf protein content differed significantly between cultivars and experiments. In the experiment with soil, protein content was higher in plants grown in vineyard soil (28.3% increment in cv. Triatlon and 11.5% in Nadine, respectively). Plants grown in nutrient solution with high copper excess had lower protein content in comparison with untreated plants, but these differences were not statistically significant. Chatterjee and Chatterjee obtained the restricted biomass of cauliflower in the presence of excess Co, Cu and Cr and assumed the poor protein formation in such conditions [5]. On the contrary, Cuypers *et al.* reported that metal application (Cu and Zn) resulted in an increase of the *Phaseolus vulgaris* primary leaf tissue protein content [30]. It seems that copper excess can influence quite differently plant tissue protein content, depending on concentration and exposure

Table 2. The physiological parameters of copper stress response in two lettuce cultivars grown outdoors in pots with vineyard and field soil.

Soil type (A)	Vineyard soil		Field soil		Analyses of variance F test		
Cultivar (B)	Nadine	Triatlon	Nadine	Triatlon	A	B	AxB
c_{Cu}	$25.24^x\pm1.11$	$22.49^X\pm1.04$	$19.50^y\pm0.64$	$19.00^Y\pm0.71$	**	ns	ns
TBARS	$6.09^x\pm0.26$	$5.50^X\pm0.43$	$5.51^x\pm0.47$	$5.50^X\pm0.27$	ns	ns	ns
PROT	$2.02^x\pm0.09$	$2.29^X\pm0.04$	$1.81^x\pm0.06$	$1.78^Y\pm0.10$	*	ns	ns
PRO	$1.05^x\pm0.08$	$1.15^X\pm0.13$	$0.86^x\pm0.10$	$0.77^X\pm0.12$	ns	ns	ns
GPOXt	$4.61^x\pm0.20$	$5.24^X\pm0.13$	$2.96^y\pm0.24$	$2.89^Y\pm0.22$	**	ns	ns
GPOXs	$2.29^x\pm0.10$	$2.30^X\pm0.08$	$1.63^y\pm0.10$	$1.62^Y\pm0.12$	**	ns	ns

(c_{Cu} - leaf Cu concentration, mg kg^{-1} DM; TBARS – lipid peroxidation level, nM g^{-1} leaf FM; PROT – leaf protein content, mg g^{-1} FM; PRO - free proline content, µM g^{-1} leaf FM; GPOXt – total guaiacol peroxidase activity, ΔA_{470} min^{-1} mg^{-1} leaf FW; GPOXs – specific guaiacol peroxidase activity, ΔA_{470} min^{-1} mg^{-1} protein; FM – fresh mass; DM – dry mass. Values are means of four replicates with four plants each ± standard errors; *significant at $P<0.05$; ** significant at $P<0.01$. x, y – treatment means with the same letter are not significant in cv. Nadine. X,Y – treatment means with the same letter are not significant in cv. Triatlon.

Table 3. The physiological parameters of the stress response in two lettuce cultivars after 48 h of 0.5 mM copper treatment in nutrient solution.

Copper treatment (A)	0.5 mM Cu		Control		Analyses of variance F test		
Cultivar (B)	Nadine	Triatlon	Nadine	Triatlon	A	B	AxB
c_{Cu}	12.77^x±1.07	15.22^X±1.62	9.05^Y±1.30	8.30^Y±0.27	**	ns	ns
TBARS	45.12^x±6.87	40.64^X±2.22	33.22^Y±4.02	35.99^Y±4.04	*	ns	ns
PROT	1.10^x±0.02	1.38^X±0.11	1.25^x±0.13	1.44^X±0.12	ns	*	ns
PRO	13.66^x±0.79	5.54^X±0.16	1.93^Y±0.10	3.33^Y±0.31	**	**	**
GPOXt	2.63^x±0.11	2.78^X±0.14	2.35^x±0.11	2.39^X±0.23	ns	ns	ns
GPOXs	2.41^x±0.15	2.03^X±0.15	1.90^Y±0.09	1.68^Y±0.18	**	ns	ns

(c_{Cu}- leaf Cu concentration, mg kg^{-1} DM; TBARS – lipid peroxidation level, nM g^{-1} leaf FM; PROT – leaf protein content, mg g^{-1} FM; PRO - free proline content, µM g^{-1} leaf FM; GPOXt – total guaiacol peroxidase activity, ΔA_{470} min^{-1} mg^{-1} leaf FW; GPOXs – specific guaiacol peroxidase activity, ΔA_{470} min^{-1} protein; FM – fresh mass; DM – dry mass; data are means of four replicates with four plants each ± standard errors; control plants had 0.05 mg Cu L^{-1} in nutrient solution); *significant at $P<0.05$; * * significant at $P<0.01$. x, y – treatment means with the same letter are not significant in cv. Nadine. X,Y – treatment means with the same letter are not significant in cv. Triatlon.

duration, plant species and genotype specificity, as well as other growth conditions.

The proline level in lettuce leaves grown in soil showed no significant differences among cultivars or treatments and the proline concentration was much lower, in general, in comparison with nutrient solution experiment. In the latest, cultivar differences, copper treatment and cultivar x treatment interaction had very significant influence on free proline accumulation in lettuce leaves ($P<0.01$). The copper stressed plants of cv. Nadine showed the highest proline concentration, however, the proline increment in leaves of Triatlon was also significant. After Claussen [10], proline accumulation does not occur unless a certain threshold stress level is exceeded. In our study, excess copper led to high accumulation of free proline in the experiment with lettuce grown in nutrient solutions, where copper was applied in the concentration of 0.5 mM. Since proline is one of the important solutes which accumulate in many organisms when they are exposed to environmental stress, it is likely that proline accumulation is related to the protection of these organisms against singlet oxygen production during stress conditions [31]. That could contribute to low TBARS content in our research. In other words, the oxidative stress in copper-treated lettuce plants in nutrient solution might have been prevented to some extent by proline accumulation. The beneficial role of proline in plant tolerance to abiotic stress suggests the need of further investigations related to dietary intake of plants enriched with proline and its possible implications on human health.

In the research of Adamo *et al.* the high activity of POD indicated that heavy metals were present at phytotoxic levels in the soils [32]. After García *et al.*, Cu concentration of 10 µM applied to sunflower in a hydroponic culture for a week caused lipid peroxidation and peroxidase responded at lower, non-toxic Cu concentrations [33]. Here, peroxidase total activity (GPOXt) was significantly lower in both tested lettuce cultivars grown on field soil as compared to plants grown on vineyard soil (Table 2), where cv. Triatlon showed higher enzyme activity than cv. Nadine (81.7% and 55.8% higher than plants in field soil, respectively). Interestingly, GPOXt activity was not influenced by cultivar or high copper concentration in nutrient solution experiment (Table 3). On the contrary, the peroxidase specific activity (GPOXs) was highly influenced by copper treatment in the same experiment (23.8% and 20.7% higher in copper treated Nadine and Triatlon plants, respectively, in comparison with control plants). In plants grown in vineyard soil, GPOXs was similarly to GPOXt higher in comparison with the plants grown in field soil, with less expressed cultivar differences (Triatlon 41.4% higher and Nadine 40.5% higher, respectively). These results suggest that specific and total enzyme activities, as stress response parameters in plants, should be evaluated and interpreted taking into account all relevant experimental conditions.

Free proline accumulation significantly correlated to GPOXs (Fig. 1) in both experiments. These two copper-induced stress parameters of tested lettuce cultivars also significantly correlated to copper accumulation in lettuce grown in pots with soil (Fig. 2), showing similar pattern of the relationship. That is in concordance

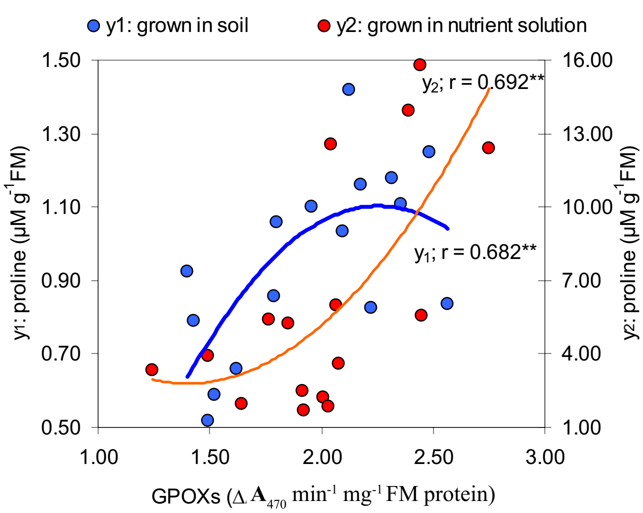

Figure 1. The relationships between guaiacol peroxidase specific activity (GPOXs; x) and free proline content in lettuce leaves grown under copper excess in soil (y_1) and nutrient solution (y_2). Soils contained total Cu 120 and 180 mg kg^{-1} (field and vineyard soil, respectively) and Cu concentration in nutrient solution was 0.5 mM (Cu treatment) and Cu 0.05 mg L^{-1} (control). FM – fresh mass; ** $P<0.01$; n =16.

with the statement of Matysik *et al.*, that under stress, accumulation of compatible solutes occurs in addition to increase in the activities of detoxifying enzymes [34].

It was reported that proline and G6PDH stimulation was correlated to phenolic content, potential polymerization of phenolics via GPOX and antioxidant activity as reflected by the free-radical scavenging assay and antioxidant enzyme response[35]. Although heavy metals can induce genes allowing increased resistance of plants, at prolonged action and/or higher doses, they usually result in death of sensitive plants because of their irreversible disturbance between the synthesis of basic metabolites and their degradation [36]. From that point of view, the role of proline in plant response to heavy metal stress needs to be further elucidated.

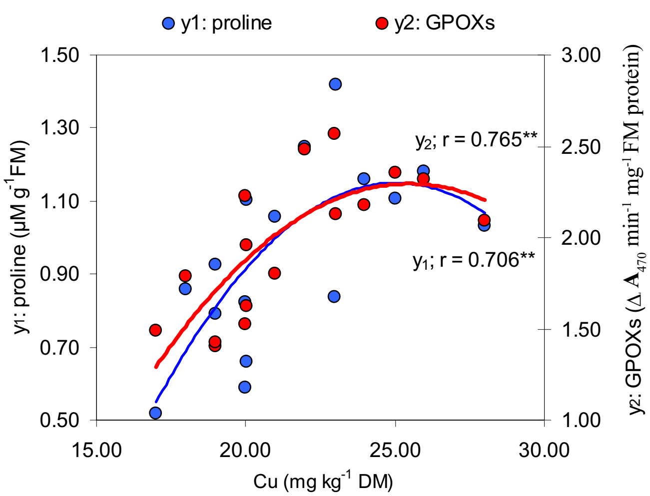

Figure 2. The relationships between copper accumulation in lettuce (Cu; x) grown under copper excess in soil and free proline content in lettuce leaves (proline; y_1) as well as guaiacol peroxidase specific activity (GPOXs; y_2). Soils contained total Cu 120 and 180 mg kg^{-1} (field and vineyard soil, respectively). FM – fresh mass, DM – dry mass; ** $P<0.01$; n = 16.

Conclusions

Based on the established copper accumulation in lettuce leaves, observed antioxidative activity (GPOX) in both experiments and increased proline concentration at higher stress level, it could be concluded that the exposure of lettuce plants to excess copper in nutrient medium resulted with altered plant metabolism due to oxidative stress. A short treatment with high copper concentration in nutrient solution resulted with significant difference between cultivars in lipid peroxidation level, showing higher tolerance of cultivar Triatlon that accumulated more copper but had lower TBARS content than cultivar Nadine. Copper stress in soil grown lettuce was not characterized by lipid peroxidation level and proline concentration, however, copper accumulation and peroxidase activities were significantly higher in the lettuce grown on vineyard soil in comparison with field soil.

The obtained results indicate similar physiological response mechanisms to copper-induced oxidative stress in lettuce grown in different experimental conditions, depending on the severity of the applied stress level and cultivar tolerance. Significant correlative relationships between free proline accumulation and peroxidase activity in leaves under influence of copper stress imply possible metabolic connections of enzymatic and non-enzymatic antioxidative response in lettuce.

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