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Physiological responses of *Vicia faba* to copper toxicity

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Abstract. *Copper plays an important role in multiple plant physiological processes including photosynthesis and protein synthesis. Excess copper in tissues leads to physiological and biochemical disturbances. In order to determine the physiological response of Vicia faba L. to copper toxicity, two varieties (Sidi Aïch and Super Aguadulce) were grown in a substrate of sand and compost (3V/V) and contaminated by different doses of Cu (0, 80, 160, 240, 320, 400 mg kg^{-1}). Dose rates ≥160 mg kg^{-1} led to the accumulation of copper in roots and shoots, in turn leading to decreases in photosynthetic activity and protein content and to the accumulation of proline, a stress indicator, and soluble sugars.*

Keywords: copper, photosynthesis, proteins, proline, toxicity, sugars, substrate, *Vicia faba* L.,

Introduction

Since the beginning of the industrial revolution, environmental pollution has accelerated as the result of industrialization, urbanization and pollution by fertilizer (Singh et al*.,* 2016). Among the main pollutants are non-biodegradable heavy metals that accumulate in soil where they are of particular concern (Asati et al*.,* 2016).

All heavy metals are potentially toxic to plants depending on their concentration and on whether they are essential to the plant. Mean copper (Cu) concentrations in different soils worldwide are reported to vary between 20 and 30 mg kg^{-1} (Alloway, 1995). In addition, the average Cu concentration worldwide is reported to be from 8 mg kg^{-1} in acid sandy soils to 80 mg kg^{-1} in heavy loamy soils. In soil, limits for copper toxicity were established between 20 and 100 mg kg^{-1} (Kabata-Pendias, 2001). Critical tissue Cu concentrations, defined as a 10% decrease in dry matter yield, were reported to vary from 5 to 30 mg kg^{-1} depending on plant species (Yang et al., 2002).

Copper is one of the most abundant heavy metals in agricultural soils. Copper-based pesticides are widely used in agricultural practices around the world (Husak, 2015). As a consequence, different food and feed crops grown on metal-contaminated soil can accumulate high concentrations of metals that pose a threat to human and animal health (Kulhari et al., 2013).

Copper, a trace element essential to plant development, is potentially phytotoxic in high concentrations. The phytotoxicity of Cu affects many morphological, physiological and biochemical processes in plants. Growth reduction is one of the most frequent responses, and the first observable response when plants are under stress from Cu toxicity (Adriano, 2001). Excess copper can reduce growth by disrupting enzymes involved in physiological processes essential to development (photosynthesis, respiration) or the induction of oxidative stress that can lead to cell death. The toxicity of copper thus results in the fixation of the metal on the thiol group of proteins, which causes inhibition of their activity and modifies their structure (Ibrahim et al*.,* 2017).

The accumulation of chlorophyll pigments is often used as a diagnostic tool for the functional state of photosystems under metal stress, as well as the accumulation of osmoregulators such as proline and soluble sugars.

The fava bean (*Vicia faba* L.) is a grain legume rich in protein, whose cultivation has long been practiced in the temperate zone of the northern hemisphere. It is mainly harvested in the form of dry seeds for human or animal consumption (Duc et al., 2015). With an estimated area of about 54 675 ha, the bean is considered the most cultivated legume in Algeria (FAOSTAT, 2018). It has an intrinsic capacity to adapt to diverse climates and provides valuable ecological and environmental services in sustainable agriculture, it allows for diversified crop rotations, and hosts many associated organisms, including pollinating insects. The ability of this species to establish symbiosis with specific rhizobia bacteria results in biological nitrogen fixation.

Environmental constraints and heavy metals in particular lead to a reduced production of fava bean due to its reaction to heavy metal toxicity (Iqbal, 2016). Thanks to its multiple agro-biological roles, fava beans remain an essential

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component in the world's agricultural production systems. This work aims to establish critical copper concentrations in the fava bean culture substrate to determine the toxicity limit and to study the physiological response of the fava bean under Cu stress. Two varieties of the bean of different origin and comportment were selected, this genotypic variability being part of the process of its adaptation to this metal stress.

Material and methods

Plant materials

The study was carried out on two varieties of the faba beans (*Vicia faba* L.): SIDI AICH, a local variety, minor bean supplied by the ITGC of Constantine, and another variety of Spanish origin, SUPER AGUADULCE.

Soil materials

The substrate used consists of a mixture of sand and compost respective proportions of (3V/V). The sand was sieved in order to remove plant and animal debris, rinsed with abundant water, dried in the open air, then mixed with compost (3V/V) and placed in different plastic pots (25 cm high and 18 cm in diameter), these pots are topped at the bottom with gravel to ensure good drainage and filled with 2.5 kg of the prepared mixture.

Conduct of the study

The experiment is conducted in a semi-automatic greenhouse at the Biotechnology Research Center (BRC), Constantine. Growing conditions are maintained for the whole experiment at temperatures of 19°C at night versus 23°C during the day with a relative humidity in the vicinity of 70%.

The seeds of both varieties were disinfected with 06% sodium hypochlorite solution for 10 minutes, and rinsed several times with distilled water to remove all traces of chlorine. Then the seeds are placed in plastic boxes on Whatman paper soaked in water and put in incubation chambers at 20°C. After four days, the germinated seeds were transplanted into the growing pots, and installed in the greenhouse and distributed on two tables for each variety, Sidi Aïch (V1) and Super Aguadulce (V2). After five days of transplanting, the plants of both varieties were treated with $CuSO_4$, $5H_2O$ with the following doses: 0, 200, 400, 600, 800 and 1000 mg kg^{-1} dry soil (ppm) (0, 80, 160, 240, 320, 400 mg Cu kg^{-1}). Each treatment had four repetitions.

Irrigation and mineral fertilization were carried out periodically, maintaining the substrate at maximum retention capacity by daily addition of water estimated by weighing the pots. Fertilization is assured by a commercially nutrient solution named ACTIVEG (Table 1). After a 35-day cultivation period, the plants were collected and transferred for a series of tests.

Table 1. Composition of the nutrient solution ACTIFEG

Chemical element	Quantity
N	1000 g
P	436.6 g
K	830 g
Mg	12 g
Fe EDTA	650 ppm
Mn	650 ppm
Mo	50 ppm
S	16 g
Cu	60 ppm
Zn	300 ppm
Boron (B)	300 ppm

Physiological measurements

Concentrations of chlorophyll pigments (Chlorophyll-a/Chl-a and Chlorophyll-b/Chl-b) were determined by spectrometry according to the procedure cited by Lichtenthaler (1987), the contents are expressed in mg/g of fresh material using the following formulas (Wang et al., 2010):

Chl-a = 12.25 × A663 - 2.79 × A645

Chl-b = 21.50 × A645 - 5.10 × A663

The method followed for the determination of proline concentration is that of Troll and Lindsley (1955), simplified and developed by Dreir and Goring (1974). The concentration is estimated in mg/g of fresh material after conversion of the optical density read on a UV- spectrophotometer with a wavelength of 528 nm.

The determination of the quantity of soluble sugars was carried out by the method of Shields and Burnett (1960). Concentrations are expressed in mg/g of fresh material by UV-spectrophotometer at 585 nm.

The protein content was determined according to the Bradford method (1976) using bovine serum albumin (BSA) as the standard. Concentrations are calculated from the UV-spectrophotometer reading at 595 nm, and are expressed in mg/g of fresh material.

The dry weight (DW) of shoot and root of plants was determined by oven drying for 48 hours at 80°C. The values are expressed in grams (g).

The determination of copper in root and shoot of the plant was carried out by atomic absorption spectrometry (AAS) according to the method described by Rehman et al. (2019).

Statistical analysis

The data were subjected to a two-factor analysis of variance (ANOVA), the means were compared according to Newman-Keuls test at $p<0.05$. Correlation between tissue copper accumulation and physiological parameters was performed using the Pearson's test at a significance level $\alpha=0.05$.

Results

Chlorophyll a and b

Increasing dose rates of Cu led to reduced chlorophyll **a** and **b**, especially beyond 160 mg kg^{-1} (Figures 1 and 2). The interaction between variety and dose rate was significant for both chlorophyll-a ($p<0.001$) and chlorophyll-b ($p<0.05$), with V1 (Sidi Aïch) being most sensitive to increasing dose rate.

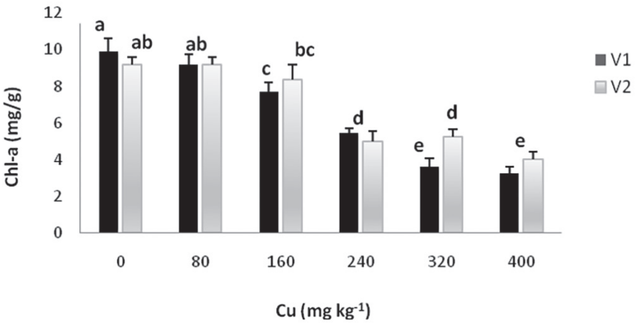

Figure 1. Chlorophyll-a content (mg/g) as a function of the dose of Cu (mg kg^{-1}) for V1 (Sidi Aïch) and V2 (Super Aguadulce), (a, b, c, d, e: groups means)

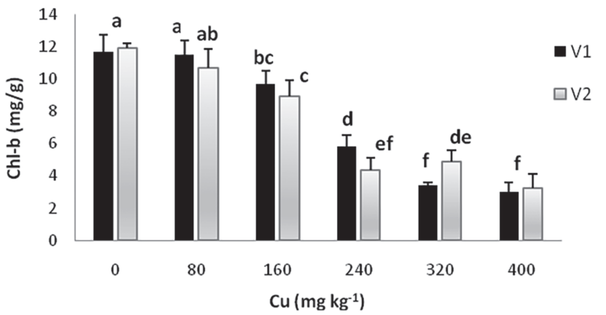

Figure 2. Chlorophyll-b content (mg/g) as a function of the dose of Cu (mg kg^{-1}) for V1 (Sidi Aïch) and V2 (Super Aguadulce), (a, b, c, d, e, f: groups means)

Proline

The proline concentration in leaves increased significantly as the dose rate of Cu increased beyond 160 mg kg^{-1} (Figure 3). There was no interaction between variety and dose rate ($p>0.05$).

Soluble sugars

The increase in the Cu concentration was accompanied by an increase in the solubles sugars content in the leaves (Figure 4). This increase was significant from a dose of 160 mg kg^{-1}. There was no interaction between variety and dose rate ($p>0.05$).

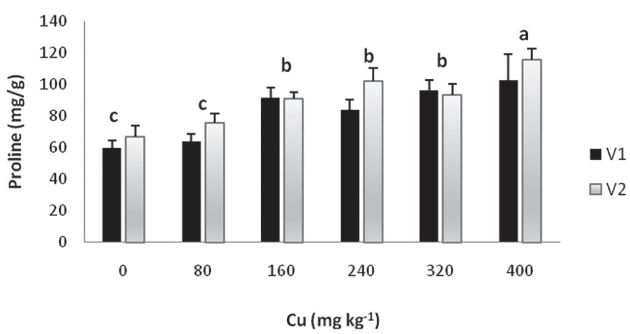

Figure 3. Proline content (mg/g) as a function of the Cu (mg kg^{-1}) for V1 (Sidi Aïch) and V2 (Super Aguadulce), (a, b, c: groups means)

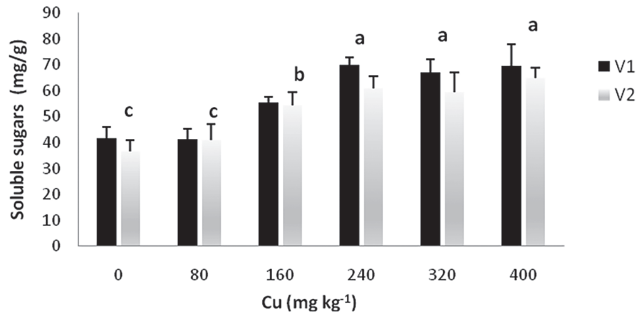

Figure 4. Soluble sugar content (mg/g) as a function of the Cu (mg kg^{-1}) for V1 (Sidi Aïch) and V2 (Super Aguadulce), (a, b, c: groups means)

Protein total

The total protein content is strongly conditioned by Cu rate ($p<0.001$), there was a decrease in protein beyond the 160 ppm dose (Figure 5). The interaction between variety and dose of copper is also significant ($p<0.01$).

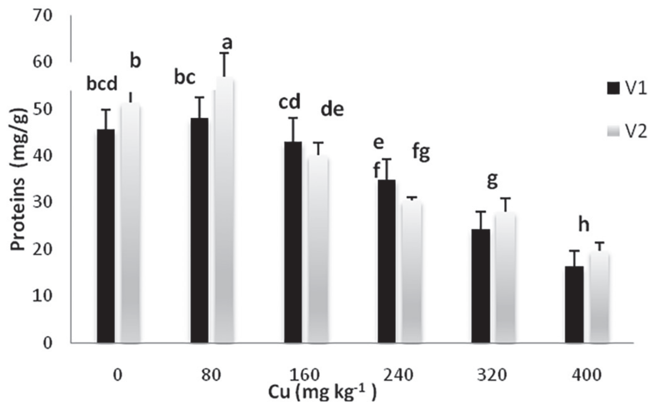

Figure 5. Protein contents (mg/g) as a function of the Cu (mg kg^{-1}) for V1 (Sidi Aïch) and V2 (Super Aguadulce), (a, b, c, d, e, f: group's means)

Root and shoot dry weight

Dry weight of roots and shoots is weakly dependent on variation in copper concentrations ($p<0.001$), Beyond 160 mg kg^{-1} dose, a decrease in the dry root and shoot weight of the bean was observed (Figures 6 and 7). No significant effect on the interaction between the variety and the copper doses ($p>0.05$).

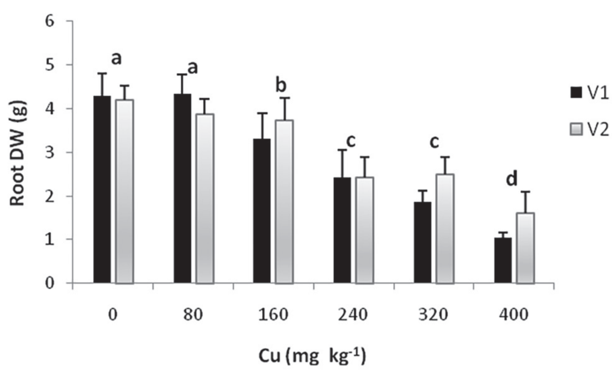

Figure 6. Dry weight of roots (g) as a function of the Cu (mg kg^{-1}) for V1 (Sidi Aïch) and V2 (Super Aguadulce), (a, b, c, d: group's means)

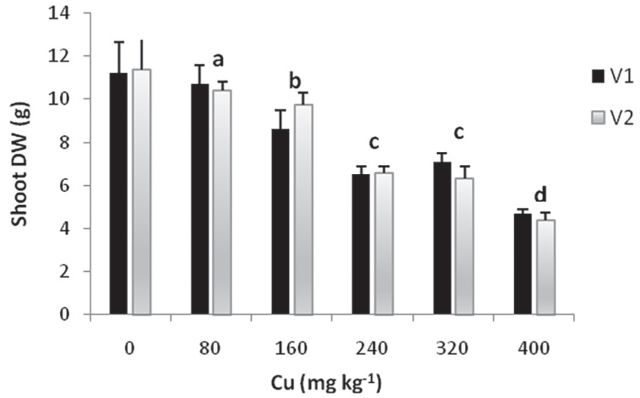

Figure 7. Dry weight of shoot (g) as a function of the Cu (mg kg^{-1}) for V1 (Sidi Aïch) and V2 (Super Aguadulce), (a, b, c, d: group's means)

Concentration of copper in root

The accumulation of copper in the roots is closely related to the Cu concentration ($p<0.001$), but the interaction between the two factors has no significant effect ($p>0.05$), indicating tested varieties behave similarly (Figure 8).

Concentration of copper in the shoot

The accumulation of copper in the shoots is influenced by the copper dose ($p<0.001$), an increasing amount of copper in plant tissue was observed in all levels of treatment (Figure 9). Also, the variety and the interaction between the two factors have a significant influence on the expression of this parameter ($p<0.05$).

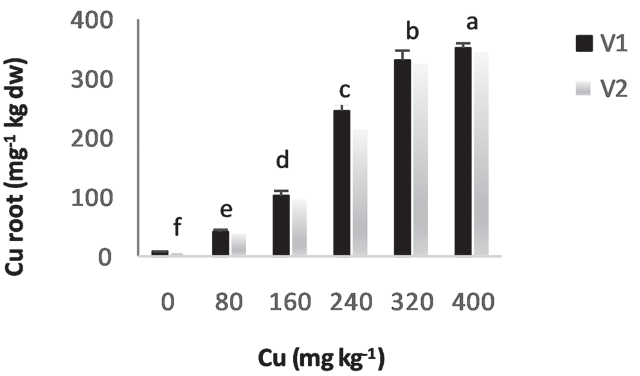

Figure 8. Cu accumulation in root (mg kg^{-1} dry weight) as a function of the Cu (mg kg^{-1}) for V1 (Sidi Aïch) and V2 (Super Aguadulce), (a, b, c, d, e, f: group's means)

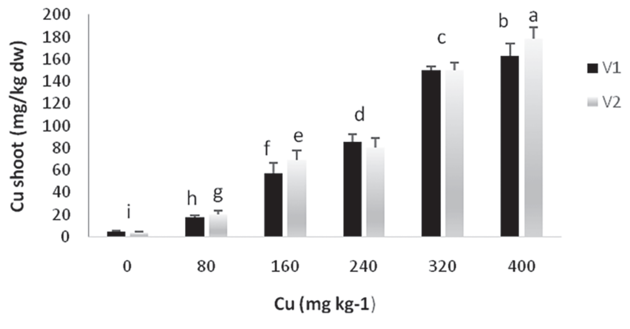

Figure 9. Cu accumulation in shoot (mg kg^{-1} d w) as a function of the Cu (mg kg^{-1}) for V1 (Sidi Aïch) and V2 (Super Aguadulce), (a, b, c, d, e, f, g, h: group's means)

Relationship between physiological parameters and Cu concentration

Cu concentrations in both roots and shoots were significantly ($p<0.001$) correlated to all of the physiological observations. The regressions were positive for soluble sugars, proline, and negative for Chl-a, Chl-b, protein and dry weight of root and shoot.

Discussion

Chlorophyll is frequently used as an indicator of photosynthetic activity (Shu et al., 2016). Chlorophyll is very sensitive to metallic stresses that decrease the total chlorophyll content a, b of the leaves (Rehman et al., 2016). Excess Cu can inhibit plant growth and affect physiological processes, including protein metabolism and photosynthesis (Kopittke et al., 2009). In this study, photosynthetic activity was affected by following exposure to copper. This is consistent with that observed in *Ocimum basilicum* L. (Georgiadou et al*.,* 2018) and barley (Eugene et al., 2020).

The reduction in chlorophyll contents might be due to the inhibited activities of various enzymes associated with chlorophyll biosynthesis (John et al., 2009). In this study, Copper accumulation in plant tissues is high from the 160 ppm Cu dose, it is negatively correlated with the contents of Chl-a and Chl-b. Metal accumulation in plants can damage the chloroplast, for example the toxicity of Cu caused a decrease in chlorophyll contents and changed in thylacoid membranes (Aggarwal et al., 2011).

At treatment 80 mg kg^{-1}, the accumulation of copper in the shoot is lower than 20 mg kg^{-1}, which corresponds to the normal copper concentration of the plant (5-25 mg/kg). Pedersen et al. (2000) reported that translocation of Cu to the aerial parts of the plant is effectively limited by the large accumulation of Cu in the roots.

From the 160 mg kg^{-1} Cu dose, the copper concentration was increased in the fava bean tissues. This is accompanied by a 10 to 23% reduction in the dry weight of the fava bean. This decrease is probably due to mineral interruptions in the plants that resulted in inhibition of cell division and elongation (Feigl et al., 2015). Nazir et al. (2019) showed that exposure to 100 mg kg^{-1} Cu for 40 days significantly decreased photosynthetic pigment by 27% and dry mass by 28.6 % in tomato plants.

Copper can also induce toxicity to plants by increasing the production of ROS. In fact, ROS was shown to affect the electron flow in photosynthetic ATC and, consequently, the photosynthetic process (Shahid et al., 2014).

Proline content was determined as a bioindicator of stress. In the present study, copper bioaccumulation was accompanied by an increase in proline. Our results are consistent with the work of (Kastori et al., 1992), where he observed an increase in proline under the effect of Zn, Pb, Cd and Cu in sunflowers. An increase in this amino acid was observed under copper stress in *Triticum aestivum* (Azooz et al., 2012), *Canavalia ensiformis* and *Coffea Arabica* (Andradea et al., 2010).

General response of plants to various kinds of stresses is the accumulation of compatible osmolytes such as proline, which protect cells against damage caused by stress (Dar et al., 2016). Proline is considered a metal chelator through thiol (-SH) groups and a protector of the subcellular structure (Azouz et al., 2011). However, it has also been shown that proline may be involved in the scavenging of ROS or the function in osmotic adjustment in some species (Szabados and Savouré, 2009).

Soluble sugars play a central role in the structure, metabolism and function of plants. They are also involved in many stress response mechanisms, both biotic and abiotic (Ramel, 2009). Beyond 160 mg kg^{-1}, a significant increase in soluble sugars was observed in fava bean plants. Similar results were also obtained in cucumbers in response to Cu stress (Alaoui-Sosse et al*.,* 2004), and in *Helianthus annuus* L. (El-tayeb et al*.,* 2006). Besides proline, total soluble sugars contribute strongly to the osmotic adjustment in plants under stress conditions. Soluble sugars may play a role in protecting chlorophylls and carotenoids against the aggressiveness of metal stress (Azooz et al., 2011). Verma and Dubey (2001) attribute this accumulation to a very possible adaptation of the plant to adjust and maintain a favourable osmotic potential.

Copper contaminants are also known to cause alterations of total proteins in plants, this alteration is probably due to the degradation of a number of proteins (Huang et al., 2020). In this study, copper stress negatively affects total protein levels, and a decrease in this element was observed in leaves subjected to high Cu concentrations compared to the control. These results are concordant with other work showing a decrease in protein content under the effect of copper in *Atriplex canescens* (Khedim, 2019) and in *Phaseolus vulgaris* L. (Yurekli and Porgali, 2006). Proteins are particularly sensitive to the action of ROS. They are also susceptible to oxidation by ROS. This oxidation causes the introduction of a carbonyl group into the protein (Srinivas et al., 2018).

These oxidation reactions, are frequently influenced by metal cations such as Cu^{2+}, it can be classified into two categories: those that break peptide bonds and modify the protein chain, and modifications of peptides by the addition of products from lipid peroxidation. These changes are such that they lead to a structural modification of proteins with major consequences (loss of catalytic function, increased sensitivity to proteases) (Levine, 2002). Several studies describe a decrease in protein levels under the effect of copper, (Georgiadou et al., 2018).

Conclusion

Copper toxicity is the source of a physiological disturbance in both varieties of the fava bean - Sidi Aïch (V1) and Super Aguadulce (V2). Copper was accumulated in the fava bean tissues with higher levels exceeding the toxicity threshold. In this experiment, the concentration160 mg kg^{-1} of soil was considered a critical dose of toxicity in the fava bean. From the 160 mg kg^{-1} concentration, the fava bean suffers the copper toxicity, its dry matter decreased from 10 to 23%, the levels of Chl-a and Chl-b being reduced in both varieties. Similarly, the total protein content recorded a 21% decrease (V2). For the plant to adapt to living under copper stress, it accumulates high levels of osmoregulatory.

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