

Original Research Article

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Tolerance of *Vicia faba* to Elevated Concentrations of Copper Ions in Nutrient Medium

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ABSTRACT

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One of the most important legume crops in the Mediterranean Basin and Arab countries is Faba bean (*Vicia faba*), which is an efficient nitrogen fixer. This study conducted to evaluate plant *Vicia faba* in response to elevated concentration of copper in nutrient media and demonstrate that this plant potentially useful as phytoremediator for moderately contaminated areas with copper. In experiments on *Vicia faba* plant investigated in a hydroponic culture with different concentration of copper (control, 50, 100, 150, 200 and 250 μ Mol / L) experiments showed that the tested species refer to indicator plants. Established that the studied plants showed stability to the toxic effects of excessive levels of copper, determined by their ability to germinate and grow as well as accumulate free proline (19 times more than control at 250 μ Mol CuSO₄ / L of media). Along with accumulation of a noticeable amount of metals in plant leaves (up to 60 μ g copper / g dry weight of plant tissue at 250 μ Mol CuSO₄ / L of media). These results characterize the studied plant species, *Vicia faba*, as potentially suitable as phytoremediator for moderately polluted areas with copper.

Introduction

Some heavy metals considered as essential micronutrients for animals, plants and many microorganisms, depending on the route and dose, as a results of metabolic interference and mutagenesis all heavy metals demonstrate toxic effects on living organism. Heavy metals can be a major problem for any biological organism as they may be reactive with a number of chemicals essential to biological processes (Kamran *et al.*, 2013). Faba bean (*Vicia faba*) is one of the most important legume crops in the Mediterranean Basin. It is known to be

an efficient nitrogen fixer of about 80% of nitrogen from the atmosphere (Huber *et al.*, 1987); which, depends on availability of *Rhizobium* spp. that colonize the legume plants (Rodelas *et al.*, 1999). It has an essential role concerning multipurpose of their usage as roasted in human diet, dried green or hay in animal feeding. Increasing in domestic consumption of dry beans due to consumer and scientific recognition of beans as a major health food. High protein level (18-41% of Dry Matter) in dried seeds have been reported (Iyad W.Musallam *et al.*,

2004), than other cultivated plants so they are used as important source for human nutrition or serves as fodder by feeding livestock with fresh stems and leaves.

As in all cultivated plants, the main objective of the faba bean breeding programme are to overcome the specific problems of this plant and to get high yield and quality. On the other hand, since genotypic and environmental factors are components determining yield and quality in plants, a primary aim should be the determination of effect of genotypic factors in selection. As the effect of environment on yield and quality in plant.

Stability to the toxic effects of elevated copper levels of various plants, due to ability of their proline accumulation has been studied (Alobaidi *et al.*, 2015). In order to clean the soil from heavy metal pollution, scientists developed a variety of technologies. One of most promising technique is phytoremediation-a method that based on the ability of the plants to clean up contaminated sites (Buravtsev, V.N. and Krylova, 2005; Krämer *et al.*, 2005).

Phytoextraction method which is most commonly used for the purpose of detoxification that is based on the ability of certain species of plants absorbs heavy metal from the soil and accumulates them in the aerial organs (Barbafieri *et al.*, 2011; Koshkin and Vagun, 2010; Chaney *et al.*, 1997; Raskin *et al.*, 1994). Phytoremediation offers a low cost and an environmentally friendly approach for decontaminating soils and waters of heavy metals.

Materials and Methods

Conditions of Plant Cultivation and Carrying out Experiments

Plants grown in different pattern depending

on the experimental setup. Petri dishes to study the effect of CuSO_4 on seeds germination, while 2 week germinated seedling transplanted into containers filled with aqueous nutrient solution (MS media) used for the estimation of the effect of elevated concentration of CuSO_4

Treatment of Seeds and Work with Seedlings

Seeds obtained from Baghdad local markets, disinfected as follow, washed with tap water for 15 minutes then sterilized by 2 % v/v sodium hypochlorite (Clorox) for 15 minutes and then washed extensively with sterilized distilled water. Petri dishes used with filter paper when working with seedlings, cultivation conducted in an incubator at 25C° . CuSO_4 solution used at a concentration of 10-300 μM , with water used as control.

Sowing and Cultivation the Initial Stage of Young Plants

Disinfected seeds sown on the surface of moist perlite in plastic containers (40h30h8sm) with holes at the bottom, placing the container on a tray. To maintain the desired humidity the top of the container closed with glass plate and removing it only after sprouting.

Replanting and Cultivation of Plants in Water Culture

At the age of 14 days, the plants removed from the perlite by using spatula as possible without damaging the roots. Then the roots washed in a small volume of water to remove the perlite particles. The plants are then cultivated in 1-liter pot, three plants per pot. Aeration and mixing of the nutrient solution carried out by continuous and uniform supply of air. In water culture plants grown in a growth chamber at temperature

around 25/20 C° (day / night), 12-hour light photoperiod. For growing in water culture, using a MS medium (Murashig and Skoog, 1962) and pH adjusted to 5.8.

Experimental Condition

In the experiments used 5-6 week plants with 3-4 fully developed leaves. Exposure started by introduction of CuSO₄ to the culture medium at concentrations of 10, 25, 50, 100 and 150 µM. Changing the culture medium performed every 5 days. Duration was 7 days. As a control, plants grown on standard medium containing 0.25 µM CuSO₄.

Measurement of Seed Germination and Seedling Root Length

Assessing the impact of excess Cu germination seedling and root growth performed using at least three seeds in each petri dishes and each variant used three petri dishes. Disinfected seeds soaked in a solution of copper sulphate in concentrations of 10 - 300 microns. Seed germination carried out for 5 days, then determined the percentage of germinated seeds and measured seedlings root length. Root length of the Germinated seeds considered at least five mm. Germination of each variant considered as a percentage - the number of germinated seeds relative to the total amount of seed.

Measurement of Plant Biomass

To evaluate the respond of the plants to the elevated concentration of CuSO₄, biomass of plants materials measured at the 7th day of exposure using sensitive electrical balance.

Determination of Metal Ion Content in Plant Tissues

Determination of the content of copper

performed by wet ashing method (14). A weighed sample of previously dried plant material (typically 50 µg) placed in a test tube filled with a mixture of nitric acid (1.6 mL) and hydrochloric acid (0.5 ml) and left overnight. The next day, the sample tubes placed in a preheated to a temperature of 110 ° C thermostat, incubated for 1 hr then a further 2 h at 180 C °. To samples added one drop of concentrated hydrogen peroxide. The next day, decolorized. Transferred into measuring tube and adjusted with distilled water to a certain volume (10-15 ml). Measuring the concentration of Cu was carried out on atomic absorption spectrophotometer «PG Instruments" (USA).

Content of Free Proline

The free proline was determined using the acid ninhydrin reagent method (15). To assess the content of proline in 1 ml of the obtained plant extract added 1 ml of glacial acetic acid and 1 ml of ninhydrin reagent, incubated in a boiling water bath for 1 hour and then quickly cooled in ice. The color intensity was determined spectrophotometrically at 520 nm on a spectrophotometer «ThermoFisher Scientific, Genesys 20" (USA).

Proline content calculated using a calibration curve by the following formula:

$$C = E * k * V / (m * 1000) \text{ where}$$

C - Concentration of proline, M/g wet weight

E - Optical density

k - Coefficient calculated from the calibration curve, 217.49

V - Volume, to which brought extract, ml

m - Mass of sample

Results and Discussion

Effects of Elevated Concentrations of CuSO₄ on Seed Germination and Seedling Growth

As described in the Materials and Methods section, disinfected three seeds of plant laid in a petri dish on filter paper, soaked with dissolved CuSO₄ solutions at a concentration of (50-300) μMol, water served as the control. The number of germinated seeds counted and measured the length of the sprouts roots on 5th day.

Influence of CuSO₄ on Root Growth During Germination of Seeds *Vicia faba*

Elevated concentrations of copper had a negative effect on seed root of *Vicia faba*, complete loss of germination occurred at (300) μMol CuSO₄ (death). *Vicia faba* greater stability clearly visible at 50 μMol CuSO₄ (table 1).

Further evaluation of the effect of copper on the growth of the seedlings roots. Elevated concentrations of CuSO₄ significantly reduces the length of seedling roots of *Vicia faba* (Table 1). Significant decrease in growth began with 100 μMol CuSO₄ and at the highest concentration (250 μMol CuSO₄) used, root growth decreased to 65 % of control. These results suggest that in the initial stages of plant *Vicia faba* showed fairly good resistance to high concentrations of CuSO₄.

Effects of Elevated CuSO₄ Concentrations on Plants Growth in Aqueous Culture

After 14-day-old seedlings of *Vicia faba*, chosen to study, germinated in pearlite, were transplanted into vessels with medium Murashige-Skoog (MS), where they grew within 3-4 weeks. Then replaced with

standard medium vessels on the same medium but supplemented with different concentrations of CuSO₄ (50 to and 250 μMol). After 10 days of stress exposure, the plants removed from the vessel and weighed to calculate the cumulative raw biomass, and then carried out the study of copper accumulation in leaf tissue and the accumulation of proline. Effect of CuSO₄ on biomass can show in (Table 2).

Accumulation of Biomass

Results presented in Table 2, which shows that growth of *Vicia faba* decrease when concentration of CuSO₄ increase. So at 150 μMol CuSO₄ reduced raw biomass weight by 44%, while 200 μMol CuSO₄ reduced raw biomass weight by 57%, and at 250 μMol CuSO₄ reduced raw biomass weight by 66% (Fig. 1).

Effect of Copper on the Content of Free Proline

The possible reasons for the differences in resistance to excessive concentrations of copper in *Vicia faba* also carried out, to study the influence of stress factors on the content of proline due to its known function as a chemical chaperone and an antioxidant. Elevated levels of CuSO₄ in the culture medium caused a significant accumulation of proline in the leaves of plants studied *Vicia faba*. Even at the concentration of 50 μMol Cu, concentration of proline in leaves was increased to 8 times more than the control in relation to the values of the plants control (Fig 2). The maximum accumulation of proline at 250 μMol CuSO₄ up to 14. μMol/g (Table 3).

Accumulation of Copper in Plant Tissues

The accumulation of heavy metals in aboveground plant organs is one of the most important parameters that determine the

phytoremediation ability of plants. Therefore, in this study it was necessary to assess the ability of the studied cultures to accumulation of copper in the plant leaves. Adding excess CuSO₄ in nutrient solution resulted in a significant accumulation of copper in the plants organs (Table 4). Greater accumulation shown at higher concentration, which reaches to about 5 times more than in the control (Fig. 3).

From this result, suggest that *Vicia faba* have moderate phytoremediation potential and ability to tolerate high concentration of CuSO₄

Pollution with Heavy Metals considered as a serious environmental problem of our time, which adversely affects both the agricultural and plant communities, as well as on human health. At present, more and more scientific and practical interest were focused on the problem of plant adaptation to damaging abiotic factors with natural and anthropogenic origin, among them special attention attracts toxic effects of heavy metals (HM). The use HM-resistant plants to clean the environment from anthropogenic pollution is advisable, that can extract and accumulate metals in the aboveground organs (phytoremediation).

Table.1 Growth of Roots of Seedlings *Vicia faba* Depending on the Concentration of CuSO₄

Variant	roots length mm	%
control	14.3	100
50	11.5	81
100	9.5	67
150	7.5	53
200	6.9	48
250	5.0	35

Table.2 Effect of CuSO₄ on Biomass of *Vicia faba*

Variant	Raw biomass (plant/g)
control	7.60
50	7.34
100	5.90
150	4.25
200	3.24
250	2.60

Table.3 Proline Content in Leaves of *Vicia faba* μMol/g Biomass

Variants	Leaves
control	0.75
50	6.25
100	8.21
150	10.22
200	12.4
250	14.6

Table 4.Accumulation of CuSO₄ in leaves μg/g dry weight

Variant	leaves
control	11.5
50	39.5
100	44.3
150	54.2
200	56.3
250	59.6

Fig.1 Percentage of Plants Biomass Accumulation in Different CuSO₄ Concentration

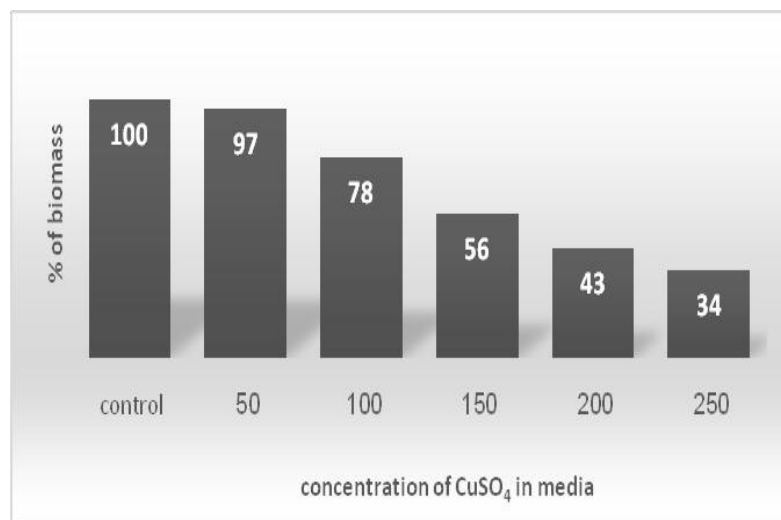


Fig.2 Proline Content in Plant Leaves at Different Concentration of CuSO₄, Control taken as a Unit

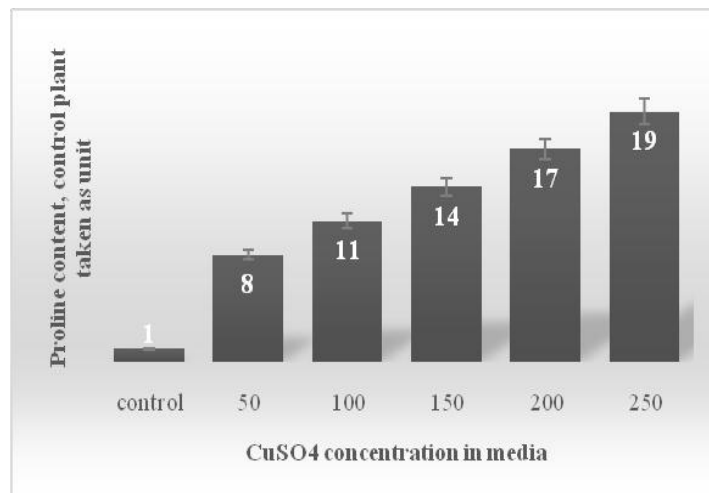
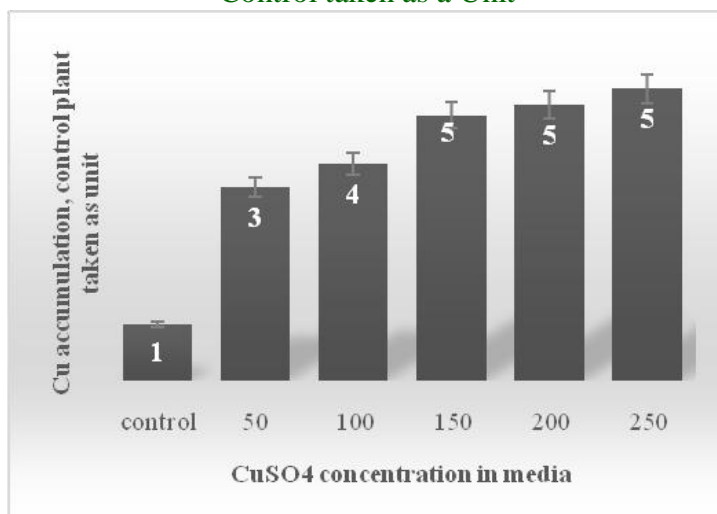


Fig.3 Copper Accumulation in Plants Leaves at Different Concentration of CuSO₄, Control taken as a Unit



For phytoremediation of contaminated areas using HM, plants have the ability to rapidly absorb metals from the soil, which is associated with the ability of the metal ion transported throughout the plant. One of the species that are promising for phytoremediation is *Vicia faba*, sufficiently high resistance to (HM), has a well-developed aboveground mass (Ben Ghnaya *et al.*, 2009; Ebbs and Kochian, 1997), allowing the accumulation of significant amounts of soil HM.

Copper is trace essential elements involved in a variety of physiological and biochemical processes in living organisms. For example, copper has important role in response for the operation of a number of enzymes and non-enzymatic proteins nature. An indispensable element of this is because when plants growing in water culture copper is an essential component of nutrient mixtures. In particular, the medium used in the experiments with *Vicia faba*, contained 0.025 milligrams/liter CuSO₄.

In this work analysis of the effect of copper on plants of *Vicia faba* been done. To characterize the plant we have studied the action of elevated concentrations of CuSO₄

on seed germination and seedling growth on young plants growing in water culture. Indicators such as growth and accumulation of plant biomass, accumulation of copper in the tissues of plants, and the content of free proline, examined in response to stress exposure to high concentrations of CuSO₄.

At the level of seed germination and the initial growth stage of the plant *Vicia faba* seedlings showed resistance to higher copper concentrations in excess, since inhibition of germination occurred at copper concentrations in excess of 300µMol. As mentioned, already at a small increase in the copper concentration in the medium was observed and the inhibition of growth of several physiological parameters.

Clear signs of the negative effect of copper is inhibition of the development of the root system (tab.1). It is known that the plants have developed efficient mechanisms for not only the absorption of any nutrient deficiency from the soil, but also the mechanisms of absorption of mineral limit elements at their highest concentrations in the environment (Kabata - Pendias and Pendias, 2001).

Accumulation of copper in plant tissues depended on the concentration of CuSO₄ in the medium, time of exposure and the type of plant. With increasing concentration of copper in the medium accumulated amount of metal in plants increased, increasing with longer exposure. Thus, the accumulation in the tissues was higher in *Vicia faba* (Table. 4).

As can be seen from the data, *Vicia faba* were very effective in Heavy Metal phytoextraction from the culture medium, which was manifested in their ability to actively accumulate Cu in the aboveground organs. Therefore, we can conclude that *Vicia faba* can be used for purposes of phytoremediation.

Many researchers (Schutzendubel and Polle, 2002; Blokhina *et al.*, 2003; Mediouni *et al.*, 2009) Furthermore, when exposed to stressors plants observed rapid inactivation constitutive pool of antioxidant enzymes.

At the present time can be considered universally accepted idea that the low molecular weight organic antioxidants in some cases are able to more effectively protect the metabolism from the damaging effect of ROS (radical oxygen species). These substances belong to some compatible osmolytes, one of which is the amino acid proline. Proline is a versatile organic tread compound. It acts as an osmolyte and antioxidant energy substrate, a source of reducing equivalents, nitrogen, and carbon, as well as a regulator of expression of stress response genes. In addition, proline exhibits chemical chaperone function, protecting the native conformation of macromolecules and membranes under stress (Kuznetsov *et al.*, 2011). It is known that the proline content is increased in the presence of heavy metals ions (Cheng, 2003).

As in the works of other researchers (Maree *et al.*, 2012) in this study, it was shown that (Table. 3) proline content increased by the action of CuSO₄. It is interesting that at low concentrations of CuSO₄ proline accumulated in the leaves of plant. Proline is a complex response of plants to the impact of stress factors, and it is important to improve plant resistance to adverse environmental conditions (Hong *et al.*, 2000; Mattioli *et al.*, 2008; Székely *et al.*, 2008). According to some authors (Kuznetsov and Shevyakova, 1997), proline is one of the most important components of the system to protect cells from the effects of stress factors. There is no doubt that the strong increase in proline content in *Viciafabais* in response to high concentrations of HM. The high level of copper accumulation in the tissues and at the same time, the high resistance of plants can occur only through the operation of a complete system of intracellular detoxification mechanisms of copper in plant organs *Vicia faba*.

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