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ORIGINAL ARTICLE

Bioremediation of copper stressed *Trigonella foenum graecum*

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Key words: alleviation, antioxidant enzymes, carbohydrate, copper, dry weight, fresh weight, mineral content, Nostoc muscorum, pigment, Trigonella foenum gracum

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Key words: alleviation, antioxidant enzymes, carbohydrate, copper, dry weight, fresh weight, mineral content, Nostoc muscorum, pigment, Trigonella foenum gracum

Medicinal plants are of great importance in the production of a whole range of medicaments. The increased need of crude drugs for the pharmaceutical industry has encouraged cultivation of many plants. *Trigonella foenum graecum* (fenugreek) is indigenous to the Mediterranean region and is cultivated in southern Europe, India and North Africa. In North Africa, the seeds are used as oral antidiabetic. The leaves are used in various folk remedies for the relief of indigestion

and general stomach discovers, chronic cough, enlargement of the spleen and as general tonic. Extracts of the seeds are incorporated into several cosmetics claimed to have effect on premature hair loss and as skin cleanser (Deni, 1995).

Heavy metals can be divided into two categories: essential (Cu, Ni, Mn, Zn, Co, Fe) which are required in trace amounts for different metabolic processes and non-essential (those are toxic even at low concentrations, e.g. Hg, Ag, Pb, Cd, Sn, etc). However, essential heavy metals are also reported to be toxic at elevated concentrations (Woolhouse, 1983). Copper is one of the heavy metals, although it is an essential microelement through interference with numerous physiological processes, when it absorbed in excess amounts, It can be toxic and induce a number of deleterious effects at morphological, biochemical, physiological and ultrastructural levels (Foyer *et al*., 1994). In general, Cu^{2+} can induce many alterations in plant cells: (1) It may bind to the sulfhydryl groups of membrane proteins or enzymes (Jouili and El-Feriani, 2003). (2) It increases the rate of lipid peroxidation (Teisseire and Guy, 2000). (3) It disturbs uptake of other essential elements (Xiong *et al.,* 2006). (4) It affects chlorophyll synthesis and electron transport so it has a toxic effect on the primary reactions of photosynthesis (Yruela, 2005). (5) As a redox radical and ROS via Haber-Wiss and Fenton reactions (Schützendubel and Polle, 2002). (6) Copper may interfere with cell permeability or binding of essential metals (Sunda and Huntsman, 1983). (7) Copper may react with free thiols (e.g. glutathione), disrupting cell function (Florence and Stauber, 1986). (8) Copper may also exert its toxicity in subcellular organelles, interfering with mitochondrial electron transport, respiration and ATP production (Viarengo *et al.,* 1981). (9) Cu can also catalyse the decomposition of H_2O_2 to produce hydroxyl radical ($OH^{\cdot}$), which may accelerate oxidative deterioration of membrane lipids (Florence *et al.,* 1984).

Potters *et al.* (2007) hypothesized that the common "stress-induced morphological response" observed under different sublethal abiotic stresses might be regarded as stress acclimation responses in order to decrease stress exposure. Plants have developed two strategies of metal tolerance exclusion and accumulation and sequestration in vacuoles and complexation by organic ligands (organic acid, amino acid and metal binding proteins). Biosorption can be defined as the ability of biological materials to accumulate heavy metals from wastewater through metabolically mediated or physiochemical pathways of uptake. Cyanobacteria are microalgae suggested to have some added advantages over other microorganisms because of their larger surface area, great mucilage volume with high binding affinity and simple nutrient requirements (Anjana *et al.,* 2007).

MATERIALS AND METHODS

Culture technique of the test blue green alga:

The pure and identified slant culture of *Nostoc muscorum* was obtained from Algal Laboratory, Botany Department, Faculty of Science, Tanta University. This slant cultures after being shaken with an amount of sterilized BG_{11} media was used as an inoculum for algal growth in liquid nutrient medium (BG_{11}) according to Rippka *et al.* (1979).

Liquid culture and growth condition:

The liquid culture was prepared by dispensing 300 ml of BG_{11} nutrient medium in 500 ml Erlenmeyer flasks then sterilized in an autoclave at 121°C and 1.5 atm. for 20 minutes. After cooling the nutrient medium in Erlenmeyer flasks, it was

inoculated by one cell or colony in sterilized laminar medium by sterilized capillary pipette. The culture flasks were aerated by air pumps and incubated at 30°C under continuous illumination provided from day light fluorescent tubes giving the photon flux density of 3000 lux. Algal cells were harvested approximately after 15 days by centrifugation and the fresh pellets were collected and rinsed three times by distilled water to remove traces of growth media before using them as fresh weight. (Katircioglu *et al.,* 2006).

Experiment Design:

During the season of growth (November and December), known number of seeds of *Trigonella foenum gracum* (cv. Baladi) were sterilised by 0.01% $HgCl_2$ for 1 min and washed thoroughly with distilled water and divided into four groups. Each group was sown in plastic pots (15 cm diameter and 10 cm depth) filled with 5 kg clay-sandy soil and 5 pots were used for each treatment. The first group of seeds was sown in untreated soil and considered as the first control; the second group was sown in soil supplemented with *Nostoc* (2.0 g kg^{-1} soil) and considered as the second control. The third group was sown in soil provided with different copper sulphate concentrations (0.4, 0.6, 0.8 and 1.0 g kg^{-1}soil) and the seeds of the fourth group were sown in a soil supplemented with a mixture of different copper sulphate concentrations (0.6, 0.8 and 1.0 g kg^{-1} soil) and *Nostoc* (2.0 g kg^{-1} soil). The seeds were left to grow and irrigated with tap water under greenhouse conditions. After 30 days of growth the plants were collected, washed with distilled water and separated into root and shoot. The fresh and dry weights of root and shoot were determined. Samples of three fresh leaves were kept frozen immediately for determination of the photosynthetic pigments and enzyme activities. Another four groups of seeds were sown in plastic pots (40 cm diameter and 45 cm depth) containing 20 kg clay-sandy soil and 5 pots were used for each treatment. Seeds of these four groups were treated as described above and left to grow for 60 days. After 60 days of growth plant samples were washed with distilled water, separated into root and shoot and measurement of fresh and dry weights and determination of pigments and enzymes activities were carried out as mentioned in the 30 days. In both stages 30 and 60 days, the remaining samples were kept in the oven at 80°C for determination of metabolic performances.

Estimation of Photosynthetic Pigments:

The plant photosynthetic pigments (chlorophyll a, chlorophyll b, and carotenoids) were determined after 30 and 60 days of growth using a spectrophotometric method as recommended by Arnon, (1949) for chlorophyll and Horvath *et al.* (1972) for carotenoids as adopted by Kissimon (1999). Pigment concentration was calculated as mg g^{-1} dry weight of leaves.

Photosynthetic Activity:

The rate of net photosynthesis was measured at both 30 and 60 day stages using an infrared gas analysis system (Botany Department, Faculty of science, Tanta University) by clipping a single leaf in a Parkinson leaf chamber of a portable ADC-LAC4 system (The Analytical Development Company Ltd, Hoddesdon, Herts, UK) at a photon flux density of about 250μEm^{-2} s^{-1} (PAR) as μ mol $m^{-1}s^{-1}$.

Preparation of enzyme extract and assay of Antioxidant Enzymes: .

A sample of 0.5 g green leaves was frozen, then homogenized in 8 ml of 50 mM cold phosphate buffer of pH7 (modified from Beauchamp and Fridovich, 1971). The homogenates were

centrifuged at 4000 rpm for 20 minutes. The supernatant was used as a raw extract for enzymatic assay.

Peroxidase (EC 1.11.1.7):

Peroxidase(POD) activity was measured according to **Kato and Shimizu (1987)**. The assay medium contained 0.1 M sodium phosphate buffer of pH 5.8, 7.2mM guaiacol, 11.8mM H_2O_2 and 0.1 ml enzyme extract. The reaction was initiated by the addition of H_2O_2 and the change in the absorbance was measured at 470 nm. Activity was calculated using the extinction coefficient (26.6 mM^{-1} cm^{-1} at 470 nm) for tetraguaiacol. Enzyme activity was expressed in units of µM of the substrate converted per minute per gram fresh weight $\mu M min^{-1} g^{-1}$ f. wt.

Superoxide dismutase (EC 1.151.1):

Superoxide dismutase (SOD) activity assayed was based on the reduction of nitro blue tetrazolium (NBT) by superoxide radicals to blue colour formazan was followed at 560nm. The SOD assay was carried out following the method of Beyer and Fridovich (1987). The reaction mixture was prepared by mixing 27 ml of 50 mmol/L potassium phosphate, pH 7.8, 1.5 ml of L-methionine (300mg/10ml), 1ml of nitro blue tetrazolium salt (14.4mg/10ml) and 0.75 ml of Triton X-100. Aliquots (1ml) of the mixture were delivered into small glass tube, followed by 20µl of enzyme extract and 10µl of riboflavin (4.4 mg/ 100ml). In the control tube, the sample was substituted by 20µl of buffer. The cocktail was mixed then the glass mixed tubes were immersed in a cylindrical glass container illuminated by two 20W fluorescent tubes and filled with three-fourth with clean water and maintained at 25°C for 7 minutes. Under the described conditions, the increase in absorbance without the enzyme extract was taken as 100 % and the enzyme activity was calculated by determining the percent inhibition per min. Fifty percent of inhibition was taken as equivalent to 1 unit of SOD activity.

Estimation of Carbohydrates:

The carbohydrate extraction and clarification of plant materials (root and shoot) was performed according to Naguib *et al.* (1968).

a) Determination of direct reducing value (DRV):

Monosaccharide was estimated in root and shoot of *Trigonella* (mg/g d. wt.) colorimetrically using modified Nelson reagent **(Naguib, 1964)**. The concentration of monosaccharide was calculated as mg glucose /g d. wt.

b) Determination of total reducing value (TRV):

Invertase enzyme was used in determination of TRV and was estimated as DRV. The difference between the value obtained (TRV) and that of the (DRV) gives an estimation of sucrose as mg glucose/g d. wt.

c) Extraction and estimation of polysaccharides (Starch):

Extraction and estimation of polysaccharides were carried out following the procedure adopted by (Naguib, 1963). The concentration of sugar was calculated as mg glucose /g d. wt.

Determination of Minerals content:

a. Extraction procedure:

The mixed acid digestion method was used for extraction element determination according to Allen *et al.* (1974). The extract was used to estimate sodium and potassium by flame photometer as described by Johnson *et al.* (1959) (Coming

Scientific Instruments, model 410) where calcium, copper and iron were estimated by the Atomic Absorption Flame Emission Spectrophotometer (Model Perkin Elmer 2380 Atomic Absorption Spectrophotometer).

b. Determination of Phosphorous:

Phosphorous was determined spectrophotometerically by the molybdenum blue method of Allen *et al.* (1974) using spectrophotometer (Model 4049 LKB Novasped). Phosphate content was determined as mg/ g d. wt. with reference to a standard calibration curve of standard phosphorus solutions.

Statistical analysis:

The obtained results were statistically analyzed using one and two ways of variance (ANOVA) to determine the degree of significance for the obtained variations by using treatments. All of the statistical methods used in this study were according to Bishop (1983), while the analysis was carried out by SPSS statistical package.

RESULTS

Root and shoot fresh and dry weights:

The changes in root and shoot fresh weights of *Trigonella foenum gracum* plants after 30 and 60 days of growth as influenced by different copper sulphate concentrations singly or mixed with *Nostoc muscorum* are presented in Figure 1. The results indicated that root and shoot fresh weights highly significant decreased with increasing the copper concentration, with the exception of a slight increase in their fresh weights at 0.4 Cu treatment. Application of *Nostoc* increased both root and shoot fresh weights at 30 and 60 days of growth. On the other hand, mixing *Nostoc* with copper had led to a pronounced increase in fresh weights of both roots and shoots compared with those of copper treatments (0.6, 0.8 and 1.0 g kg^{-1} soil) after 30 and 60 days of growth. It was found that at 30 and 60 days of growth root dry weight was highly significant increased by treatment the soil with copper concentration of 0.4 g kg^{-1} soil singly, whereas increasing copper concentrations beyond 0.4 g kg^{-1} soil gradually decreased dry weights of both root and shoot. Addition of *Nostoc* markedly increased dry weights of roots and shoots at 30 and 60days of growth when compared with the control. Mixing *Nostoc* with copper concentrations of 0.6, 0.8 and 1.0 g kg^{-1} soil, increased dry weights of roots and a slight increase of shoot dry weight in comparison to copper singly (Figure 2).

Photosynthetic activity:

The photosynthetic activity was slightly affected at different copper concentrations, with the exception of a reduction of the rate at 1.0 g kg^{-1} soil copper treatment after 30 and 60 days of growth. Addition of *Nostoc* singly or mixed with copper increased the photosynthetic activity when compared with the control (Figure 3).

Photosynthetic Pigments:

The data indicated that emendation of copper to the soil at a concentration of 0.4 g kg^{-1} soil induced a pronounced increase in chl.a, chl.b, at 30 and 60 days of growth, compared with the control. Increasing copper concentration beyond 0.4 g kg^{-1} soil clearly showed a decrease of chl.a, chl.b and carotenoid contents with increasing copper concentration at 30 and 60 days of growth compared with the control (Figure 4). Addition of *Nostoc* highly significant increased chl.a, chl.b and carotenoids content at 30 and 60 days as compared with the control. On the other hand, emendation of *Nostoc* to the soil with different concentrations of copper had recorded an increased content of chl.a,

chl.b and carotenoids compared with the corresponding values of Cu singly after 30 and 60 days of growth.

Antioxidant Enzymes:

After 30 and 60 days of growth, activity of both peroxidase (POD) and superoxide dismutase (SOD) was significantly enhanced with increasing copper concentration especially at 1.0g Cu/kg soil treatment (Figure 5). The POD and SOD activities in presence of *Nostoc* were reduced at 30 and 60 days of growth when compared with the control. However, inoculation of *Nostoc* with copper concentrations of 0.6, 0.8 and 1.0 g kg^{-1} highly significantly decreased both antioxidant enzyme activities compared with copper singly at 30 and 60 days of growth indicating the alleviating effect of *Nostoc* on Cu toxicity.

Carbohydrates content:

The different carbohydrate fractions at 30 and 60 days of *Trigonella* growth tended to increase remarkably of both roots and shoots at different copper concentrations singly with the exception of starch content which was decreased when compared with the control. Meanwhile, application of *Nostoc* singly, had led to a great increase in D.R.V and sucrose, at 30 and 60 days of growth, in root and shoot, respectively with respect to the control. The data also showed that mixing *Nostoc* with the test levels of copper concentrations (0.6, 0.8 and 1.0 g kg^{-1} soil) resulted in a pronounced increase in D.R.V. and sucrose content compared with its counterpart in copper. It is clear from the results that, application of *Nostoc* singly, decreased starch content in root and shoot, respectively as compared with the control. On the other hand, addition of *Nostoc* in a mixture with copper resulted in a pronounced decrease in starch content compared with its corresponding value in copper (Figurer 6).

Mineral content:

Potassium (K^+), calcium and phosphorous content was gradually decreased in root and shoot with increasing copper concentration with the exception of their increase in shoot at 0.4 Cu treatment when compared with the control. However, K^+ content in shoot was stable at 0.6 and 0.8 Cu treatments. Inoculation of *Nostoc* singly increased K^+ level in root and shoot, respectively, while it was slightly increased calcium content in root and markedly increases in shoot as compared with the control. On the other hand, mixing *Nostoc* with copper at concentrations of 0.6, 0.8 and 1.0 g kg^{-1} soil increased K^+, Ca^{2+} and phosphorous content in root and shoot, respectively compared with its corresponding values of copper singly. Moreover, the results indicated that iron content was gradually increased in root and shoot either when Cu was applied singly or mixed with *Nostoc muscorum.* However, the magnitude of increase was great in root, particularly with mixture of *Nostoc* and Cu at 0.8 and 1.0 Cu treatments compared with its counterpart in copper treatment. The data also revealed that copper content was increased parallely to the increase in Cu concentration both in root and shoot, but it was lower in shoot than root. At 0.4 Cu treatment, Cu was absent from the shoot, whereas it was present in root in a concentration comparable to that in the control. Application of *Nostoc* with Cu decreased Cu content in root and shoot at 0.6, 0.8 and 1.0 Cu treatments, respectively compared with its counterpart in Cu treatment (Figure 7).

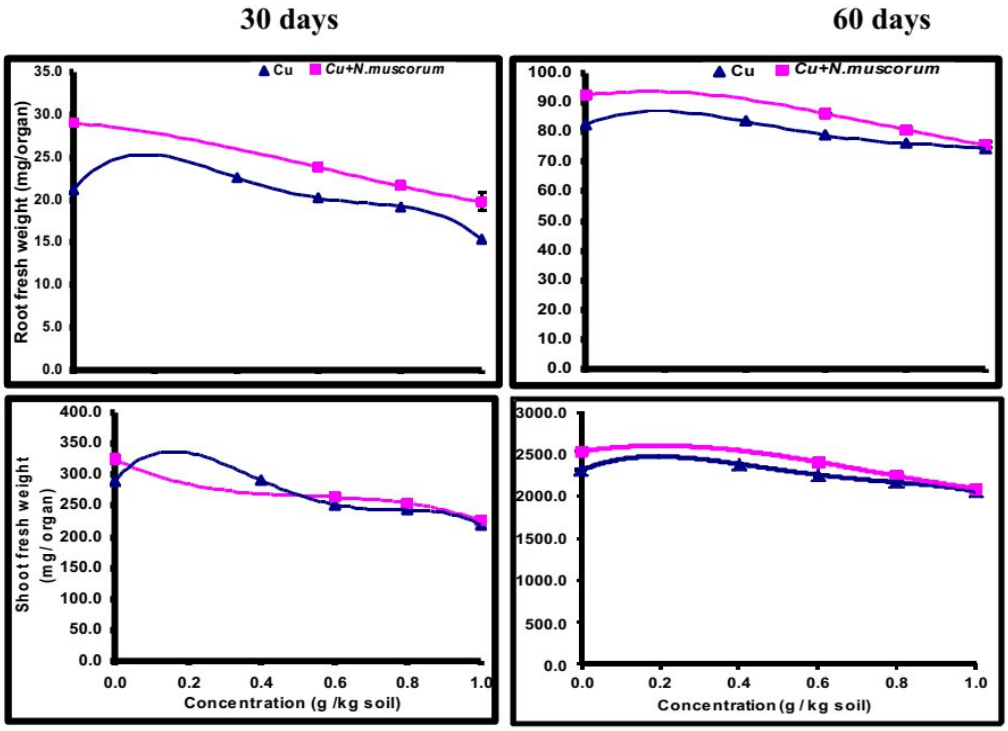

Figure 1. Effect of different concentrations of copper sulphate (g kg^{-1} soil) singly or mixed with *Nostoc muscorum* (2 g kg^{-1} soil) on root and shoot fresh weights (mg/organ) of *Trigonella foenum gracum* plants after 30 and 60 days of growth.

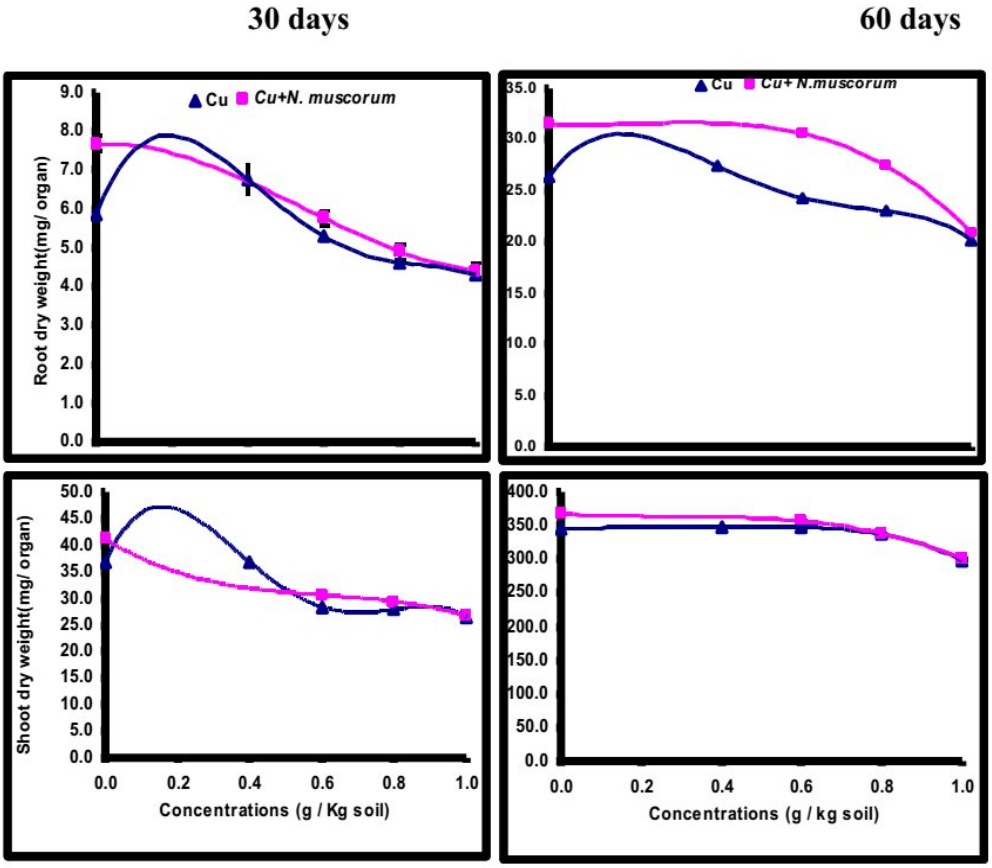

Figure 2. Effect of different concentrations of copper sulphate (g kg^{-1} soil) singly or mixed with *Nostoc muscorum* (2 g kg^{-1} soil) on root and shoot dry weights (mg/organ) of *Trigonella foenum gracum* plants after 30 and 60 days of growth.

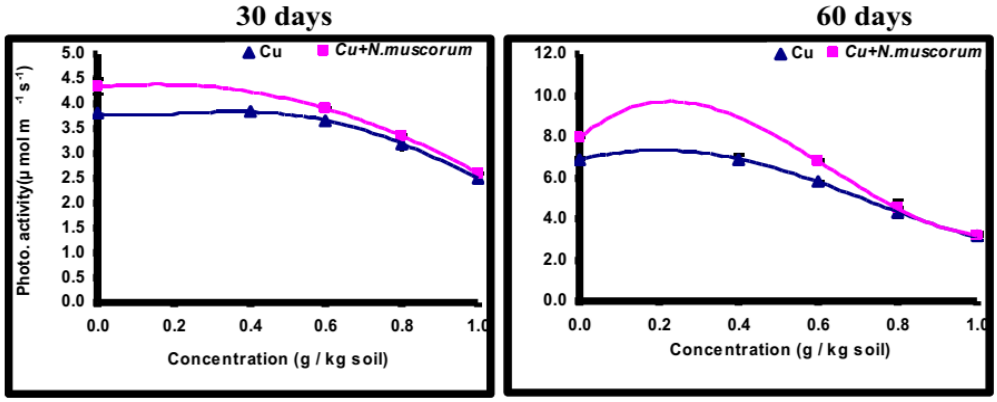

Figure 3. Effect of different concentrations of copper sulphate (g kg^{-1} soil) singly or mixed with *Nostoc muscorum* (2 g kg^{-1} soil) on photosynthetic activity (µ mol m^{-2} s^{-1}) of *Trigonella foenum gracum* leaves after 30 and 60 days of growth.

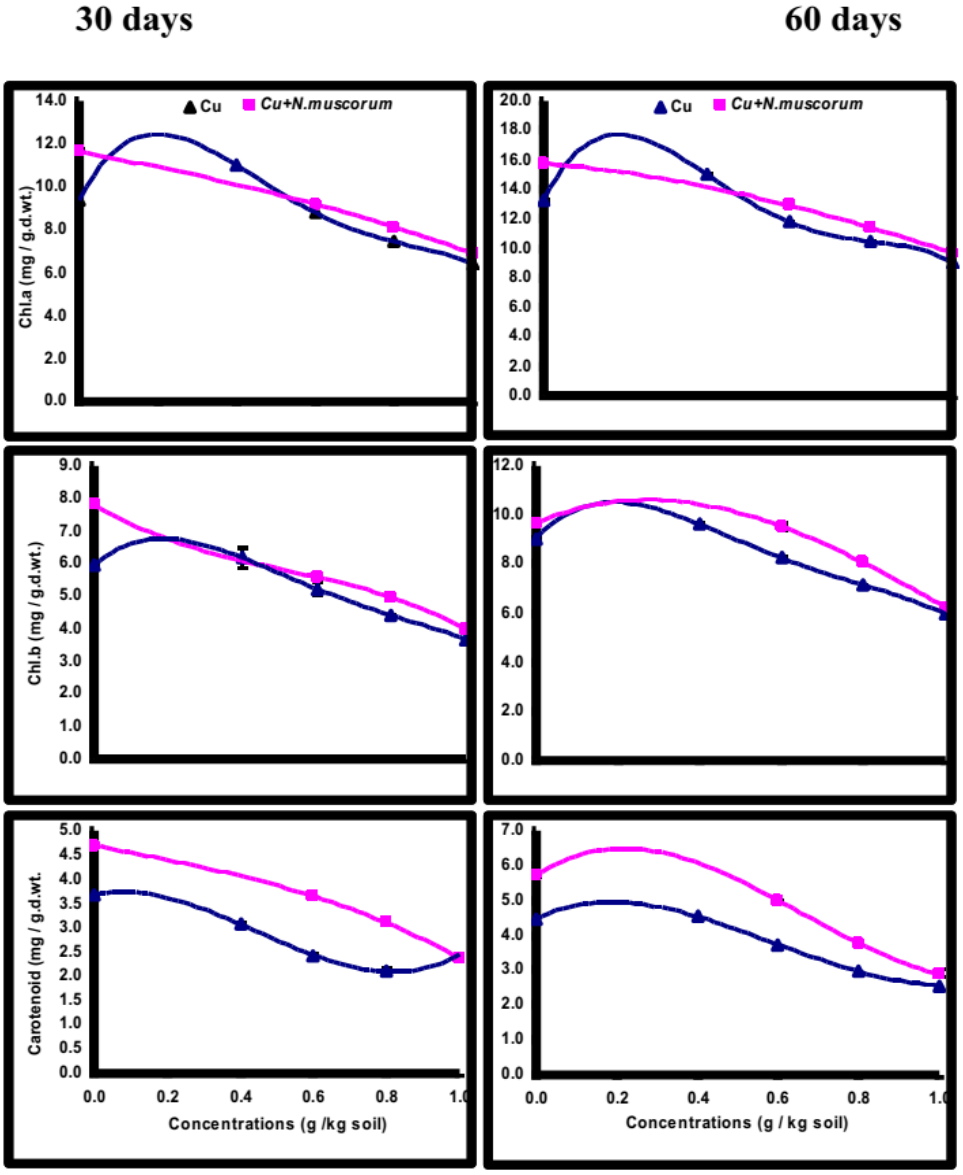

Figure 4. Effect of different concentrations of copper sulphate (g kg^{-1} soil) singly or mixed with *Nostoc muscorum* (2 g kg^{-1} soil) on photosynthetic pigments (mg/g d. wt.) of *Trigonella foenum gracum* leaves after 30 and 60 days of growth.

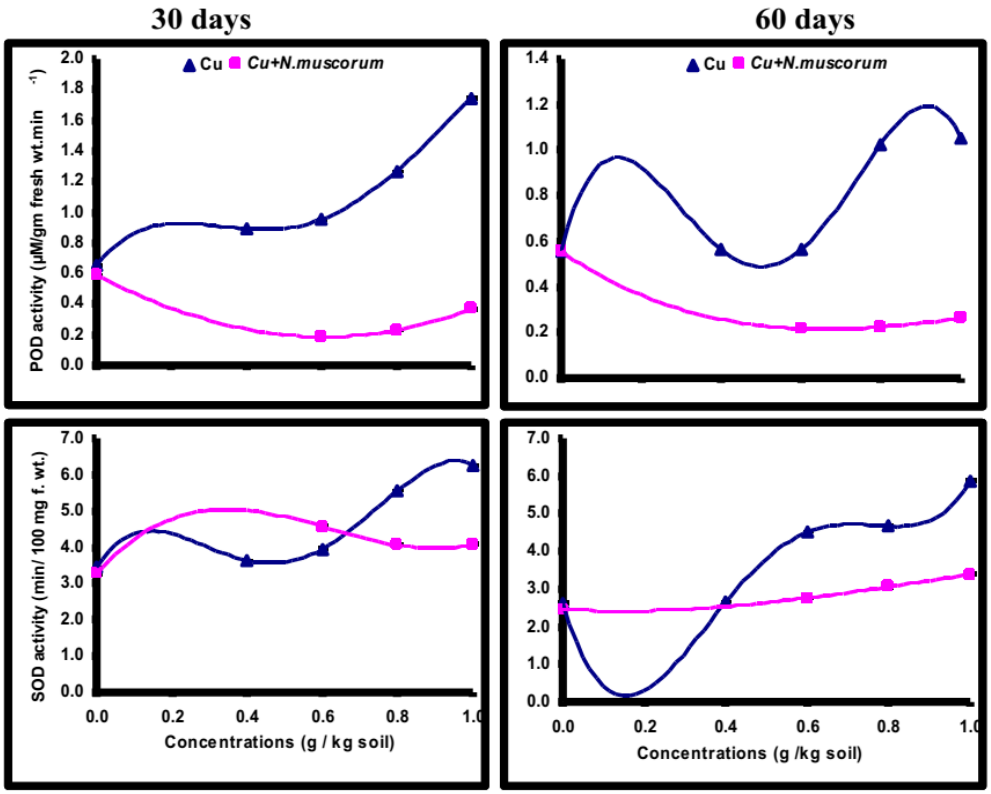

Figure 5. Effect of different concentrations of copper sulphate (g kg^{-1} soil) singly or mixed with *Nostoc muscorum* (2 g kg^{-1} soil) on the activities of peroxidase and superoxide dismutase of *Trigonella foenum gracum* plants after 30 and 60 days of growth.

DISCUSSION

Results of this study indicated that root and shoot fresh and dry weights of *Trigonella foenum gracum* plants were significantly decreased with increasing copper concentration from 0.4 to 1.0 g kg^{-1} soil. These results were in agreement with those of Nenova *et al.* (2009) who showed that both root and shoot fresh weights of two lines of wheat were decreased with increasing copper concentration (10^{-5} and 10^{-6}M). The decline in biomass production (fresh and dry weights) in the present study could be explained, in part, by inhibition of both cell elongation and division by heavy metals which may be related to damaged root epidermal cells and root cell membranes (Tanyolac *et al.*, 2007). The fresh and dry weights of root and shoot of *Trigonella* plants were significantly increased by application of *Nostoc muscorum* to the soil either singly or mixed with copper. The increase in shoot fresh and dry weights was reported by presoaking of cotton seeds in cyanobacterial filtrate of *Nostoc muscorum* (Likhitkar and Tarar, 1995) and sorghum grains in *Anabaena oryza* filtrate (Dowidar, 2002). Also, Haroun and Hussein (2003) showed that fresh and dry weights of *Lupinus termis* were significantly increased by soaking the seeds in *Cylindrospermum muscicola* and *Anabaena oryza* culture filtrates separately.

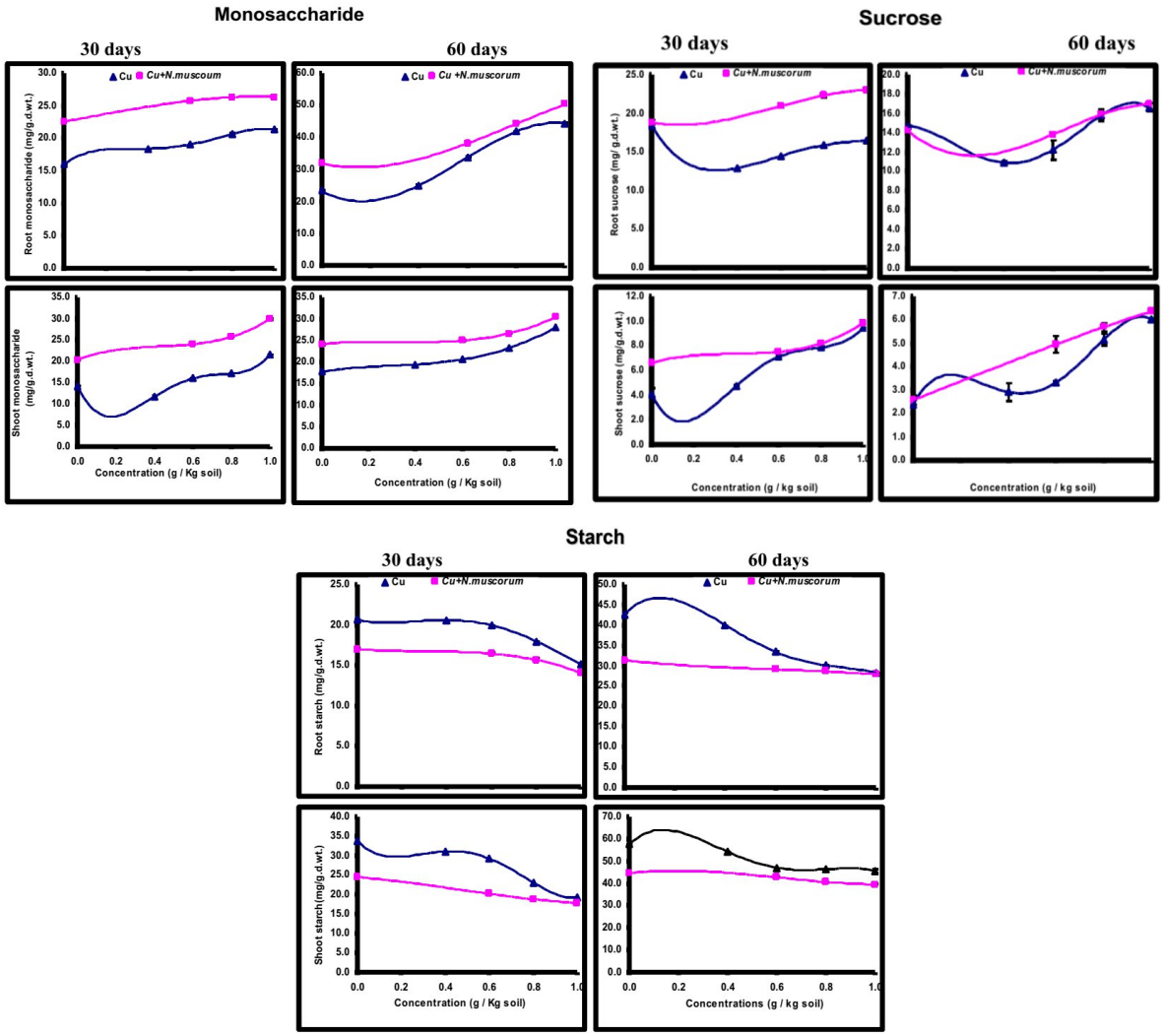

Figure 6. Effect of different concentrations of copper sulphate (g kg^{-1} soil) singly or mixed with *Nostoc muscorum* (2g kg^{-1} soil) on carbohydrates content (mg/g d. wt.) of *Trigonella foenum gracum* plants after 30 and 60 days of growth.

Moreover, Osman *et al.* (2010) demonstrated that the both root and shoot dry weights of pea seedlings were increased by addition of *Nostoc entophytum* and *Oscillatoria angustissima* as fresh material to the soil. Cyanobacteria are known to produce different growth regulators such as auxin-like (Soliman *et al.,* 2000), cytokinin-like (Rodgers *et al.,* 1979) or gibberellin substances like (Singh and Trehan, 1973). In addition, increased amylase activity induced by gibberellin or by phenolic compounds present in the algal extract (Monerri *et al.,* 1986) may also account for the promotion of germination and growth.

In this study, there was a gradual decrease in the photosynthetic pigments (chl.a, chl.b and carotenoids) with increasing copper stress. These data were in accordance with those obtained by Martins and Mourato (2006) and Yurekli and Porgali (2006) on tomato and bean plants respectively, in which they reported that chlorophylls content (chl.a and chl.b), were severely affected by copper toxicity in the first plant and decreased in the second at 100 µM Cu^{2+}.

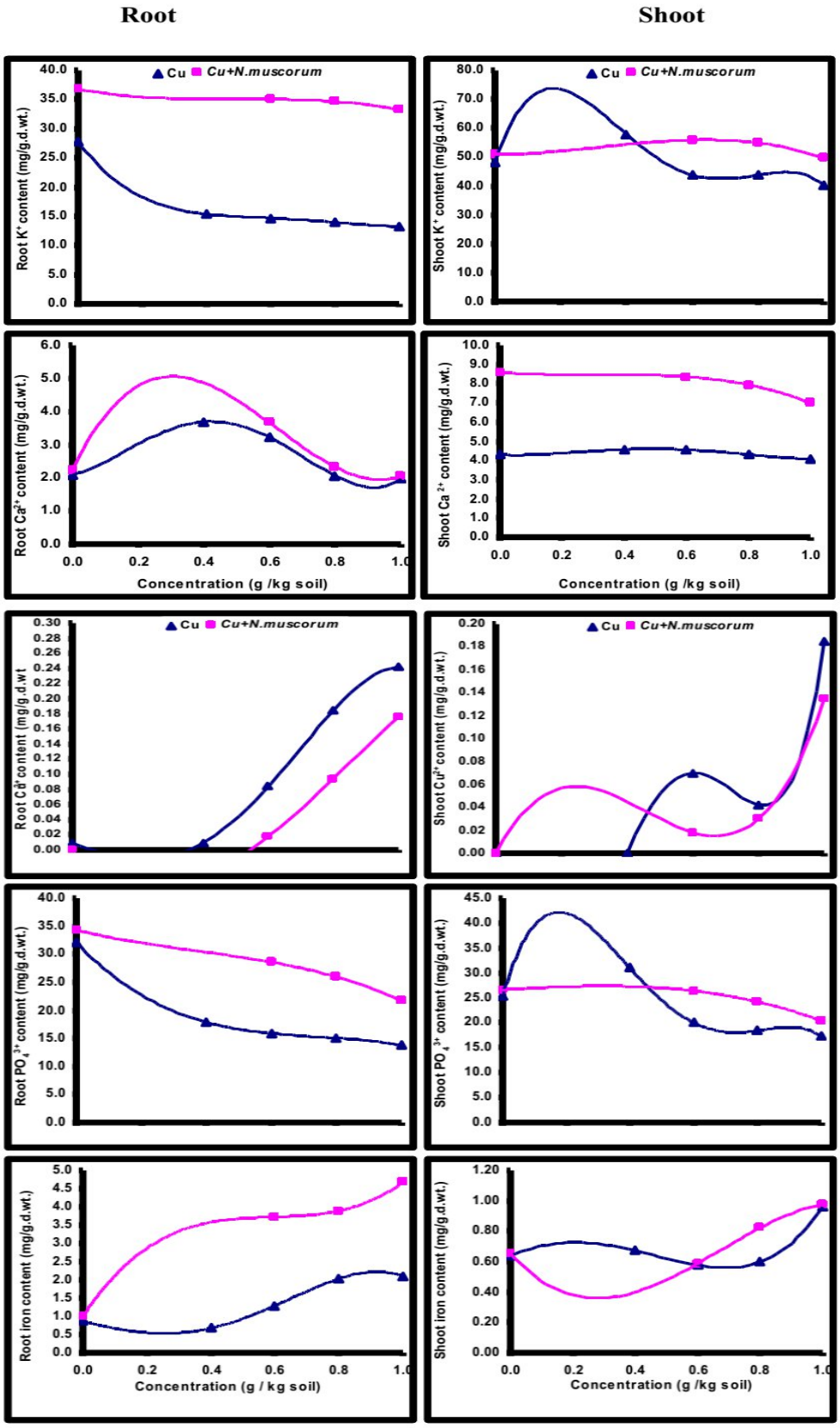

Figure 7. Effect of different concentrations of copper sulphate (g kg^{-1} soil) singly or mixed with *Nostoc muscorum* (2 g kg^{-1} soil) on mineral contents (mg/g d. wt.) of *Trigonella foenum gracum* plants after 60 days of growth.

The decrease in the content of chl.a and chl.b with the increase in Cu concentration in this study is probably related to inhibition of pigment accumulation and retardation of chlorophyll integration into the photosystems (Caspi *et al.,* 1999). Inhibition of pigment accumulation may result from chlorophyll degradation and destruction by Cu via the effect of Cu on chlorophyll binding protein. Moreover, reduction in chlorophyll content may be attributed to Cu-induced blocking of key enzymes acting in chlorophyll synthesis via its interference (oxidation) with functional SH-group of these sulfhydroxyl-requiring enzymes. These enzymes are ALA-dehydrates (Fernandes and Henriques, 1991), AL`-synthase, and photochlorophyllide reductase (Mohanty *et al.*, 1989). The present study showed that the carotenoids content was significantly decreased under higher Cu concentrations in which case was in accordance with Nenova *et al.* (2009) of two lines of wheat. This may be a due to degradation of β-carotein and formation of Zeaxanthins as a result of oxidative stress which is a marker of the tissue aging, induced by stress factors of environment (Hendry and Grime, 1993). Data of the concurrent investigation revealed that photosynthetic activity was reduced with the increase of Cu concentration. Consistent with our results, it has been reported that excess copper affects photosynthesis in an indirect way, causing a slow down of electron transport and decreased efficiency of photophosphorylation (Gora *et al.,* 1985). The reduction of photosynthetic activity may be attributed to oxidative stress induced by Cu and PSII is a possible target for inhibition by reactive oxygen species (Kim *et al.,* 1993). Our results indicated that photosynthetic pigments were increased by addition of *Nostoc* to soil in a mixture with copper. This result was consistent with Bhowmik *et al.* (2010) and Lakshmi and Annamalai (2008) who found that the chlorophyll content of two species of *Phaseolus* and *Withania somnifera* was markedly increased after addition of blue green algae. The increase of chlorophyll content by mixing *Nostoc* with copper may be related to presence of phenolic compound (Yang *et al.*, 2004) or to growth regulators (GA_3) present in algal suspension which stimulate accumulation of chlorophyll via the increase of Mg^{2+} and/or inhibition of chloropyllase activity (Dreakeiwicz, 1994). On the other hand, carotenoids content was increased in the present study after addition of *Nostoc* in a mixture with copper. This result was consistent with Osman *et al.* (2010) who found that carotenoids content of pea seedlings was significantly increased in response to treatment with two blue green algae. Furthermore, results of the present investigation showed that photosynthetic activity was increased by mixing *Nostoc* with copper as compared with copper singly. This result was in agreement with Dowidar (2002) who claimed that photosynthetic activity of sorghum plant was stimulated by soaking its grains in *Anabaena oryza* culture filtrate and it was correlated with an increase in chlorophyll. Stimulation of the photosynthetic activity in this study may be attributed to the increase of chlorophyll due to presence of growth regulators (GA_3) produced by algae (Abd El-Baky *et al.,* 2008).

Copper, a redox active metal, can catalyze the formation of harmful free radicals such as hydroxyl, peroxyl and alkoxyl radicals, resulting in oxidative stress. The present data showed a significant increase in the activity of peroxidase (POD) enzyme with the increase of copper stress. This result was in agreement with Posmyk *et al.* (2009) and Sharma and Singh (2013) who found a significant increase

of POD activity of red cabbage and chickpea seedlings, respectively stressed with Cu^{2+}. On the other hand, many studies have showed that excess Cu may cause rise or drop in peroxidase activity, the effect being both Cu concentration-and time–dependent variable according to plant species (Peng *et al.,* 2006). POD activity is always considered a useful biomarker for sublethal metal toxicity in plant species (Passardi *et al.,* 2005). The recorded rise of POD activity, in this study was a sign of oxidative stress. There has been evidence that Cu induces a rise in endogenous H_2O_2 level in leaves. H_2O_2 will rapidly diffuse across the membrane and be toxic as it acts as both an oxidant as well as a reductant (Foyer *et al.,* 1997). Passardi *et al.* (2005) suggested that increased POD activity in *Jatroph curcas* might be sufficient to protect protein, chlorophyll and lipids of some parts of plants against ROS attack. The present work evidenced an increased SOD activity in response to excess copper. This result was consistent with those obtained by Gao *et al.* (2008). Copper facilitates the production of superoxide anion due to its redox nature. Therefore, the production of O_2 anions, whose detoxification is necessary for the growth of organisms, is increased, and become converted into peroxide by the increased activity of SOD(Sultan *et al.,* 2007) which explains the recorded rise of SOD activity in this study. Our findings suggested that SOD is involved in the oxidative stress defence system of *Trigonella* playing a protective role by detoxifying free radicals Li *et al.* (2009). Both POD and SOD activities were reduced by addition of *Nostoc muscorum*. The recorded reduction in the activity of both enzymes might be due to the increase of carotenoids and calcium. Carotenoids are non-enzymatic antioxidant substances, which scavenge singlet oxygen in the PSII reaction centre and protect membrane lipids against peroxidation (Havaux *et al.,* 2005a). Also, the role of Ca^{++} in antioxidant enzyme signal transduction was reported by Agarwal *et al.* (2005) who demonstrated that Ca^{++} acts as a second messenger and causes a transient increase in H_2O_2, which in turn induces antioxidant enzyme activity leading to a decrease in ROS on long- time basis.

Presence of heavy metals alter carbohydrate accumulation and distribution in plant. The results indicated an increase in the carbohydrate fractions with increasing copper concentration with the exception of starch. This result was compatible with that of Deef (2007) who found that carbohydrate fractions increased under copper treatment (50-3200 ppm) as compared with the control, except polysaccharides, which were decreased with increasing copper concentration. The inhibitory effect of Cu on the polysaccharides may be attributed to the increase of respiration of the plant organs. Meanwhile, the increase of D.R.V (accumulation of reducing sugars) is related to enhancement of amylolytic activity by heavy metal, which leads to an increase of soluble sugar than polysaccharides (Mohamed, 1994). The increase of soluble sugars in the root than shoot could possibly provide an adaptive mechanism in maintaining favourable osmotic potential (Verma and Dubey, 2001). The present results indicated that the addition of *Nostoc muscorum* in a mixture with Cu^{2+} increased the carbohydrate contents with the exception of starch. This result was in harmony with Osman *et al.* (2010) who found that the direct reducing value (D.R.V) and total reducing value (T.R.V) of pea seedlings were significantly increased by inoculation of soil with two blue green algae. The marked increase in the carbohydrate fractions of *Trigonella* plants, which was associated with

enhancement of the photosynthetic rate, may be attributed to the increase of the rate of photosynthetic electron transport and stimulation of pigment biosynthesis.Growth bioregulators of cyanobacterial filtrate may be involved directly or indirectly in saccharides and nitrogen metabolism (Haroun and Hussein, 2003).

Excess heavy metals usually lead to the loss of important mineral nutrients. The present work showed a decrease of the mineral content of root and shoot of *Trigonella* (K, Ca and P) with the exception of Cu and Fe. This result was in agreement with those of Manivasagaperumal *et al.* (2011) who demonstrated that higher copper concentration decreased the content of K, Ca, P and N with the exception of Fe and Cu. The slight decrease of K content recorded in the shoot may be due to K efflux as part of Cu tolerance mechanism. The recorded decrease of N, P and Ca in the shoot of *Trigonella* exposed to copper may be due to increased competition and/or to breakdown of membrane function (Deef, 2007). Excess copper resulted in lowering phosphorus content of *Trigonella* plant and revealed a close relationship between phosphorus and copper. High concentration of copper suppresses phosphorus metabolism by lowering the content of inorganic phosphorus via P-Cu interaction mechanism of *Trigonella* (Manivasagaperumal *et al.,* 2011) suggesting a negative correlation between copper and phosphorus (Mateos-Naranjo *et al.,* 2008). Fe content was increased in the root and shoot of *Trigonella* in response to Cu treatment and this was in harmony with Kováčik *et al.* (2009) who found that Fe content increased in root of *Matricaria chamomilla* in response to Cu and Cd metals. Copper content was increased in root and shoot with increasing Cu concentration. This result was in agreement with Martins and Mourato, (2006) who postulated that Cu accumulated in roots and leaves of tomato, respectively with time and increase of Cu concentration. The accumulation of more Cu in root than shoot of *Trigonella* may be due to prevention of translocation of phytotoxic amounts of heavy metals from the roots to the shoots, which is one of the mechanisms of Cu tolerance that plant may use (Fernandes and Henriques, 1991). The root accumulated higher copper content than shoots because (i) they are the first organ that contacts the metal, (ii) Cu has low mobility inside the plant and (iii) it may be a consequence of the preferential accumulation in roots (Panou– Filotheou and Bosabalidis, 2004). The increased Cu content of *Trigonella* shoot may be related to lateral transport, resulting from Cu diffusion into the xylem parenchyma. Total P content increased with mixing *Nostoc* with Cu. The increase in the P content may be partially attributed to production of organic acids produced by variety of cyanobacteria, leading to conversion of non-available P into available P. Soil microorganisms can also make P available to plant by producing chelating substances, which lead to solubilization of phosphates (Halder *et al.,* 1991). In addition, cyanobacteria may be a reservoir of P, which releases P due to cell death and lysis (Yanni, 1991).

In this study, *Nostoc muscorum* proved to have the efficiency of removal of the toxic level of copper from the soil, and this was in agreement with El-Sheekh *et al.* (2005) who found that *Nostoc muscorum* and *Anabaena subcylindrica* had efficiency in removing heavy metals (Cu, Co and Pb) from sewage and industrial waste water effluents. The efficiency of metals absorption by *cyanobacteria* depends on the nature and charge of the cell wall polysaccharides, which contain high

amount of uronic acids, and consequently, a high copper complexing capacity compared to the less charged polysaccharides inside the cell. Moreover, carboxyl groups on algal cell biomass are also responsible for binding to various ions. In addition, cyanobacterial cells may respond to metals such as copper, lead and cadmium through passive accumulation in cells. Thus, they remove harmful metals from the environment (Gardea-Torresdey *et al.,* 1990).

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