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Role of cyanobacteria in amelioration of toxic effects of copper in *Trigonella foenum gracum*

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Abstract

The present work aimed to study the effect of different concentrations of copper sulphate (0.4, 0.6, 0.8 and 1.0 g kg^{-1} soil) on *Trigonella foenum gracum* and assessing the role of *Nostoc muscorum* when added at concentration of 2.0 g kg^{-1} soil as live pellet or mixed with copper at concentrations of 0.6, 0.8 and 1.0 g kg^{-1} soil. This experiment was conducted in green house and the plants were grown in clay-sandy soil (2:1 w/w) amended either with different copper concentrations or *Nostoc* mixed with copper. The influence of *Nostoc* on *Trigonella* plant in presence of copper was evident in the increase of its yield parameters compared with copper treated plants. Biochemical analysis of the seeds revealed that the content of total carbohydrate (111.9 mg/g dry weight), total soluble protein (147.6 mg/g dry weight) and total lipids (68.6 mg/g dry weight) were significantly increased when *Nostoc* was applied with copper , whereas, total alkaloids (11.0 mg/g dry weight) and phenolics (10.6 mg/g dry weight) were significantly decreased. Protein profile of seeds indicated de novo synthesis of two polypeptides with molecular weight of 44 and 67 KDa which may be associated with the tolerance of *Trigonella* to copper toxicity.

Keywords: alkaloids, *Nostoc muscorum,* phenolic compounds, protein pattern, yield parameters.
Abbreviations: Cu_copper, PAGE_polyacrylamide gel electrophoresis, PMSF_phenyl methyl sulphonyl fluoride, ANOVA_analysis of variance.

Introduction

Copper (Cu) is one of heavy metals, although it is an essential microelement through interference with numerous physiological processes (Lanaras et al., 1993). When 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). Sonmez et al. (2006) studied the effect of copper applications as CuSo4. They applied copper in three different levels (10, 1000 and 2000 mg Cu Kg^{-1}) to a calcareous soil and measured the yield and growth of tomato plants. They found that the total yield, fruit number, root dry weight and plant height were decreased with increasing Cu application to soils. Deef (2007) showed that the application of copper at concentrations of 50 and 200 ppm gradually increased the sugar fractions. However, the high concentrations gradually diminished the sugar fractions. Zhang et al. (1996) showed that exposure of spinach plants to excess copper resulted in a significant decrease in the acyl lipid contents and induced changes in the lipid and fatty acid composition in the chloroplast membranes. Deminevska-kepova et al. (2004) exposed barely plants to excessive supply of Cu (1500 μM). They found that the highest Cu concentration (1500 μM) reduced the total soluble protein of the leaf. Ahsan et al. (2007) showed that increasing copper concentration induced significant changes in the protein pattern of germinated rice grains. The SDS-PAGE revealed 25 protein spots differentially expressed in copper treated samples. Posmyk et al. (2009) investigated the change in phenolic compounds content in red cabbage seedlings exposed to copper stress (0.5 and 2.5 mM). They found that the level of phenolic compounds increased in both low (o.5 mM) and high (2.5 mM) copper concentrations. Biosorption can be defined as the ability of biological materials to accumulate heavy metals from waste water through metabolically mediated or physiochemical pathways of uptake (Volesky and Holan, 1995). 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). The exocellular polysaccarides of *Nostoc* had been suggested to alleviate the heavy metal toxicity by biosorption (Dephilippis et al., 2003). El Sheekh et al. (2005) investigated the ability of *Nostoc muscorum* and *Anabaena subcylindrica* for removal of heavy metals from sewage wastewater. They found that Nostoc muscorum was able to remove Cu^{2+}, Co^{2+}, Pb^{2+} and Mn^{2+} from wastewater by 64.4, 22.2, 84.6 and 64.1% respectively. This investigation aimed at assessing the influence of *Nostoc muscorum* on the inhibitory effects of copper stress imposed on *Trigonella foenum graecum* as manifested in its growth and some metabolic activities as a trial to alleviate the copper toxicity.

Results

All the yield parameters of *Trigonella* seeds represented by the number of pods per plant, number of seeds per pods, weight of pods per plant, weight of seeds per pods and weight of 1000 seed were significantly decreased by the gradual increase in copper concentration compared with the control (Fig 1). Application of *Nostoc* in mixture with copper had an ameliorative effect on copper stress exerted on all the previous yield parameters. The content of total carbohydrate and total soluble protein of the seeds of the yield were increased with increasing the copper concentration, while, the

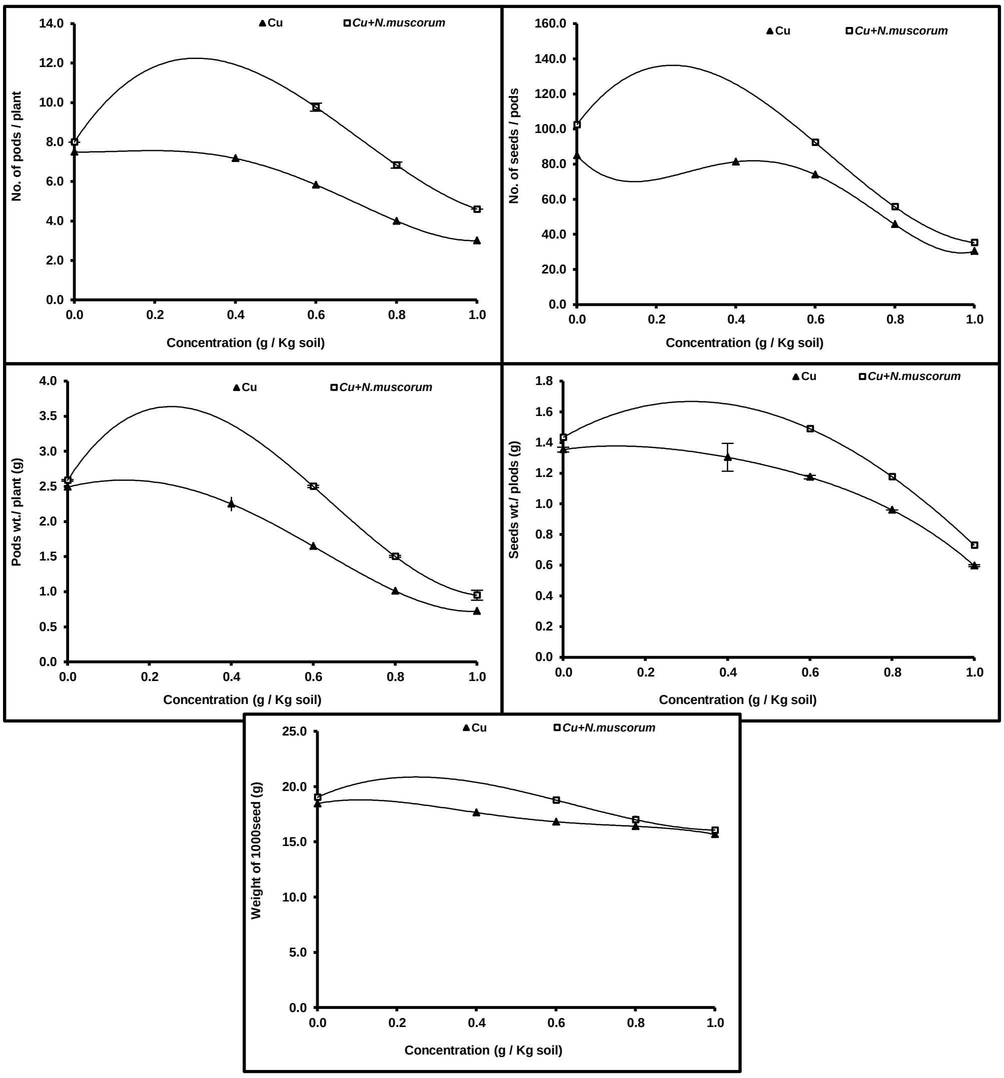

Fig 1. Effect of different concentrations of copper sulphate (g kg^{-1} soil) singly or mixed with *Nostoc muscorum* (2 g kg^{-1} soil) on yield parameters (No. of pods/plant, No. of seeds/pods, Pods weight/plant, Seeds weight/pods and Weight of 1000 seed) of *Trigonella foenum gracum* seeds.

content of total lipids and total protein were decreased parallel to the increase of copper concentration when compared with the control. Inoculation of *Nostoc* in mixture with different copper concentrations significantly increased the content of total carbohydrate, total soluble protein and total lipids when compared with its counterpart in copper treatment. The total protein content was significantly increased only at copper concentration of 0.6 g kg^{-1} soil mixed with *Nostoc* but decreased at copper concentrations of 0.8 and 1.0 g kg^{-1} soil mixed with *Nostoc* when compared with its corresponding value of copper treatment (Fig 2). Figure 2 also showed that the content of total alkaloids and total phenolics of the seeds were gradually increased with increasing the copper concentration either singly or in a mixture with *Nostoc.* However, the content was decreased in presence of *Nostoc* as compared with its counterpart of copper treatment. The protein profile of *Trigonella* seeds revealed the appearance of two newly formed polypeptides with molecular weight of 44 and 67 KDa at all copper treatment either singly or mixed with *Nostoc*. Moreover, treatment of 1.0 g kg^{-1} copper mixed with *Nostoc* resulted in the disappearance of two polypeptides with molecular weight of 32 and 33 KDa present in the control (Fig 3).

Discussion

Heavy metals make a significant contribution to environmental pollution as a result of human activities (Nedelkoska and Doran, 2000). Elevated levels of both essential and non-essential heavy metals in the soil are known to cause curtailing of nearly all growth parameters (An, 2004) and productivity (Wu et al., 2004). Excess copper negatively affects growth of the rice plant and the damage was more severe in root than shoot (Thorny et al., 2012). Our results revealed that the yield parameters of *Trigonella* seeds decreased with increasing copper concentration. This was in agreement with Kasim (2006) who reported that total number of grains / plant and weight of 1000 grains of *Sorghum bicolor (L.)* Moench were severely reduced in response to 10^{-5} M $CuSO_4$. The decrease in yield parameters, in our study may be attributed to change in the structure and consequently the function of the vascular and stomatal apparatus (Kasim, 2006).

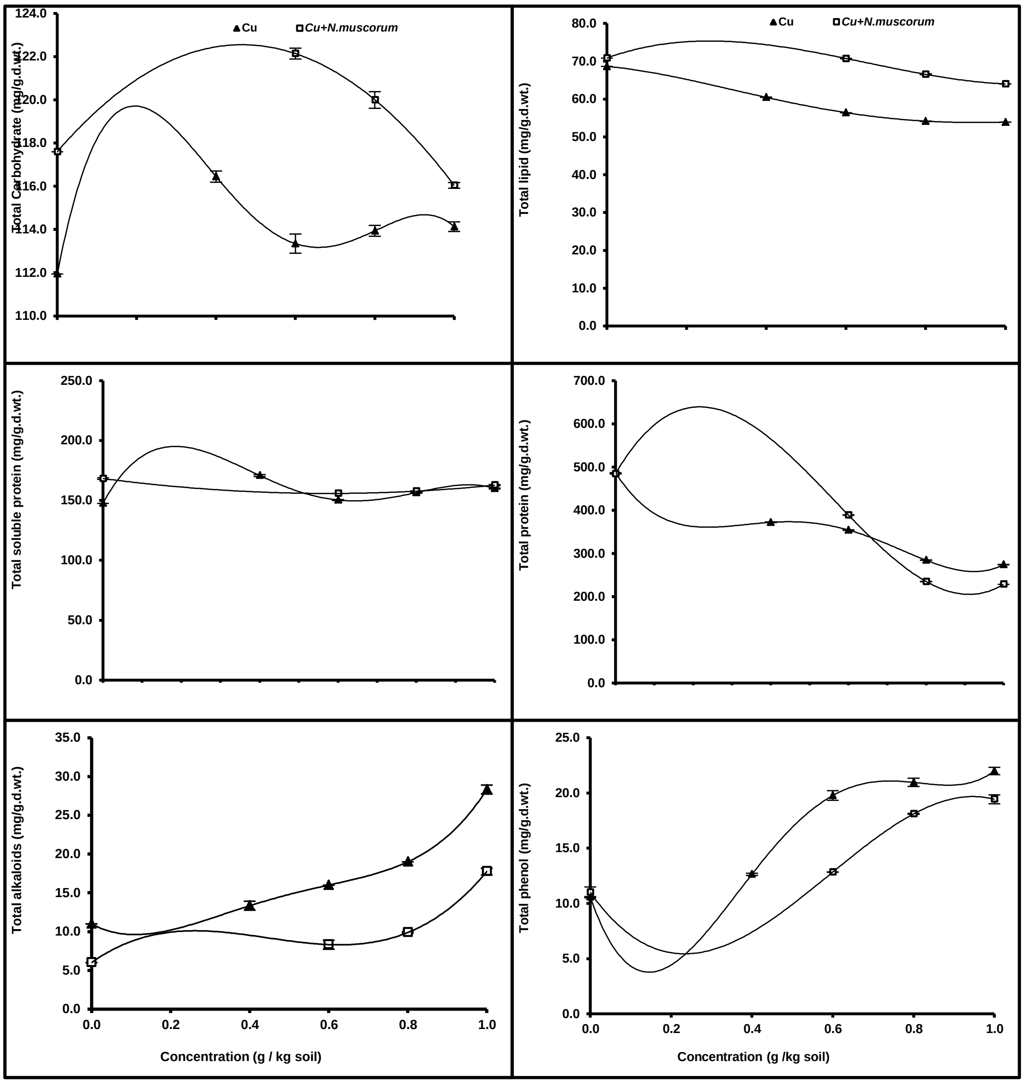

Fig 2. Effect of different concentrations of copper sulphate (g kg^{-1} soil) singly or mixed with *Nostoc muscorum* (2 g kg^{-1} soil) on total carbohydrate, total lipid, total soluble protein, total protein, total alkaloids and total phenol (mg/g dry weight) of *Trigonella foenum gracum* seeds.

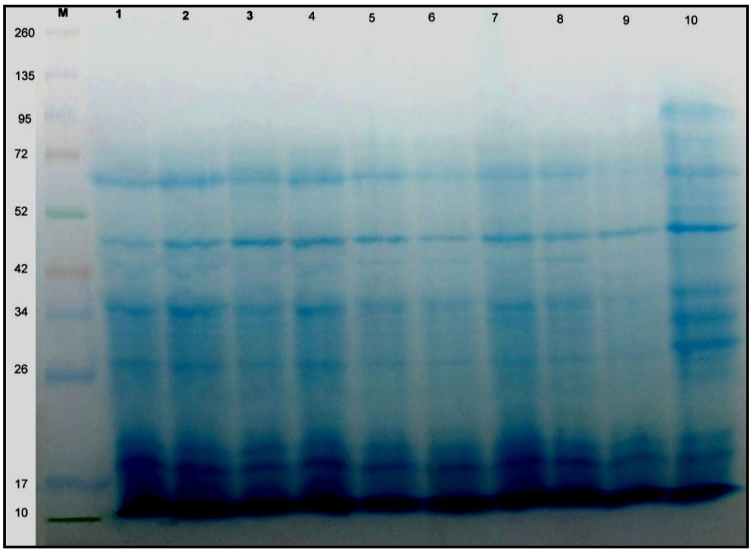

Fig 3. SDS-PAGE of protein extracts of *Trigonella foenum gracum* seeds under different copper sulphate concentrations (g kg^{-1} soil) singly or mixed with *Nostoc muscorum* (2 g kg^{-1} soil). Lane (M) = Marker. Lanes: 1=Control, 2= 0.4 Cu, 3= 0.6 Cu, 4=0.8Cu, 5= 1.0 Cu, 6= 00+ *N .muscorum*, 7= 0.6+ *N .muscorum*, 8= 0.8+ *N .muscorum*,9=1.0+ *N .muscorum*

This may affect uptake of essential nutrients such as N, P and K which leads to a consequent decrease in growth parameters (Gangamrutha, 2008). The present work showed also higher yield parameters with application of *Nostoc* compared with those of copper treatment. This increase in yield parameters may be related to the growth promoting substances secreted by blue green algae as extracellular products (Ordog et al., 2004). Supplementation of the soil with different copper concentrations induced a highly significant increase in total carbohydrate. This may be associated with the increased content of soluble sugars via the enhanced amylolytic activity (Mohamed, 1994). The present results agreed with those of Roitto et al., (2005) who found an increase of soluble sugars of *Pinus sylvestris L*. when exposed to copper. Coupling *Nostoc* with copper increased the carbohydrate content particularly the soluble sugars in the seeds due to the direct effect of growth regulators (GA3) on photosynthesis or their indirect effect on the amylolytic enzymes. Lipids are the most effective source of storage energy, function as insulators of delicate internal organs and hormones and play an important roles as the structural constituents of most of the cellular membranes (Singh et al., 2002). The total lipids of *Trigonella* seeds were decreased with increasing copper concentration. The decrease may be due to an enhanced rate of catabolic and/ or suppression of lipid bio-syntheis induced by copper.Olga et al., (2012) found that copper stimulated lipid peroxidation and enhanced membrane permeability and made changes of lipids in the membranes of chloroplasts, mitochondria and microsomes. The soluble protein content in the plant cells is an important indicator of their physiological state in response to a wide variety of stressors (Singh and Tewari, 2003). In the present study, total soluble protein content of *Trigonella* seeds was increased by increasing copper concentration, while the total protein content was decreased. The decrease in the content of total protein with concomitant increase of total soluble protein of the seeds was most probably due to enhanced activity of proteolytic enzymes (Antonov et al., 1981) and degrading storage protein into its subunits thus increasing soluble protein on the exposure of total protein (Matto and Conlon, 1985). Addition of *Nostoc* either singly or in a mixture with Cu increased total soluble and total protein content in the seeds. This increase may be due to the increase of the nitrogen fixation and nitrate reductase activity of cyanobacteria or uptake of NH4, amino acids and peptides by *Trigonella* seeds produced by *Nostoc* (Haroun and Hussein, 2003; Osman et al., 2010). The total alkaloid in the present study was increased with increasing copper concentration. This increase may be attributed to metal stress, which may induce the alkaloid accumulation, which act as self-defense of plant (Fathalla et al., 2011). The decrease of alkaloid content when *Nostoc* mixed with copper may indicate the alleviation of the inhibitory effect of copper stress on *Trigonella*. The present investigation showed that addition of *Nostoc* to soil contaminated with Cu decreased phenolic compounds in the seeds and alleviated the inhibitory effect of copper. This decrease may be attributed to phenolic compounds produced by algae and transported to the seeds of *Trigonella*. These transported phenolic compounds may be effective in the inhibition of all phases of the peroxidative process: first neutralizing free radicals, then blocking the peroxidation catalysis by oxidizing agent and finally through interruption of lipid radical chain reactions (Cervato et al., 2000; Athukorala et al., 2006) consequently decreasing phenolic compounds in *Trigonella* seeds. Expression of stress proteins in plants is known to be an important adaptive strategy of environmental stress tolerance. They are highly water-soluble and heat stable, associate to cytoplasmic membranes and organelles (Wahid and Close, 2007). In the present study, it was possible to demonstrate that the stress protein expression was induced in the seeds of *Trigonella* exposed to copper singly or combined with *Nostoc*. Some of these induced stress protein bands were newly synthesized in the treated plant and some others disappeared indicating that such heavy metal is highly effective inducing a major re-shuffle of the protein profile (Kasim, 2005). The two newly formed polypeptide bands in the seeds determined under copper stress either singly or combined with *Nostoc* were 44 and 67 KDa. The disappearance of protein bands in response to *Nostoc* may be attributed to changes in the gene expression (Popova et al., 1995) caused by hormones of the cyanobacteria. Disappearance of the two polypeptides with molecular weight of 32 and 33 KDa at treatment of 1.0 g kg^{-1} copper mixed with *Nostoc* may be explained either by delay of protein synthesis (Krupa, 1988) or precipitation of these protein as a result of heavy metal treatment. In addition, the disappeared proteins may act as a precursor for some antioxidant molecules in conjunction with protein (Heath et al., 2006).

Materials and methods

Plant materials

The experimental plant used in the present investigation was *Trigonella foenum gracum* (fenugreek) cv. Baladi. The seeds of the plant were obtained from the Egyptian Ministry of Agriculture, Giza, Egypt.

Culture technique of the tested blue green alga(Nostoc muscorum)

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}). The composition of BG_{11} nutrient medium is according to Rippka et al. (1979). The liquid culture was prepared by dispensing 300ml of BG_{11} nutrient medium in 500 ml Erlenmeyer flasks then sterilized in an autoclave at 121^{o}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 (Stein, 1973). The culture flasks were aerated by air pumps and incubated at 30^{o}C under continuous illumination provided from day light fluorescent tubes giving the photon flux density of 3000Lux. 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).

Pot experiment

During the season of growth (November 2010), known number of seeds (1000 seed) of *Trigonella foenum gracum* (cv. Baladi) was sterilized by 0.01% $HgCl_2$ for 1min. and washed thoroughly with distilled water and divided into four groups. Each group was sown in plastic pots (40cm diameter and 45 cm depth) filled with 20kg 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 supplemented 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 1.0g kg^{-1} soil) and *Nostoc* (2.0 g kg^{-1} soil) combinations. The seeds were left to grow and irrigated with tap water under greenhouse conditions. At the end of growth season (120 day-old) plants were collected and the following measurements were carried out (1) Yield parameters (number of pods/plant, number of seeds/ pod, pods weight/ plant, seeds weight/ pod and weight of 1000 seeds) (2) Analysis of total carbohydrate, total lipid, total soluble protein, fatty acids, protein content and profile and some secondary metabolites in the harvested seeds.

Biochemical analysis

Estimation of total carbohydrate content in the seeds

The carbohydrate content was measured by hydrolyzing polysaccharides into simple sugars using dilute HCL and estimating the resultant monosaccarides. Monosaccharides were estimated in 100 mg of dried seeds by the method given by Hedge and Hofreiter (1962) using spectrophotometer (Model 404 g LKB Novasped. The concentration of monosaccharides was calculated as mg/g dry weight.

Determination of total lipid content

This was performed by the sulfophosphovanillin method described by Knight et al. (1972). The amount of total lipid was calculated as mg/g dry weight from a calibration curve made with triglyceride (tristearin).

Determination of total protein

Extraction of total protein was carried out by adding 10ml of 0.5 N NaOH to about 100 mg of oven dried seeds which was left over night. The extract was reached to 50ml with distilled water (Rausch, 1981). The determination of total protein was done as procedure described by Hartree (1972).

Determination of total soluble protein

Total soluble protein content was determined in borate buffer extract using the method described by Bradford (1976). The concentration of protein was calculated as mg g^{-1} dry weight.

Quantitative estimation of alkaloids

Alkaloids were measured in 1g dry seeds powder using the method described by Harbone (1973). The weight of alkaloids was expressed as mg g^{-1} dry weight.

Quantitative estimation of total phenolic content

Extraction of total phenol was done according to Proestos et al. (2005) and the total phenol of the aqueous methanol extract was determined according to the Folin Ciocalteu's method (Zheng and Wang, 2001). Standard curve was prepared using different concentrations of gallic acid. Results were expressed as mg g^{-1} dry weight.

Qualitative characterization of protein using SDS-PAGE

A sample of 0.5g frozen seeds was homogenized with 1ml of extraction buffer (25mM Na- acetate, PH 4.5 and 1m M phenyl methyl sulphonyl fluoride [PMSF]), vortexes and left for 2 hours at $4^{O}C$. The extract was centrifuged at 10.000 rpm at $0^{o}C$ for 15 min. and the clear supernatant was used as the total protein extract. Characterization and molecular mass determination of proteins were carried out using one dimensional SDS-polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli (1970).

Statistical analysis

The obtained results were statistically analyzed using one and two ways analysis of variance (ANOVA) to determine the degree of significance for the obtained variations by the used treatments. Each treatment was replicated five times. All of the statistical methods used in this study were according to Bishop (1983).

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