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RESEARCH ARTICLE

In vitro evaluation of excess copper affecting seedlings and their biochemical characteristics in *Carthamus tinctorius* L. (variety PBNS-12)

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Abstract The present study was focused to recognize the changes in the Safflower (*Carthamus tinctorius* L. variety PBNS-12), when exposed to different concentration of copper (25, 50 and 100 μM) along with control (0.5 μM) for 10 and 20 days. This experiment used Hoagland's nutrient solution to meet the external nutrient conditions, which includes micro and macronutrients equivalent to soil solution with copper sulphate ($CuSO_4$. $5H_2O$) as a metal stress. The plant samples were harvested after 10 and 20 days. The effect of increased concentrations of copper was indicated by the reduction in overall growth with reduced fresh and dry weight. Copper stress caused significant increase in the non- enzymatic antioxidants (polyphenols and flavonoids) in leaves of treated safflower seedlings as compared to the control. Also, enhanced accumulation of proline was observed in the safflower leaves. In response to excess copper concentration, the level of MDA content was found to be increased. The results showed that the copper has time and dose-dependent effects on safflower seedlings.

Keywords Safflower · Copper stress · Morphology · Proline

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Abbreviations

ROS	Reactive oxygen species
MDA	Malondialdehyde
TI	Tolerance Index
FW	Fresh weight

Introduction

Safflower (*Carthamus tinctorius* L.) commonly known as Kusum in Hindi belongs to family Asteraceae (Al Chami et al. 2015). It is a multipurpose crop and has long history of cultivation. India is the largest producer of Safflower in the world producing around 206,000 t of seeds annually (Kizil et al. 2008). Safflower has been mainly grown for the dye which is commonly known as Carthamin, until the cheaper dye aniline became available. Safflower is commercially grown for the seeds for producing the edible oil. The safflower oil is highly rich in linoleic acid known to reduce the blood cholesterol level (Dajue and Mundel 1996), therefore, it is mixed with other vegetable oil to upgrade them nutritionally. This crop is widely known for its great pharmaceutical properties as it is used for treatment of cardiovascular diseases, menstrual problems, atrophic gastritis, rheumatism, and chronic nephritis (Yar et al. 2012).

In past few years, the accumulation of heavy metal in environment has increased through various anthropogenic activities (Zengin and Kirbarg 2007) which mainly include the copper containing fertilizers, pesticides, fungicides etc. (Sonmez et al. 2006). Copper (Cu) is an essential micronutrient which is required for normal growth and development of plants (Li et al. 2015). It is a vital component involved in various processes, including the electron transfer reactions of respiration and photosynthesis or the removal of superoxide radicals (Adrees et al. 2015). However, excess copper can induce alteration in photosynthetic and respiratory processes, enzyme activities leading to chlorosis, inhibition of root growth (Bouazizi et al. 2010) by causing injury at cellular level by formation of reactive oxygen species (ROS) (Alaoui-Sossé et al. 2004; Lombardi and Sebastiani 2005). Also, copper toxicity leads to disturbances in uptake of essential nutrients (Patsikka et al. 2002) and oxidation of lipid membrane. The high level of copper is also known to inhibit metabolic activities such as germination, root, leaf and stem growth, photosynthesis, biomass, and pigment content (Mallick et al. 2010). To detoxify the effect of ROS, plants have evolved the antioxidative defence mechanism which includes various enzymatic and non-enzymatic compounds (Solanki et al. 2011). Also, the accumulation of proline is a very common response in abiotic stress (Azooz et al. 2012). Thus, it is reasonable to link the metabolism of proline and heavy metals stress in plants. The aim of our study was to observe the effects of different concentrations of copper on morphological and biochemical responses in Safflower seedlings (variety PBNS-12) after 10 and 20 days of copper treatment.

Materials and methods

In this study, seeds of the Safflower (*Carthamus tinctorius* L.) variety PBNS-12, were collected from Directorate of Oilseed Research Institute, Hyderabad, India. Seeds were sterilized with 0.1 % $HgCl_2$ for 3 min, and then rinsed several times with distilled water and germinated in Hoagland's medium (pH 6.8) in a growth chamber at 25 ± 2 °C, with 18 h photoperiod under fluorescent white light (40-50 $\mu mol^{-2}s^{-1}$ light intensity). To provide heavy metal stress, different concentrations of copper in the form of copper sulphate ($CuSO_4.5H_2O$) were added separately to the medium. Seedlings were harvested after 10 and 20 days to determine morphological and biochemical parameters. After harvesting, seedlings were kept at −80 °C and were used for further analysis. Each experiment was repeated three times.

The 10 and 20 days old seedlings were harvested and root and shoot lengths were measured. The plant samples were dried in an oven at 110 °C for 24 h and dry seedlings weight were recorded. Copper tolerance index (TI) was determined by the formula given by Azooz et al. 2012. The free proline was determined by ninhydrin method (Bates et al. 1973). The quantitative estimation of flavonoids was done using modified aluminium chloride colorimetric method (Chang et al. 2002). Also, total phenolics were determined using Folin-Ciocalteu reagent (Singleton and Rossi 1965). The level of products of lipid peroxidation was measured according to method given by Heath and Packer 1968.

Statistical analysis

All the experiments were conducted three times. All values reported in this work are mean ± SE of three replicates. To test the significance of experiments, the chemical treatments and their interactive effect on morphology and biochemical characteristics, the data was statistically analyzed using analysis of variance (ANOVA). The significance of the data was determined through the Tukey's multiple range test at two levels ($P \leq 0.05$ and $P \leq 0.01$) through the software SPSS.

Results and discussion

Effect on copper on morphology of seedlings and tolerance index

Copper (Cu) is an essential micronutrient for normal growth and metabolism but it becomes toxic at higher concentration. It can cause interference in growth of young seedlings, and root elongation by causing damage to root epidermal cells and root cell membranes (Xiong and Wang 2005; Tanyolac et al. 2007). The significant reduction in these parameters with increasing concentration of Cu indicated that high Cu concentration produced toxic effects within 10 and 20 days of growth (Fig. 1). It proved that there was a relationship between increased Cu concentrations and reduced growth of seedlings.

The graphical presentation of data (Figs. 2, 3, 4 and 5) indicated the changes in root length, shoot length, fresh weight and dry weight. After harvesting of safflower seedlings, it was observed that 25 μM copper treated seedling's roots were found to be increased by 25.3 % (10th day) and 13.96 % (20th day) while in 50 μM and 100 μM treated seedling's roots were found to be decreased by 61.9 %, 87.3 % (10th day) and 53.98 %, 81.99 % (20th day) respectively as compared to the control (100 %) (Fig. 2). Also, the shoot length was found to be significantly affected at 100 μM concentration of copper as compared to other copper treated seedlings (Fig. 3). The results showed that the shoot length was found to be reduced at all the three treatments by 6.68 %, 13.34 % and 70 % as compared to the control in 10th days harvested seedlings. In 20th day harvested seedlings, the shoot length was found to be slightly increased by 4.54 % in 25 μM copper treated seedlings, while it was reduced by 9.09 % and 54.54 % in other two treatments as compared to control. The dry and fresh weight was also found to be significantly decreased. The data represents that the fresh weight (Fig. 4) was found to be reduced by 14.5 %, 37.97 % and 70.88 % in the treated seedlings with increased copper concentration (25 μM, 50 μM and 100 μM) respectively in 10th days harvested seedlings as compared to control (100 %). While in 20th day harvested seedlings, the fresh weight was found to be elevated by 19.88 % in 25 μM treated

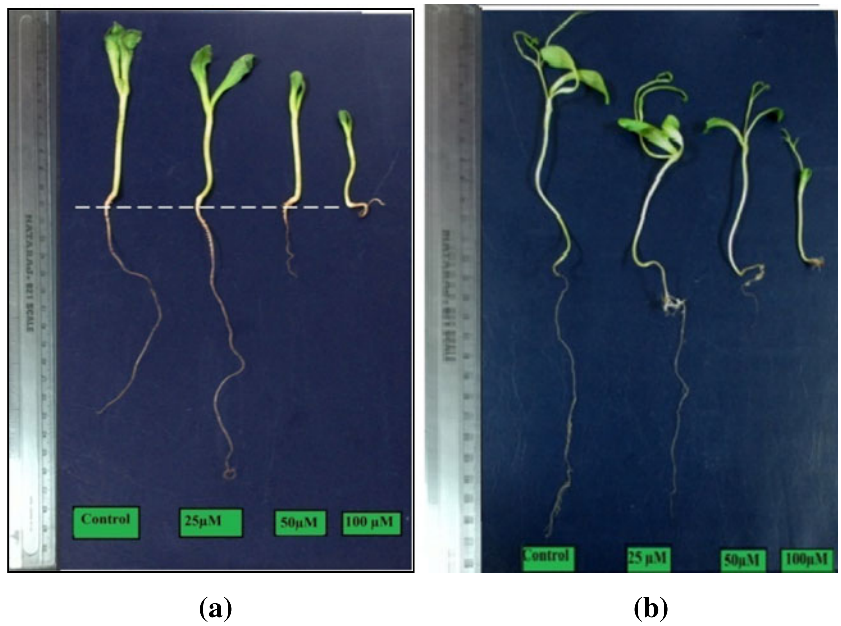

Fig. 1 Effect of various concentrations of copper on morphology of safflower seedlings. The seedlings were collected after 10th day (a) and 20th day (b) respectively

seedlings. But with the increased concentration (50 μM and 100 μM), it was found to be significantly decreased by 24.40 % and 58.81 % respectively as compared to control. The dry weight (Fig. 5) was found to be increased in 25 μM copper treated seedlings by 17.14 % and decreased by 11.42 % and 28.57 % with increase in copper (50 μM and 100 μM) treatment as compared to control. Therefore copper ultimately leads to reduction in overall growth (Sonmez et al. 2006). In 10th days harvested seedlings, the tolerance index (TI%) was found to be increased by 19 % in 25 μM copper treated seedlings while it get decreased by 9 % and 28 % in 50 μM and 100 μM Cu concentration as compared to control (100 %). While in 20th day harvested seedlings, the TI% was found to be decreased by 15.5 %, 38 % and 61.5 % in all the Cu treatment (25 μM, 50 μM and 100 μM) respectively as compared to control (Fig. 6). It has been proved that the application of high level of copper inhibits the root growth before affecting the shoot growth. It is because the roots are in an environment where copper is in excess (Lexmond and Van der Vorm 1981). Therefore copper binds to root surface and decreases its trans root potential and which is very essential for water and ions uptake (Arduini et al. 1994). Our studies are supported by the findings of the (Xiong et al. 2006; Dey et al. 2014). The reduction in biomass may be due to interference in photosynthesis, protein formation and carbohydrate translocation (Wani et al. 2007) Fig. 6.

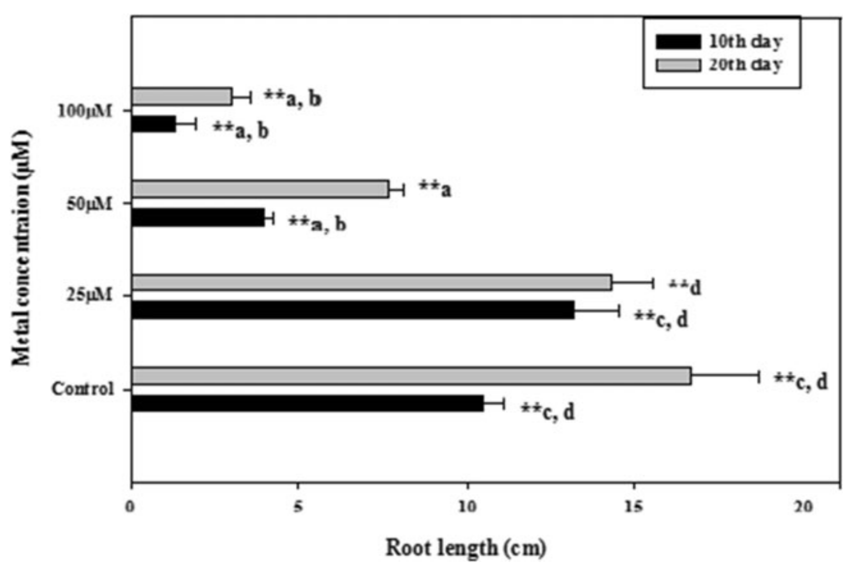

Fig. 2 The effect of elevated copper on safflower seedling's root length (variety PBNS-12). The values are the average of three replicates. Horizontal bars represent the standard error (** values significant at $P \leq 0.01$)

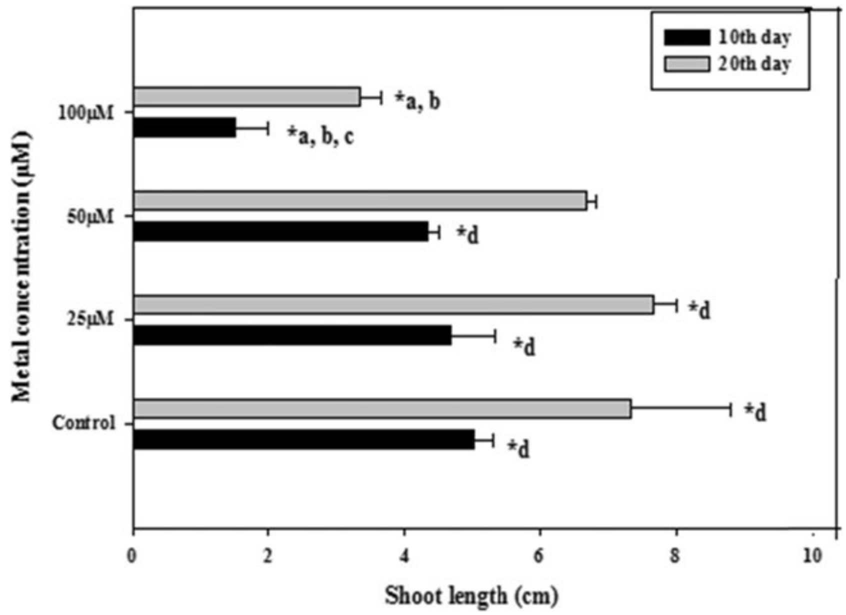

Fig. 3 The effect of elevated copper on safflower seedling's shoot length (variety PBNS-12). The values are the average of three replicates. Horizontal bars represent the standard error (* shows that values are significant at $P \leq 0.05$)

Lipid peroxidation

In this experiment, we evaluated the possible role of excess copper in lipid peroxidation which can be described as the destruction in lipids containing any number of carbon double bonds due to oxidative stress (El-Beltagi and Mohamed 2013). It is used as an indicator of damage in cells and tissues (Ahmed et al. 2010). In response to copper toxicity, the malondialdehyde (MDA) content found to be increased in leaves of both 10th and 20th days copper treated safflower seedlings (Fig. 7).

In 10th and 20th day harvested seedlings, the MDA content was found to be increased by 32.7 %, 47.54 %, 51.6 % (10th day) and 9.87 %, 22.22 %, 108.02 % (20th day) with increase in copper concentration (25 μM, 50 μM and 100 μM) respectively as compared to control (100 %). The MDA which is product of lipid peroxidation, results from the oxidation of lipid membrane. Because Cu is considered as a very strong catalyst of free radical formation and can initiate the oxidative breakdown of polyunsaturated fatty acids (Mediouni et al. 2006). Therefore, the peroxidation of lipid membranes is the most considerable symptoms in most plants and animals. This may be due to induction of significant increase in lipoxygenase (LOX) activity. Our studies are in line with the results shown by (Yurekli and Porgali 2006; Zhao et al. 2010), in which they also observed the increased lipid peroxidation in presence of copper toxicity.

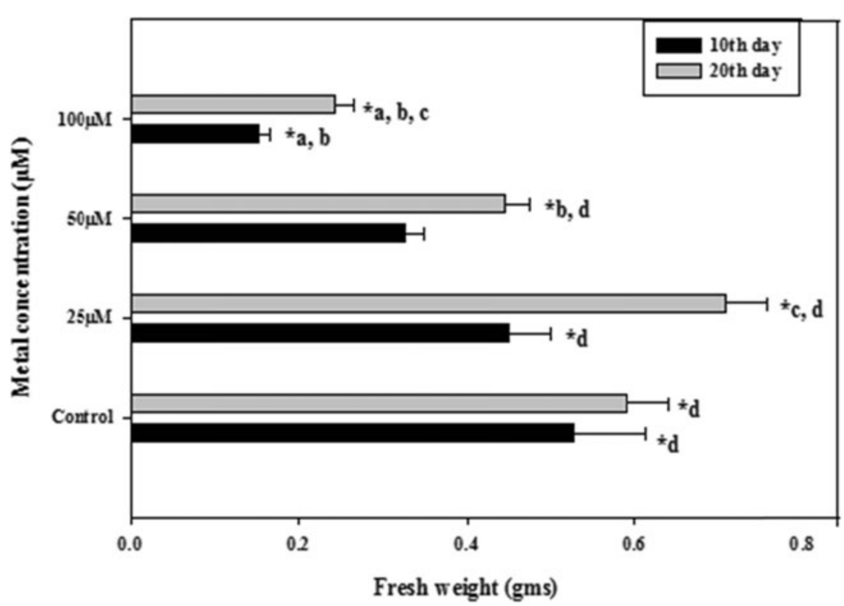

Fig. 4 The effect of elevated copper on fresh weight of safflower seedlings (variety PBNS-12). The values are the average of three replicates. Horizontal bars represent the standard error (* shows that values are significant at $P \leq 0.05$)

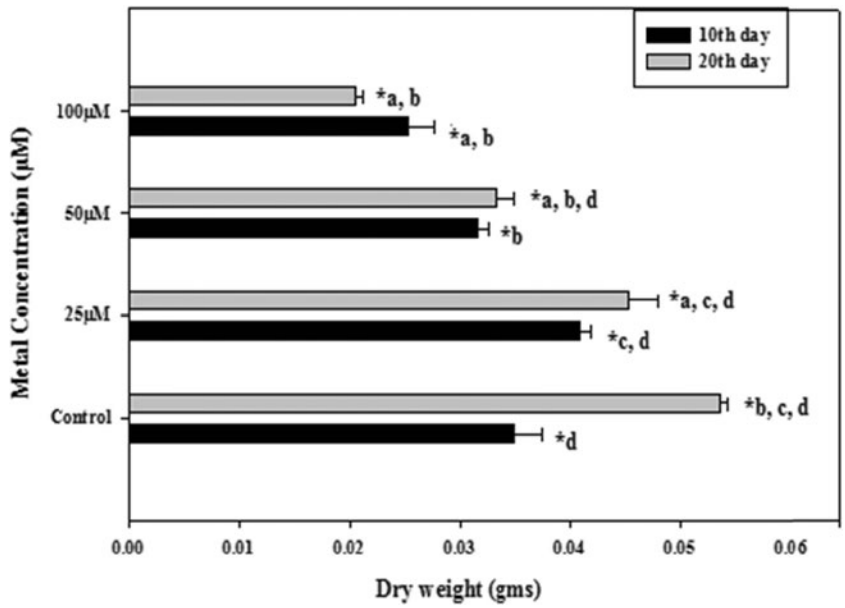

Fig. 5 The effect of elevated copper on dry weight of safflower seedlings (variety PBNS-12). The values are the average of three replicates. Horizontal bars represent the standard error (* shows that values are significant at $P \leq 0.05$)

Effect of copper on polyphenols and flavonoids content

During abiotic stress, phenolic compounds acts as metal chelator by directly scavenging the molecular species of the reactive oxygen. The antioxidative property of polyphenols is mainly due to their redox properties, which allow them to act as reducing agents, hydrogen donors, singlet oxygen quenchers and metal chelators (Sakihama et al. 2002). In our study, with increased concentration of copper, the polyphenol content enhanced in leaves of harvested seedlings as compared to control in both 10th and 20th days (Fig. 8). The data shows that the polyphenol content was found to be increased by 66.01 %, 77.26 % and 104.4 % with increase in copper treatment in 10th day harvested leaves as compared to control. Also, in 20th day harvested leaves, the polyphenol content was found to be increased by 12.33 %, 24.05 % and 37.81 % with increase of copper treatment as compared to control. Our findings are supported by the result shown by (Hamid et al. 2010), which demonstarted that phenolic compound is found to be increased with increase of heavy metal stress. Similarly, Ganeva and Zozikova 2007 reported that free phenols increased under copper stress. Accumulation of phenols under heavy metal stress protects the plant from damage and increase the stability of the cell membrane (Sgherri et al. 2004).

Flavonoids are widely distributed group of plant phenolics and are commonly found in leaves, floral parts and pollens (Ahmad et al. 2009). In this experiment, the involvement of flavonoids in copper tolerance in safflower seedlings was

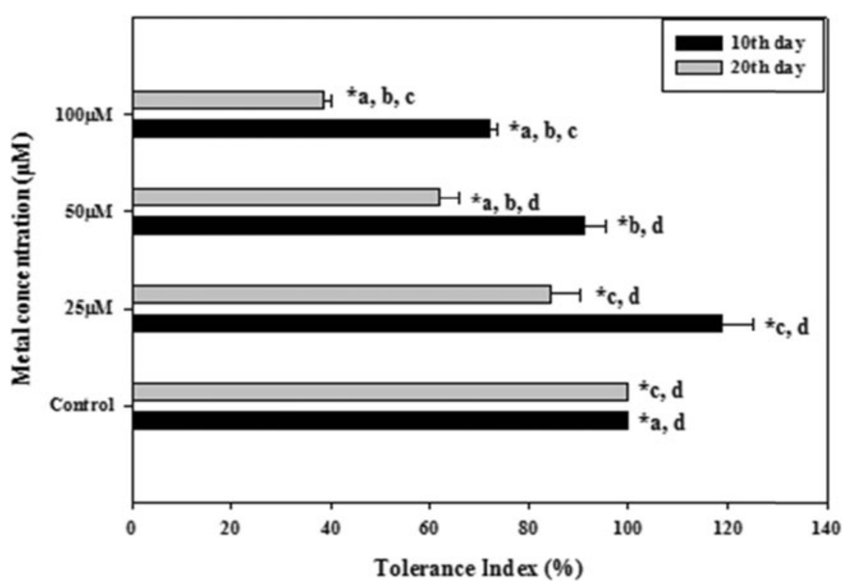

Fig. 6 The effect of elevated copper on tolerance index (TI) in safflower seedlings (variety PBNS-12). The values are the average of three replicates. Horizontal bars represent the standard error (* shows that values are significant at $P \leq 0.05$)

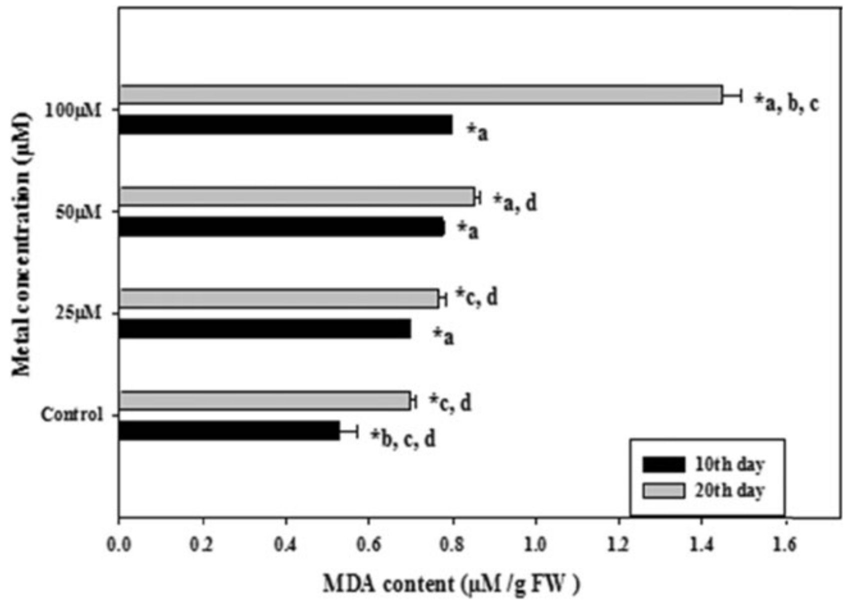

Fig. 7 The effect of elevated copper on malondialdehyde content in safflower seedlings (variety PBNS-12). The values are the average of three replicates. Horizontal bars represent the standard error (* shows that values are significant at $P \leq 0.05$)

investigated. In response to copper as heavy metal stress, flavonoids content was found to be increased in both days of treated safflower seedlings leaves as compared to control (Fig. 9). The results showed that the flavonoids content increased by 8.98 %, 15.73 %, 34.71 % as compared to control in 10th days harvested leaves with increase of copper concentration. Also, in 20th day harvested leaves, the flavonoids content increased by 40.97 %, 47.68 % and 123.14 % as copper treatment increases as compared to control (100 %). Flavonoids possess the antioxidative activity, as they scavange the ROS by locating and neutralizing radicals before they damage the cell structure (Boguszewska and Zagdańska 2012). Flavonoids are known to form complexes with heavy metal, therefore leading to addition in plant defence mechanism (Korkina 2007).

Proline content

Our result indicates that proline content is found to be accumulated in high concentration as copper treatment increases (Fig. 10) in both the days. It was observed that proline content was found to be increased by 88.84 %, 121.6 % and 188.35 % with increase of copper treatment as compared to control (100 %) in 10th day harvested seedlings. While in 20th day harvested leaves, the proline was found to be increased by 5.74 %, 8.02 % and 35.8 % with increase in copper concentration as compared to control. Therefore accumulation of copper in safflower seedlings appears to be a significant defence tool against the reactive oxygen species. Our results are favoured by the findings of Vinod et al. 2012.

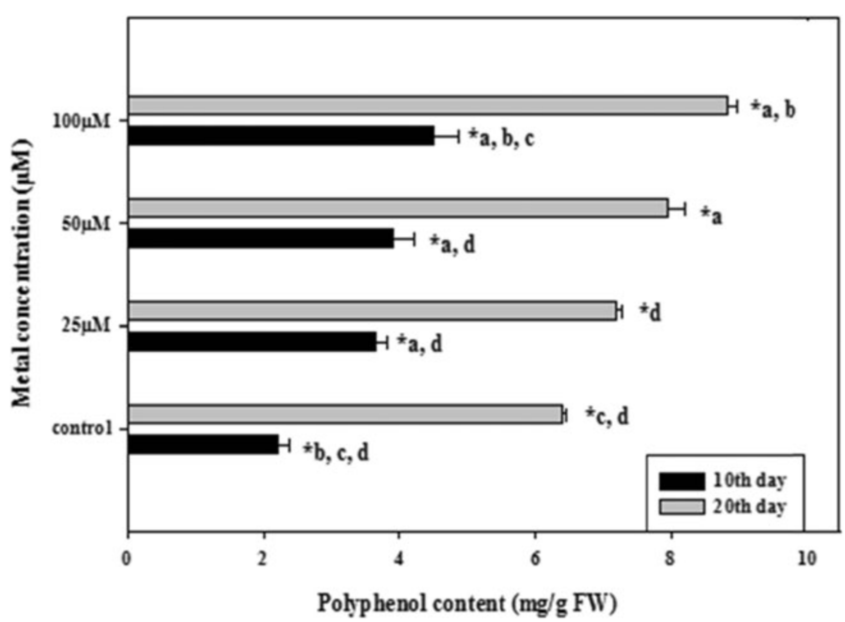

Fig. 8 The effect of elevated copper on polyphenol content in safflower seedlings (variety PBNS-12). The values are the average of three replicates. Horizontal bars represent the standard error (* shows that values are significant at $P \leq 0.05$)

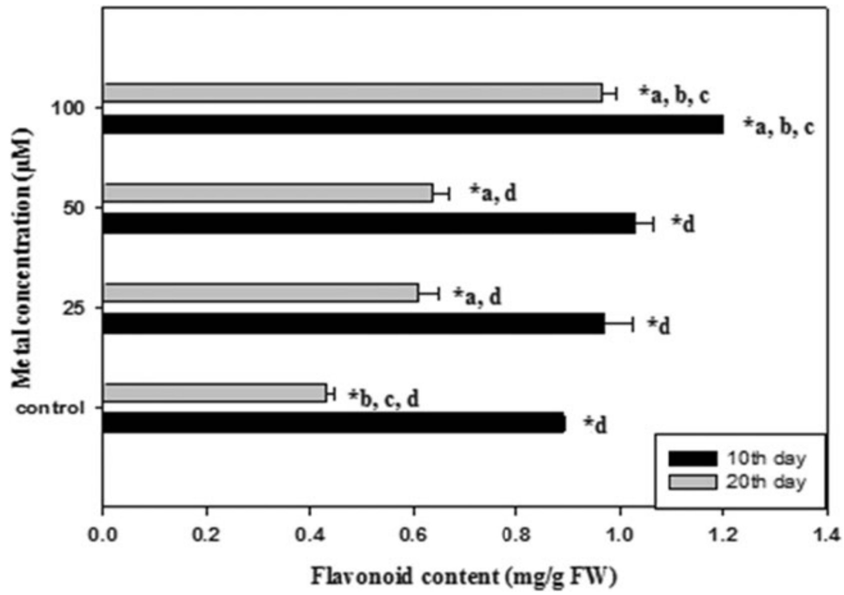

Fig. 9 The effect of elevated copper on flavonoid content in safflower seedlings (variety PBNS-12). The values are the average of three replicates. Horizontal bars represent the standard error (* shows that values are significant at $P \leq 0.05$)

Proline is an amino acid which is found to be accumulated in wide variety of organisms ranging from bacteria to higher plants. It has been already proved that heavy metal copper induces the accumulation of proline (Zengin and Kirbag 2007). Proline plays an important in osmoregulation, protection of enzymes against denaturation (Kuznetsov and Shevyakova 1997), stabilization of the machinery of protein synthesis, regulation of cytosolic acidity, as well as in non-enzymatic free radical detoxification (Alia and Saradhi, 1991). Increased activity of an enzyme (Δ^1-pyrroline-5-carboxylate synthetase (P5CS) which is responsible for proline biosynthesis in presence of copper stress has been reported in higher plants (Chen et al. 2001).

Conclusion

In conclusion, our results revealed that Cu^{2+} had a favourable effect in early growth of safflower seedlings. Long time exposure (10th and 20th day) under various concentration of copper (25, 50 and 100 µM) were reflected by oxidative damage along with reduction in the morphology of PBNS-12 safflower's seedlings. The adverse effect was indicated by the decrease in the biomass production along with an increase in lipid peroxidation. The stimulatory effect of copper was reflected by the biosynthesis of various non-enzymatic antioxidants (flavonoids, polyphenols etc) which may play important role in providing resistance to seedlings against copper toxicity. Therefore, it could be suggested that elevated levels

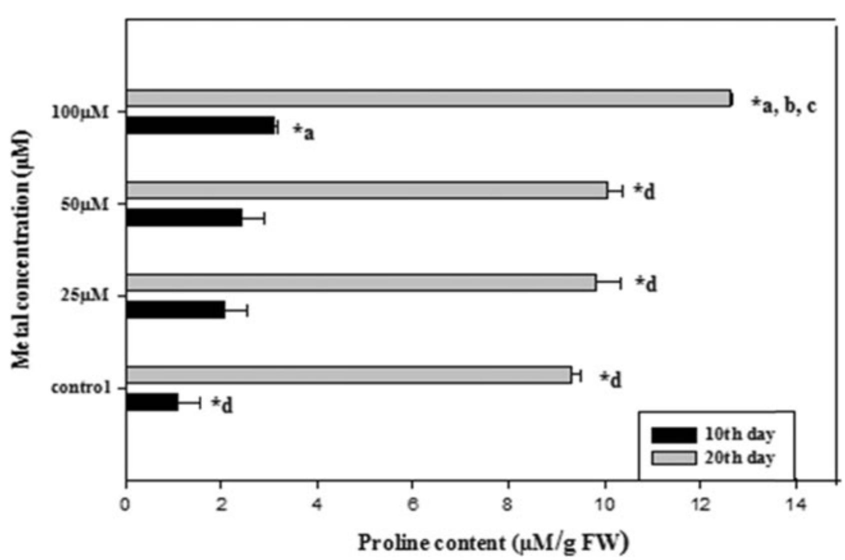

Fig. 10 The effect of elevated copper on proline content in safflower seedlings (variety PBNS-12). The values are the average of three replicates. Horizontal bars represent the standard error (* shows that values are significant at $P \leq 0.05$)

of all these parameters, at least in part, were responsible for development of resistance against Cu^{2+} stress in PBNS-12 safflower. Further, this variety of crop has the ability to grow in copper polluted area by altering their various physiological processes.

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