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GROWTH AND NON-ENZYMATIC ANTIOXIDATIVE STUDIES IN *IN-VITRO* GROWN SAFFLOWER (CV A-1) SEEDLINGS UNDER COPPER STRESS

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ABSTRACT: In the present study, we were interested in evaluating the effects of copper on morphological features and non- enzymatic antioxidants in the safflower (cv. A-1). The safflower seeds were grown in Hoagland's solution supplied with the 0.5 µM (control), 25, 50 and 100 µM $CuSO_4$ for 10th and 20th days. The seedlings were harvested after different days (10th and 20th days). The presence of 50 µM and 100 µM concentration of Cu stimulated impaired root growth after 10th and 20th days, while 25 µM Cu was found to be less in inhibiting the root growth. Also, the shoot growth was found to be adversely affected with increased copper concentration. The malondialdehyde content, nonenzymatic antioxidants (flavonoids and polyphenols), and proline were found to be accumulated in both days harvested leaves of safflower's seedlings. Thus the conclusion reveals that the increase of copper above threshold point affects the plants in a negative manner.

Keywords: Safflower, Copper stress, Proline, Lipid peroxidation

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INTRODUCTION: Safflower (*C. tinctorius* L.) also called Kusum in Hindi belongs to family Asteraceae [1]. It is a multipurpose crop and has a long history of cultivation. India is the largest producer of Safflower in the world [2], producing around 206,000 tonnes of seeds annually [3]. 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 to produce high-quality edible oil [4].

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Now a day this crop has attracted its uses in pharmaceuticals being rich in medicinal properties. It is widely known for its great natural antioxidant properties [5, 6]. The heavy metal pollution in the environment affects the normal functioning of the soil ecosystem and plant growth [7]. These metals are not degraded and hence persist in the environment for a long time and enter the food chain [8]. Copper (Cu) is an essential micronutrient for the growth and development of the plants [9]. The increased level of Cu in the environment arises from mining; the uncontrolled use of Cu containing fungicides, pesticides, and bactericides to control pests and diseases [10].

The great concern of our study is that safflower is susceptible to fungal attack, which mainly includes the wilt caused by *Fusarium oxysporum* f. sp. *Carthami* [11]. So, it is very important to take a

control measure which constitutes the treatment of safflower seeds with copper-containing fungicides (*e.g.,* Copper oxychloride). But copper can be detrimental for the growth of plants when applied to the fields above the recommended amounts. The excess concentration of Cu leads to the generation of reactive oxygen species (ROS) via the Haber-Weiss and Fenton reactions [12]. These ROS cause damage to plants by the peroxidation of lipids, DNA mutation, and disruption of chlorophyll content [13, 14].

To overcome the effects of ROS, plants possess the antioxidative defense mechanism, which includes enzymatic and non-enzymatic compounds. The enzymatic compounds include Catalase (CAT), superoxide dismutase (SOD), peroxidase (POD) [15, 16], while the non-enzymatic antioxidants includes the ascorbate, carotenoids, glutathione [17, 18, 19]. To cope with the abiotic stress, plants produce various antioxidants such as phenolic compounds, flavonoids, proline [20, 21, 22]. The objective of the current study was to find out the effect of Cu accumulated with A-1 (Spiny) variety of safflower for its morphological and biochemical response under threshold concentration (Cu stress) in the environment.

MATERIAL AND METHODS:

Plant Material: The seeds of safflower (variety A-1) were obtained from the Directorate of Oilseed Research Institute (ICAR), Hyderabad, A.P. (India).

Plant Culture and Copper Treatment: The experiment was conducted in the June to December 2015 in the Department of Bioscience and Biotechnology, Banasthali University, Banasthali (Rajasthan), India. The seeds were washed with running tap water for 15 min and subjected to surface sterilization with 0.1% Mercuric chloride for 3 min and then rinsed with sterile deionized water (each time for 3 min) for four times under sterilized conditions. The uniform seeds were inoculated in pots containing Hoagland's medium (pH 6.8), which served as control as well as nutrient solutions supplemented with copper sulphate ($CuSO_4.5H_2O$) of various concentrations (25, 50 and 100µM) which served as treatment solutions. Pots were kept for growth of seedlings in a growth chamber at 25 ± 1°C under 60-80% relative humidity and 16 h photoperiod with 40-50 $\mu mol^{-2}s^{-1}$ light intensity in a controlled environment. For biochemical and morphological estimations, the seedlings were harvested after 10^{th} and 20^{th} days and washed thoroughly with distilled water. Harvested seedlings were deep freezed and stored at -80°C for further analysis. To minimize the experimental errors, the experiments were carried out at least three times.

Seedlings Growth Analysis: After the 10^{th} and 20^{th} days, the seedlings were removed smoothly from the pots and length of root and shoot length of seedlings were measured using a meter scale. Fresh weight of the seedlings was taken immediately after harvesting of the seedlings. Dry weight of seedlings was determined by placing the samples in hot air oven at 60°C till they dried to constant weight. While the tolerance index (TI%) was calculated as the ratio of dry weight of Cu treated seedlings and controlled seedlings according to the formulae given by [23, 24].

$$TI\ (\%) = \frac{\text{Dry weight of copper-treated plants} \times 100}{\text{Dry weight of copper-untreated plants (control)}}$$

Chlorophyll Content Determination: 0.2 gm leaves were homogenized in 10 ml of 80% acetone. The extract was centrifuged at 5000g for 10 min. The upper phase was transferred into a new tube, and its absorbance was taken at 663, 646, 470 nm (UV-VIS spectrophotometer, *Shimadzu*, Singapore) respectively with 80% acetone as a blank. The chlorophyll a, b and carotenoid content were measured according to the method of [25, 26].

Total Polyphenol Determination: The total polyphenol content was determined according to the method of the using Folin- Ciocalteu reagent [27]. The absorbance of the sample was taken at 725 nm against a blank by spectrophotometer. Gallic acid was used as the standard for preparing the standard curve. The results were expressed as mg of gallic acid equivalent per gram of fresh weight.

Flavonoid Determination: The aluminum chloride method was used for flavonoid determination [28]. The absorbance of the sample was taken at 510 nm against a blank by spectrophotometer. Quercetin was used as a standard curve for preparing the

standard curve. The results were expressed as mg of quercetin equivalent per gram of fresh weight.

Proline Determination: The accumulation of proline was determined according to the ninhydrin method [29]. The absorbance of the sample was monitored at 520 nm by spectrophotometer using toluene as blank. The known concentration of proline was used for preparing the standard curve. The results were expressed as µM of proline per gram of fresh weight.

Lipid Peroxidation Determination: The level of lipid peroxidation in leaves was analyzed by the formation of malondialdehyde (MDA) content after the reaction of TBA (Thiobarbituric acid) [30] and amount was calculated with a coefficient of 155 mM^{-1} cm^{-1}.

Statistical Analysis: Each value was represented as Mean ± SE from three triplicates. All of the data were subjected to one-way analysis of variance (ANOVA), taking $p<0.05$, $p<0.01$ and $p<0.001$ as significant according to Tukey's multiple range test to detect the significance using SPSS software (version 16.0).

RESULTS AND DISCUSSION:

Effect of Copper on Growth Parameters: The seedlings of Indian safflower (*Carthamus tinctorius* L. cv A-1) seeds were used for the current research study to understand their ability to tolerate the excess concentration of Cu through the morphological and biochemical parameters. At the end of the experiment (10^{th} and 20^{th} day), the seedlings were harvested to determine the root, shoot length, fresh, and dry weight. The previous research study also revealed that excess Cu affects the seed germination and growth of seedlings in the presence of Cu [32, 33].

Also, the inhibited growth and reduction in biomass are very common responses to heavy metal toxicity generally observed in higher plants. The decline in biomass may be due to the interference of heavy metal in cell division and elongation [34]. Some Scientists [35] also reported that heavy metal affects the ultrastructure of meristematic cells and varying the ribosomal RNA precursor biosynthesis, ultimately affecting the plant growth. It has been proved that the effect of Cu toxicity is principally on root morphology [36].

FIG. 1: (A) AND (B) REPRESENTS THE EFFECT OF COPPER ON MORPHOLOGY OF SAFFLOWER SEEDLINGS HARVESTED AFTER 10^{th} AND 20^{th} DAYS RESPECTIVELY

The Cu tends to accumulate in the root tissue with little translocation to shoots because Cu is sparingly movable in plants [10]. Root systems, though genetically determined [37], are very plastic and can be affected by several environmental factors, including metals [38]. Our plants did not show any symptoms of chlorosis. Our results have similar findings with the research work [39], in which they observe the stunted root growth and absence of root hairs. Our results are also supported by the findings of [40, 41]. According to **Fig. 2E**, the metal tolerance index was found to be increased by 21% and 41.74% in 10^{th} and 20^{th} days seedlings respectively at 25µM, while it was found to be decreased by (4.8% and 12%) (9.09% and 20%) at 50 µM and 100 µM respectively in 20^{th} days seedlings respectively. The seed germination was affected in the presence of increased Cu

concentration (data not shown). The results showed that shoot length was found to be less affected as compared to the roots both in 10th and 20th days **Fig. 2A**, **B**. We evaluated that there was an increase in 1.12% of root length in 25µM while it was found to be significantly reduced by 21% and 47% with increased concentration of Cu (50 µM and 100 µM) as compared to non-treated (100%) safflower seedlings in 10th days harvested seedlings. While in 20th day's harvested seedlings, root length was found to be significantly reduced (29%, 37%, and 63%) with an increased concentration of Cu (25, 50 and 100 µM), respectively as compared to control. There was a net reduction in the fresh and dry weight **Fig. 2C**, **D** which have been used as an indicator of heavy metal toxicity in various studies. The fresh weight was found to be increased by 6% and 10.4% in 25µM at 10th and 20th days harvested seedlings respectively along with the control. It was found to be reduced by 9.6% and 18.8% in the 10th day while 7.4% and 38% in 20th days harvested seedlings concerning control in 50 µM and 100 µM Cu concentration respectively.

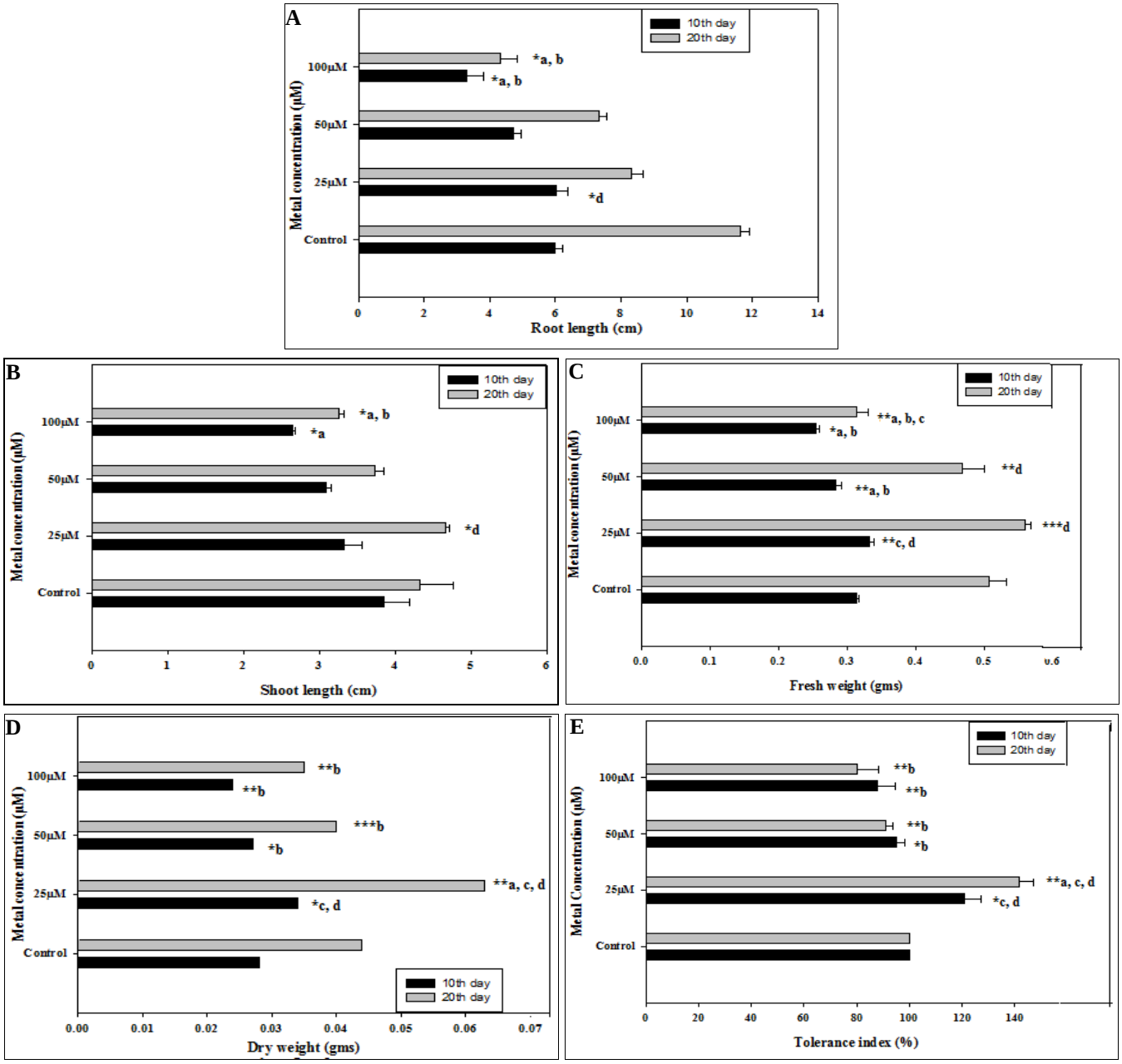

FIG. 2: EFFECT OF DIFFERENT CONCENTRATION OF COPPER ON ROOT AND SHOOT LENGTH (A, B), FRESH AND DRY WEIGHT (C, D) AND TOLERANCE INDEX (TI%) (E) ON SAFFLOWER SEEDLINGS COLLECTED AFTER 10TH AND 20TH DAYS OF GROWTH. Values indicates the mean ± SE (n=3). Data with different letters were significant at (*= $p<0.05$, ** = $p<0.01$, ***= $p<0.001$) these levels.

The dry weight was found to be 21.4%, and 43.18% increased at 25 µM Cu concentration along with control at 10th and 20th days harvested seedlings. While it was found to be significantly reduced by 3.57% and 14.2% in 10th days and 9.09% and 20.45% in 20th days harvested seedlings at 50 µM and 100 µM Cu concentration respectively.

Effect of Copper on Chlorophyll Content: All Cu concentrations in the nutrient solutions caused a significant reduction in the chlorophyll a, chlorophyll b, total chlorophyll. The highest reduction in chlorophyll content was observed in 100 µM Cu treated safflower seedlings **Table 1**.

TABLE 1: THE CONTENT OF PHOTOSYNTHETIC PIGMENTS (CHLOROPHYLL a, b) IN DIFFERENT TREATMENTS OF Cu

Days	Metal treatment (µM)	Chl a (mg/g FW)	Chl b (mg/g FW)	Total chlorophyll (mg/g FW)
10th day	Control	0.161±0.010	0.066±0.005	0.228± 0.015
	25	0.147±0.013	0.054±0.001	0.201± 0.014
	50	0.077±0.001	0.039±0.004	0.116± 0.005
	100	0.072±0.002	0.036±0.001	0.109± 0.003
20th day	Control	0.856±0.041	0.350±0.025	1.206± 0.066
	25	0.813±0.017	0.232±0.004	1.045± 0.021
	50	0.787±0.006	0.116±0.020	0.904± 0.026
	100	0.763±0.004	0.030±0.037	0.794± 0.041

In plants, chlorophyll content is determined to assess the alteration in pigment content, which is a visual symptom during stress [42, 43]. In plant tissues, more than half of Cu is found in the chloroplasts [44]. However, the photosynthetic pigments are sensitive to excess Cu. According to various reports, the effect of excess Cu is reported in terms of reduction in chlorophyll pigments in plants [45, 46, 47] A very common symptom to metal toxicity is observed by the inhibition in biosynthesis of chlorophyll [48] which may be due to result of inhibition of the enzymes such as δ-aminolevulinic acid dehydratase (ALA- dehydratase) [49, 8] and protochlorophyllide reductase [50]. The other reasons for the decrease in pigment may be the destruction of chloroplast membrane by lipid peroxidation and replacement of Mg^{2+} with heavy metals in chlorophyll structure [51].

Effect of Copper on Flavonoid and Polyphenol Content: As depicted in **Fig. 3A** and **B**, we evaluated the changes in flavonoid and polyphenol content in safflower seedlings after 10th and 20th days. There was an increase in flavonoid content in 10th day (9.99%, 34.7% and 64.8%) while in 20th days harvested seedling's leaves (20.4%, 88.2% and 97.8%) with increase in Cu concentration (25, 50 and 100 µM) respectively as compared to control (100%).

Also the polyphenol content, which was found to be abruptly increase in 10th day (12.9%, 49.4%, and 70.7%) and 20th day (39.2%, 67.5% and 118.6%) harvested seedling's leaves with increase in Cu concentration (25, 50 and 100 µM) respectively as compared to control (100%).

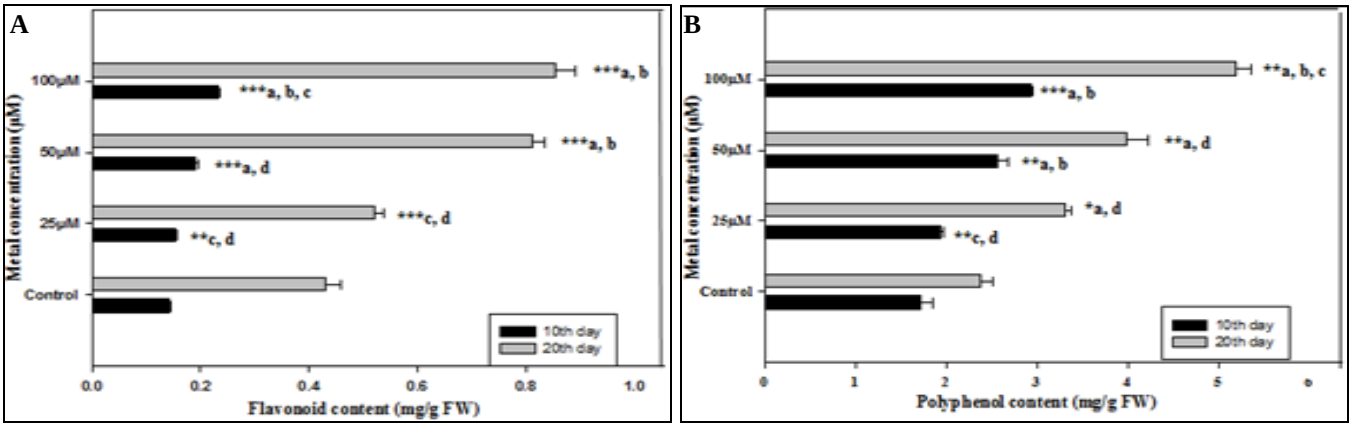

FIG. 3: EFFECT OF COPPER ON FLAVONOID CONTENT AND POLYPHENOL (A AND B) RESPECTIVELY IN SAFFLOWER LEAVES COLLECTED AFTER 10th AND 20th DAYS OF GROWTH. Values represent mean ±S.E. (n = 3). Data with different letters were significant at (*=p<0.05 **= p<0.01 *** = p<0.001) these levels.

According to the previous studies, it has been suggested that the phenolic compounds are found to generally increase with the response to heavy metals stress. They play a magnificent role in antioxidation by metal chelation and scavenging the ROS [52]. These compounds have noteworthy ability to chelate the transition metal ions and the inhibiting the superoxide-driven Fenton reaction [53]. It has been reported that the polyphenols have the chelating potential for metals such as Cu, therefore making it unavailable to plants, and this property is beneficial in case of Cu toxicity. Flavonoids are the most widely and distributed group of plant phenolics [54]. Flavonoids act as a suppressor of enzyme lipoxygenase, responsible for converting polyunsaturated fatty acids to oxygen-containing derivatives [55-56], thus serving in inhibiting the lipid peroxidation.

Effect of Copper on Proline Content: Fig. 4 shows a significant gradual increase in the accumulation of proline in both the day's treated safflower leaves. However, at 100 µM Cu concentration, the level of proline content was significantly higher (216%) as compared to other concentrations (25 µM and 50 µM), which were found to be increased by 26% and 52% in 10th-day safflower seedling's leaves respectively. While in 20th days leaves, it was found to be appreciably elevated with increased concentration of Cu (28.3%, 138.4%, and 229.1%) respectively.

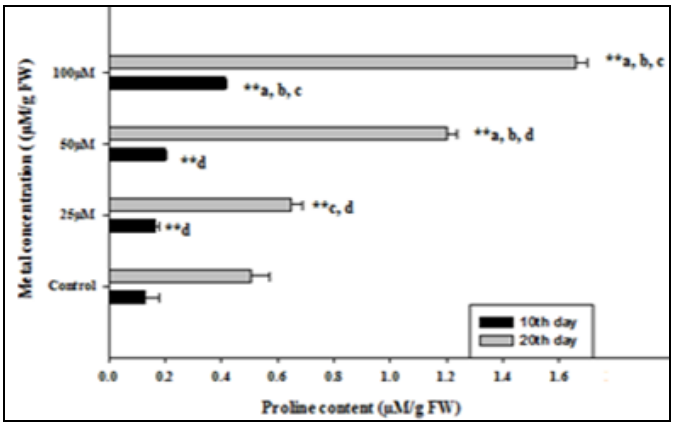

FIG. 4: EFFECT OF COPPER ON PROLINE CONTENT IN SAFFLOWER LEAVES COLLECTED AFTER 10th AND 20th DAYS OF GROWTH. Values represent mean ±S.E (n = 3). Data with different letters were significant at (**= p<0.01) these levels.

Proline is an amino acid which is found to be involved in protecting the plants from stress by absorption of OH radical. Proline has a remarkable affinity to form a complex with cupric ions, which is cooperative in reducing the Cu toxicity. In response to abiotic stress, the proline is accumulated due to increasing its denovo synthesis or decrease degradation [57]. Proline helps in the survival of plants by acts as osmoregulant, protection of enzymes from denaturation [58], thus protecting the plant from stress [59] and stabilization of protein synthesis [60]. In our study, the accumulation of proline in all treatments depicts that proline is involved in the detoxification of ROS. Our results are in agreement with the other researcher's results [61, 62].

Effect of Copper on Lipid Peroxidation: The exposure of different concentration of Cu (25, 50 and 100µM), which leads to about 16%, 36% and 56% increase in MDA content in 10th days harvested leaves while in 20th days harvested seedling's leaves it was found to be increased by 4%, 18% and 84% as compared to control (100%) respectively **Fig. 5**.

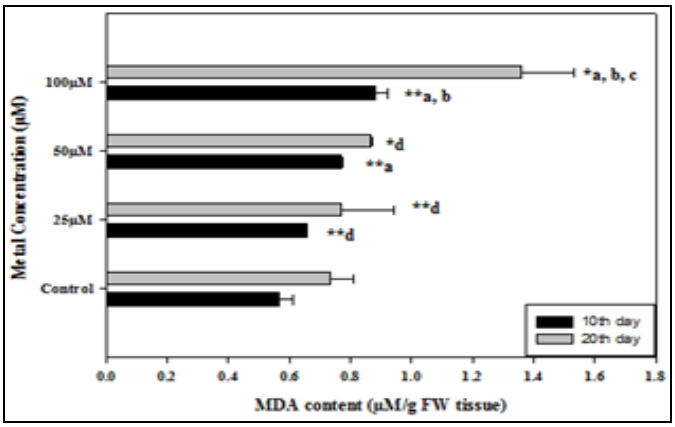

FIG. 5: EFFECT OF DIFFERENT CONCENTRATION OF COPPER ON THE LEVEL OF MDA CONTENT IN SAFFLOWER LEAVES COLLECTED AFTER 10th AND 20th DAYS OF GROWTH. Values represent mean ±S.E (n = 3). Data with different letters were significant at (*= p<0.05, **= p<0.01) these levels.

Due to the generation of free radicals or enzymatic activities, the process of lipid peroxidation occurs [63]. Due to these reactions, mainly free poly-unsaturated fatty acids (linoleic and linolenic acids) are oxidized [64]. These free radicals also cause damage to free and membrane-bound fatty acid, which ultimately leads to the degradation of membranes. Malondialdehyde (MDA) which is a product of lipid peroxidation [65], can be considered as an indicator of heavy metal stress. Therefore the production of MDA and other aldehydes during the

Cu toxicity is a very common process. It is cleared that different Cu concentration induces the changes in the level of MDA content in both the day's treated leaves of safflower seedlings. The present study is supported by the other researchers who have observed an increased MDA content under Cu stress [66, 67].

CONCLUSION: Since safflower is highly susceptible to fungal infection, mainly *Fusarium sp*. *Carthami*, therefore, the usage of Cu containing fertilizers, pesticides, and fungicides has abruptly increased in the field. With our study, it can be concluded that the *C. tinctorius* cv. A-1 is sensitive to Cu toxicity. There was an adverse effect on the morphology of seedlings with an increased concentration of Cu due to increase in Cu toxicity, which leads to the production of ROS. The oxidative damage caused by the ROS generated due to excess concentration of Cu is evidenced by the increase of the level of malondialdehyde. However, in our research study, various non-enzymatic components were found to be involved in antioxidative defense mechanism. Proline was found to be accumulated, which is believed to play a significant role in tolerance during abiotic stress by scavenging the ROS. Flavonoid and polyphenols were accumulated to mitigate the harmful effects of ROS and helps the treated safflower seedlings to survive by keeping the detrimental reaction to the minimum extent in the medium.

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CONFLICT OF INTEREST: Nil

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