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Bioscience Research

Print ISSN: 1811-9506 Online ISSN: 2218-3973

Journal by Innovative Scientific Information & Services Network

RESEARCH ARTICLE BIOSCIENCE RESEARCH, 2020 17(2): 1156-1165. **OPEN ACCESS**

Effect of heavy metals on micropropagated banana plantlets under aseptic conditions

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The imbalanced nutrition with increased concentrations of heavy metals retard the growth of plants. Under metallic stresses, antioxidant enzymes like as peroxidase (POD) and catalases (CAT) are led to change the non-enzymatic plant defense system for plant survival. In this study, the effects of Cu^{2+} assessed on various growth attributes of micropropagated banana plantlets under *in-vitro*. Exact 4-weeks old plantlets from MS_3 [MS_o (Murashige and Skoog salts), 15 µM BAP, 2.0 g l^{-1} phytagel] medium sub-cultured on Cu^{2+} stressed MS_4 (MS_3 + 1.25 mg L^{-l} $CuCl_2$), MS_5 (MS_3 + 2.50 mg L^{-l} $CuCl_2$) and MS_6 (MS_3 + 5.0 mg L^{-l} $CuCl_2$) including the control (MS_3) medium for 3-weeks. With the increasing level of copper concentration number of plantlets and its biomass decreased, while chlorophyll ratios (Chl a/Chl b), catalase, peroxidase and free amino acids (proline and glycine-betaine) increased. The antioxidant enzymes showed increase in their activities including the non-enzymic antioxidants like as glycine betaine (GB), ascorbic acid (AsA), proline, carotenoids, phenolics and flavonoids also increased ($p \leq 0.05$) with increase in Cu^{2+} levels. In conclusion, both enzymatic and non-enzymatic antioxidants are raised in the multiplying banana shoots, which may be taken as the reliable indicators of copper stress either prior or on to the plant wilting.

Keywords: *Musa* spp., micro propagation, Cu^{2+}, enzymic antioxidants, free prolines, antioxidants

INTRODUCTION

The banana (*Musa* spp.) is the most important and 2nd largest fruit plant used all over the world after citrus (Madhulatha et al., 2004). It annual production is over 102 million tons (FAO, 2018). The banana-fruit is rich in carbohydrates and minerals that is equally beneficial diet from children to adults (Vuylsteke et al., 1996). However, with the passage of time, its production is decreasing due to serious impacts of the number of biotic and a-biotic stresses (Hussain and Singh, 2015; Villanueva and Adlakha, 1978). Therefore, the optimal soil nutritional conditions are essential for good growth and yields of plants.

The heavy metals are inorganic in nature and causing soil contamination. These metals remain hazardous for all living organism either into soil or water (El-Hassanin et al., 2018). Even these are essential elements for the survival of all life-forms. With the passage of time, the concentrations of heavy metals are increasing due to improper management of the emissions from expanding industries (Appenroth, 2010; Arora et al., 2008). However, their increasing accumulations are creating very serious toxic problems for both human health as well as his environment. Excess heavy metals are developing characteristics feature in the living systems which is especial the

elevation of peroxidases and catalase including the free amino acids (Nadgórska-Socha et al., 2013) as well as elevated stress causes inactivation of other different cellular enzymes (Gadd and White, 1989). It also has effects in the form of carcinogenic and mutagenic for plants (Buszewski et al., 2018) due to the metal's immobilization by plant (Lopareva-Pohu et al., 2011).

Meanwhile, among the metals, copper (Cu^{2+}) is very essential transition metal involved in various plant physiological processes. Due to its existence in multiple oxidation states in living systems, it is a structural regulatory element of mitochondrial respiration, photosynthetic electron transport, oxidative stress response, hormone signaling and cell wall metabolism (Jacoby et al., 2012; Marschner et al., 1986). Copper also acts as cofactor for the activation of many enzymes (i.e. superoxide dismutase, amino oxidase, cytochrome C-oxidase, lactase etc.). Plant shows specific symptoms in the deficiency of copper like as wilting in young leaves and reproductive organs. The copper is not readily accumulating in the plant organs like as other metals, if so, it is concentrating in the medium that would be highly toxic to the growing plants (Fernandes and Henriques, 1991; Oliva et al., 2010).

Under *in vitro* plant micro propagation, cultures have balanced plant nutrition with optimal potential, which allows the plant to grow maximum in numbers, in shorter time and space with less labor (Haq and Dahot, 2007). It has been well proven that no need of soil conditions to grow plants if all necessary plant nutrient elements are supplied under aseptic conditions (Al-Amin et al., 2009). The *in-vitro* micropropagated plantlets are considered as healthy and disease free (Dobránszki and Teixeira da Silva, 2010). The aseptic plant propagation is a useful tool to examine the stress of heavy metals at cell level and to analyze its effects on plant morphogenesis closely (Wang et al., 2006; Zayed et al., 2019).

The purpose of present study is to assess various growth attributes of micropropagated banana (*Musa* spp., cv. Basrai) plantlets under different concentrations of Cu^{2+} metal stress. Its toxic effects in the growth medium causes specific alterations in morpho-biochemicals of both roots and shoots of the plantlets. The effects of elevated copper concentrations on banana growth and then plant defense responses are still unclear. On the basis this study the farmers can be suggested about the toxicity of copper contaminated soil and type of soil suitability for banana cultivation.

MATERIALS AND METHODS

Collection and preparation of plant materials

The healthy suckers of banana (*Musa* spp.) cv., Basrai (3-4 weeks old) were selected in the open-air banana form-house. They were taken out from soil safely (without injury). The inner portions of banana-sucker (5-6 cm sized meristem-region with ¾ sucker and ¼ pseudo-stem) excised with fine knife and these used as explant. They were dis-infected from microbes by dipping in ethanol (90%) for 1 min than washed with running tap-H_2O. After that they stirred in 30% Robin bleach (5.25% NaOCl) for 30 min. They were washed for 3-times with sterile dH_2O by stirring on magnet stirrer for 3 min each time.

Cultures of banana micropredation

The explants after sterilization cultured on MS_{2a} [MS_0 (Murashige and Skoog 1962) basal salts, B_5 vitamins (Gamborg, Miller, and Ojima 1968), sucrose (3%)] organ induction medium supplemented with different plant hormones like as 10 µM indole acetic acid (IAA) and 8 µM 6-Benzylaminopurine (BAP). The banana cultures were incubated for 3-weeks at standard plant growth supporting environmental conditions. These cultures were sub-cultured on MS_{2b} [MS_0, 15 µM BAP, 1.0 g l^{-1} phytagel] medium for shoot induction for 2-weeks. After that these organogenized cultures sub-cultured on fresh MS_3 [MS_0, 15 µM BAP, 2.0 g l^{-1} phytagel] plant nutrient medium for shoot elongation and further multiplication (Haq and Dahot, 2007).

Micropropagation cultures with heavy metal treatment

When banana plantlets were 4-weeks old, these multiplying plantlets were sub-cultured on differentially heavy metal stressed cultures from the MS_3 plant micro-propagation cultures. These Cu^{2+} stressed cultures represented as MS_3 (control), MS_4 (MS_3 + 1.25 mg L^{-l} $CuCl_2$), MS_5 (MS_3 + 2.50 mg L^{-l} $CuCl_2$) and MS_6 (MS_3 + 5.0 mg L^{-l} $CuCl_2$). The metal stressed cultures incubated to grow for further 4-weeks on copper stressed plant micropropagation cultures.

Medium constants, sterilization and incubation conditions

All the cultures maintained for this study were contained 3 % sucrose which is used as carbon source for the cultured materials and 20 µM L-

cysteine for reduction in phenolic oxidation in injured tissues. The pH of these cultures adjusted from 5.7 to 5.8 before auto clavation (121°C and 20 lbs for 15 min) of the medium. The 4-replicates of each treatment maintained throughout this experiment. These cultures incubated for specific growth and time period at 25±1°C under light (16 h day^{-l}) conditions (except MS_2 cultures) maintained with artificial fluorescent lamps light intensity (~2000 lux) and RH 35%.

Banana cultures harvest and data collections

After 4-weeks of heavy metal (Cu^{2+}) treatment, the micro-propagated plantlets taken out from their containers. The plantlets washed under running tap-H_2O for the removal of entangled culture-medium. These plantlets were dried with toilet tissue-paper. The data was collected from the harvested plantlets.

Measurements of morphological parameters

The dried plantlets placed in the clean petri-dish and it morphological data was collected like as number of plantlets per explant were counted, shoot height (cm) measured with scale and fresh shoot biomass (g) also noted on digital balance than this fresh stuff was incubated at 72°C to dry them in electric oven for 2-days. Finally, dry biomass of plantlets from each culture also measured.

Measurements of chlorophyll contents

For the determination of photosynthetic pigments, 1.0 g fresh weight of shoots/leaflets samples taken in glass test tube with lid and 3 ml DMSO reagent (dimethyl sulfoxide) was added. The mixture was incubated for 45 min at 65°C in water-bath. It was stirred with hand with adding of 2 ml DMSO more for 30 seconds. In final, its OD was read at 445, 663 and 670 nm and these values used in the equations for chlorophyll a, chlorophyll b and total carotenoids determination respectively (Arnon, 1949; Lichtenthaler, 1987). Based on the fresh weight used as sample for both chlorophylls and carotenoids determination their units expressed in mg g^{-1}.

Analysis of bio-chemical contents

From the fresh shoots, various bio-contents like as free proline (Bates, Waldren, and Teare 1973), glycine-betaine (Grieve and Grattan, 1983) and total phenolics (Ozyigit et al., 2007) determined spectrophotometrically. Similarly, total flavonoids (Woisky and Salatino, 1998), antioxidants (Prieto et al., 1999) and ascorbic acid (Tabata and Morita, 1997) also analyzed. The total sugars determined by mixing 1 ml sample, 2.50 ml H_2SO_4 (conc.) and 5 µl phenol (80%). After 10 min its absorbance was taken at OD485 (Ciha and Brun, 1978; Dubois et al., 1956). The reducing sugar also analyzed by mixing 1 ml sample, 2.0 ml 3, 5-Dinitrosalicylic acid (DNS) reagent and heated in boiling water-bath for 15 min than OD540 was read (Miller, 1959). Furthermore, for total proteins, 2.5 ml alkaline copper reagents and 1 ml sample mixed thoroughly than after 10 min 0.25 ml folin reagents (1:1, w/v) added and its OD750 was read (Bradford, 1976).

Peroxidases and catalases activities

The peroxidase (POD) and catalase (CAT) activities were measured as exact 200 mg sample was homogenized in 10 ml 0.1M phosphate buffer (pH 6.8). Its 5.0 ml (remaining used for other biochemical analysis) centrifuged at 17,000 g for 15 min in refrigerated centrifuge. The supernatant is used as a crude enzyme source. The activities of both peroxidases and catalases assayed according to Chance and Maehly (1955) method with few modifications. For catalases analysis, 5 ml assay mixture [300 µM phosphate buffer (pH 6.8), 100 µM H_20_2] mixed with I ml crude enzyme (diluted twicely). The reaction mixture was incubated at 25°C. After 1 min, reaction stopped with 10 ml H_2SO_4 (2 %). It was titrated at room temperature against 0.01 N $KMnO_4$ until faint purple color appeared against water control. For peroxidases assay, 5 ml assay mixture [125 µM phosphate buffer (pH 6.8), 50 µM pyrogallol, 50 µM H_20_2] mixed with 1 ml enzyme extract (20 times-diluted). This mixture incubated at 25°C for 5 min. After which reaction stopped with 0.5 ml H_2SO_4 (5 % v/v). For purpurogallin quantification by taking its OD420. The tannases activity (Sharma et al., 2000; Rodríguez et al., 2008) assayed with spectrophotometric method at OD520 by formation of chromogen after reaction between rhodanine and gallic acid on standard curve of gallic acid. Pre-treated 100 µL crude enzyme mixed with 100 µL methyl gallate (0.025M), which prepared in phosphate buffer (0.05M, pH 6.5). exact 150 µL methanolic rhodanine [prepared 0.667% rhodanine in 100% methanol(w/v)] added to the mixture. After incubation at 30°C for 5 min, 100 µL KOH (0.5M) poured to the reaction mixture.

Statistical analysis

The collected data from each treatment with 4-replicates of the present study subjected for ANOVA (analysis of variance) and Duncan's Multiple Range (DMR) test at 5 % level of significance (Snedecor and Cochran, 1983). All these statistical analyses were computed with computer based statistical software "COSTAT" package (*CoHort* Software, Berkeley, USA).

RESULTS AND DISCUSSION

In nature, plants are the important immobile components, while salts especially heavy metals are transferring the environmental systems from biotic to abiotic. With the passage of time the contamination of heavy metals in the soils is increasing with different unmanaged industrial drained systems. It is a serious environmental problem, which interacts with biological targets including plants that limits the bioavailability of earth (Chojnacka et al., 2005; Misra et al., 2009). The *in-vitro* plant exposure to heavy metals leads to strict the production of ROS (Reactive Oxygen Species) as well as alters the antioxidant responses (Gratão et al., 2005). On the propagation of plants under heavy metals stress changes both enzymatic and non-enzymatic antioxidant systems for plant defenses. In present study, copper has exemplified the adjustments of various morphogenic roles during aseptic banana micropropagation.

PLANT MICROPROPAGATION AND ITS BIOMASS

The exposure of plant micropropagation to differential increasing stresses of copper chloride ($CuCl_2$) under aseptic conditions causes significant changes in growing plant biomass (Fig 1). The maximum plant multiplication rate observed in MS_3 (control) cultures, while decreased from lower (MS_4) to higher concentrations of the copper (MS_6) supplemented cultures. The plant cultures treated with 5.0 mg L^{-1} copper chloride observed tallest, while both fresh and dry weights of the cultures decreased with the increase in metal stresses. Various reports are suggesting that the increase in copper (Cu^{2+}) concentrations in MS salts, the rate of plant micropropagation increased, while its 200-folds increase lead to decrease the numbers of plantlets, its height and biomass also (Prażak and Molas, 2015). Even it is also reported that increase in copper ions in MS cultures have positive influence on initiation of organs (organogenesis) in many plant species. It can also induce the unorganized cell growth (callus production) along the cell differentiation (Nas, 2004). Similar results have also been observed in melon (*Cucumis melo* L.) as reported by Garcia-Sogo and Moreno (1991). They also recommended 1.0 mg copper sulphate as favorable concentration callusing, while it has noted that when copper applied in banana multiplication cultures in its copper chloride form has negative effects ($p \leq 0.05$).

CHLOROPHYLL CONTENT

A significant reduction in total chlorophyll contents observed in the copper stressed banana cultures than control plantlets or shoots. A proportional decrease in green pigments showed by the cultures in response to the increase in Cu^{2+} concentrations from the control (MS_3) cultures (Table 1). The decrease in chlorophyll contents, while increase in chl a/chl b ratios and increase in total carotenoids confirms the toxic effects of copper stress on the photosynthetic pigments likely to other heavy metals (Garrison et al., 2013; Hangarter and Stasinopoulos, 1991). It is also reported that the shoots possess lower chlorophyll contents but higher Chl a/Chl b ratio means that the chlorophyll b contents are losing consequently due to the reduction in capacity of light harvesting complex under copper stress (Küpper et al., 2003; Spiller and Terry, 1980; Vassilev et al., 2002). On other hand, inactivation of or degradation of photosynthetic enzymes may result to decrease in chlorophyll contents (Thapar et al., 2008). Other studies reported that Cu^{2+} have affinity with plastocyanin (electron transport chain) of PS-I. Its deficiency reduces the efficiency of plant photosystems (PS-I and PS-II) due to less or no formation of plastocyanin (Baszyñski et al., 1988; Henriques, 1989; Terry and Droppa, 1983). Meanwhile, abundance copper in leaves induces inhibition directly to these important reaction centers especially PSII (Küpper et al., 2002, 2003). The higher levels of Cu^{2+} interferes with biosynthesis and activity of these changing photosynthetic pigments lead to damages in their structures and losses functions (Feigl et al., 2015; Küpper et al., 2009).

Biochemicals of micropropagated shoots

With the increase in Cu^{2+} levels have directly proportional relations with free proline, glycine betaine, phenolics and reducing sugars. The total sugars and proteins levels decreased among the copper stressed cultures inversely (Table 1). Maximum prolines, glycine betaine and phenolic

contents (p≤0.05) observed in 5.0 mg L^{-1} stressed copper cultures (MS_6).These alterations in the reducing agents of the growing plantlets under metal stressed conditions indicates the increasing toxic effects on their physiological attributes. Such effects lead to decrease in biomass of plantlets.

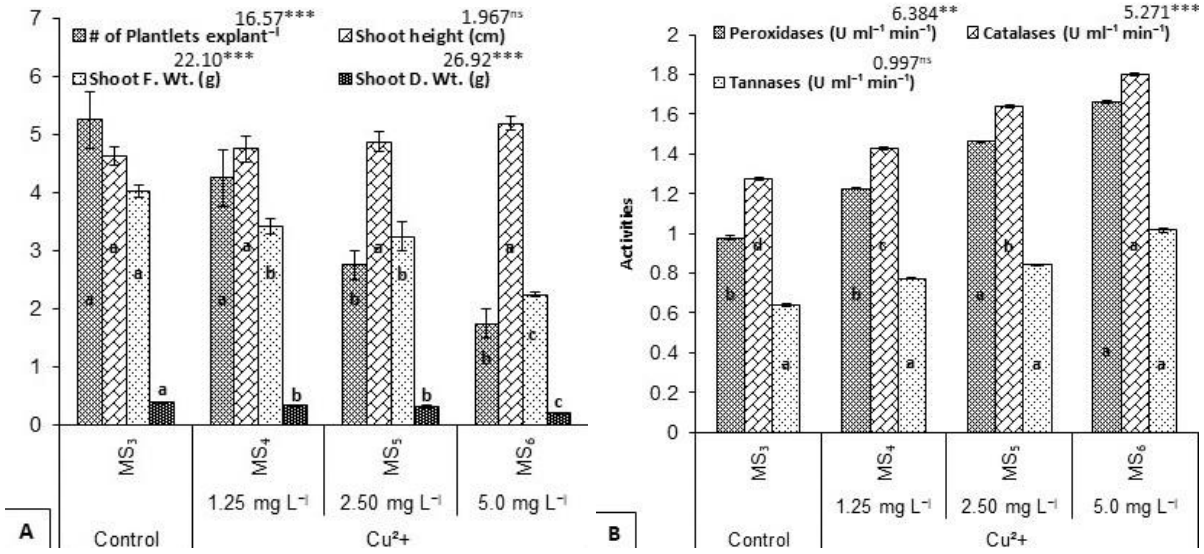

Figure 1: Effect of copper (Cu^{2+}) chloride on rate of shoot micro propagation, its biomass (A) and various enzyme activities (B) of banana (*Musa* spp.) cv., Basrai developed under *in vitro* cultures. The graphs are presented from means values of 4-replicates with its standard error, while letters i.e. a,b,c,d … used for DMRT, *, **, * for data significance and ns for non-significance at 0.05 (5%) level**

Table 1: Effect of copper (Cu^{2+}) chloride on physio-biochemical attributes of banana (*Musa* spp.) cv., Basrai micro propagated under *in vitro* cultures.

#s.	Characteristics	Control	Cu^{2+} stressed medium			p-signicance
		MS_3	MS_4	MS_5	MS_6	
A. Chlorophyll contents and carotenoids (mg ml⁻¹)						
01.	Chlorophyll a (Chl a)	2.132±0.028[a]	2.073±0.022 [a]	1.966±0.018[b]	1.901±0.008[b]	25.88***
02.	Chlorophyll b (Chl b)	0.898±0.006[a]	0.769±0.006[b]	0.722±0.009[c]	0.648±0.004[d]	276.0***
03.	Chl a/Chl b	2.374±0.019[a]	2.695±0.049[b]	2.725±0.043[b]	2.936±0.015[c]	45.70*
04.	Chlorophyll ab (Chl ab)	3.031±0.033[a]	2.843±0.017[b]	2.688±0.020[b]	2.549±0.010[b]	4.557*
05.	Total carotenoids	3.970±0.043[d]	3.724±0.022[c]	3.521±0.026[b]	3.339±0.014[a]	64.63***
B. Shoot bio-contents (mg ml⁻¹)						
01.	Total sugars	13.270±0.061[a]	10.47±0.146[b]	9.030±0.124[c]	7.570±0.113[d]	437.8***
02.	Reducing sugars	2.299±0.056[c]	2.792±0.156[b]	3.664±0.087[b]	5.939±0.299[a]	89.73***
03.	Total proteins	12.191±0.105[a]	8.873±0.160[b]	5.691±0.070[c]	4.730±0.090[d]	920-.8***
04.	Ascorbic acid	0.559±0.005[d]	0.649±0.029[c]	0.874±0.005[b]	0.987±0.004[a]	170.5***
05.	Free proline	0.852±0.005[d]	1.242±0.009[c]	1.558±0.004[b]	1.787±0.005[a]	4661***
06.	Glycine betaine	0.233±0.007[d]	0.462±0.005[c]	0.644±0.006[b]	0.841±0.005[a]	20.86***
07.	Total phenolics	5.886±0.055[d]	7.931±0.034[c]	10.11±0.030[b]	13.65±0.070[a]	1469***
08.	Flavonoids	3.104±0.038[d]	3.898±0.035[c]	5.487±0.031[b]	7.068±0.028[a]	3620***
09.	Antioxidants	0.228±0.005[d]	0.421±0.005[c]	0.568±0.005[b]	0.667±0.004[a]	172.2***

These values are the means of 4-replicates with standard error, while letters i.e. a,b,c,d … used for DMRT, *, **, *** for data significance and ns for non-significance at 0.05 (5%) level.

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Under heavy metal stresses, plants tissues accumulate various compatible osmolytes in response to the applied stress (Serraj and Sinclair, 2002). These compatible solutes are highly soluble and low molecular weight organic compounds, which are non-toxic usually even at their higher concentrations in the cell. These typical solutes have plant protection ability by contributing the osmotic adjustment for ROS detoxification, to maintain membrane integrity and stabilization of enzymes/protein against applied stress in the cell (Ashraf and Foolad, 2007; Strange and Yancey, 2020). These osmolytes include free proline, various reducing sugars, trehaloses, polyols and quaternary ammonium compounds (glycine betaine, proline betaine, alinine betaine and pipecolate betaine (Ashraf and Harris, 2004; Rhodes and Hanson, 1993), while phenomenon of proline accumulation is well known under heavy metal stresses (Sharma and Dietz, 2006; Hameed et al., 2015). Apart from cellular osmotic adjustments, proline involves in stabilizing membranes organelles and proteins with buffering the cellular redox potentials (Ashraf and Foolad, 2007).

Influences in antioxidant systems under Cu^{2+} stressed cultures

The ascorbic acid, flavonoids and antioxidants showed increase in concentrations with the increasing copper stresses (Table 1). These could also be involved in adjustments of the physiological processes under the copper stress. With the development of Cu stress on the plant tissues, many other oxidative stresses also get generated in the cells and these as a whole lead to inhibit the photosynthetic reactions (Rocchetta and Küpper, 2009). Like as it has reduced transpiration photosynthetic rates (Küpper et al., 2009). The toxic effects of metal appear to increase lipid peroxidation and protein carbonylation with development of antioxidant defense systems (Devi and Prasad, 2005; Gallego et al., 1996; Tripathi and Gaur, 2006).

The antioxidant enzymes including the peroxidases, catalases and tannases ($p \leq 0.05$) have showing directly proportional relationships with increasing levels of copper stresses (Fig 1). Mostly, plants on exposure to metals lead to the activation of antioxidative systems, while its direction is variant, which depend on plant species, its tissues and type of imposed metal stress (Hossain et al., 2012; Gupta et al., 2015). The prime role of antioxidant enzymes is to contribute for tissue protection from the toxicity of metals in plants as well as in fungi (Krieger-Liszkay et al., 2008; Utarbayeva et al., 2018). According to these results have shown the important roles of antioxidant enzymes for scavenging of hydrogen peroxide (H_2O_2) produced under toxic metals stress in the plant cultures. The major role of antioxidant enzymes is to protect the plant tissues the production of reactive oxygen species (ROS). The increase in the levels of antioxidant enzymes systems are leading to break down the concentration of (H_2O_2) in cells/tissues. This increasing level of enzyme activities are lessing the damages due to ROS.

CONCLUSION

The aseptic plant multiplication depends on the balanced supply of nutritional salts and optimal environmental conditions. Any fluctuation in the required amount of salts could be realized in appearance of growing plants. The copper has applied in excess in doses than its normal requirement in the banana propagation cultures. The copper stress reduces the plant growth (multiplication rate and its biomass) significantly, which is probably divert the plant tissues to invest excess net-energy in the preparation of antioxidant defense system. Likely to that with increasing the metal concentrations lead to increase activities of peroxidases and catalases. These enzymes are involved in the inhibition of metallic oxidative cellular damages (lipid, protein and DNA etc.) and loss of intrinsic membrane properties (ions fluidity, ionic transport) with ROS formation. This study could be helpful in future for the recommendation of soil either contaminated with metals or not for the cultivation of a crop with safe and optimal productions.

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

ACKNOWLEGEMENT

The authors are thankful for the administration of the institute for providing experimental station and for financial support for the completion of the work

AUTHOR CONTRIBUTIONS

First two authors IH and MDK designed and performed this research project and contributed equally. The collected data analyzed statistically by NZG. GY & IH also supervised this experiment. FAB reviewed the text including literature,

structure of paragraph and tables. All authors have read the research paper and approved its final version for publication.

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