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Full Length Research Paper

Study of copper phytotoxicity on *in vitro* culture of *Musa acuminata* cv. 'Bantala'

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The present study aimed to investigate the effect of different concentration of copper on growth and development of *Musa acuminata* cv. Bantala grown *in vitro*. The results showed that 1.0 µM copper stimulated root induction, elongation and shoot growth when compared with the control (0.1 µM copper). In addition, higher level of copper (100 µM) has toxic effect on banana leaves with regard to stunted growth, curling leaf and complete inhibition of root formation. Copper exposure increased photosynthetic pigment contents, decreased carbohydrates and protein contents at 100 µM of copper. This investigation will help to estimate the copper tolerant plants for phyoremediation programme.

Key words: Copper, phytotoxicity, banana micropropagation.

INTRODUCTION

Heavy metals are essential elements for all forms of life. However, accumulation of excess heavy metal in soil and water creates serious problems for the environment and human health. The characteristics feature of excess heavy metals are poisoning, and resulting in the inactivation of enzyme systems at elevated concentration (Gadd and White, 1989). Higher levels of heavy metals uptake by plant cause carcinogenic and mutagenic effects (Goyer and Mehlman, 1977).

Copper (Cu) is an essential transition metal that is involved in many physiological processes in plants, because it can exist in multiple oxidation states *in vitro*. It acts as a structural element in regulatory proteins and participates in photosynthetic electron transport, mitochondrial respiration, oxidative stress response, cell wall metabolism and hormone signaling (Marschner, 1995; Raven, 1999), and also as cofactor in many enzymes, such as superoxide dismutase, Cytochrome C oxidase, amino oxidase, lactase and plastocyanin polyphenol oxidase. When copper is not available, plant develop specific deficiency symptoms, most of which affect young leaves and reproductive organs. Thus, plants require Cu as an essential micronutrient for normal growth and development. As a result of the formation of organocopper complex, excess Cu may be highly toxic (Marschner et al., 1986). Compared to other heavy metals, Cu is not readily bioaccumulated and so, it is unusually dangerous for human and animal health, but it is highly toxic to plants (Fernandes and Henriqus, 1991).

The aim of the present study was to investigate the effect of various Cu concentration on roots and shoots of banana cultured *in vitro*. The toxic effect of super-optimal concentration of Cu in rooting medium of *in vitro* plantlets on biochemical parameters was also studied. Earlier studies showed that Cu can interact with a wide range of physiological and biochemical processes in plant species (Jouili and Ezzedine, 2003; Draobekiewicz et al., 2004). However, the effect of elevated Cu levels on growth and defense responses of *Musa acuminata* cv. Bantala are still unclear. Keeping in view the importance of Cu in plant system, it is desirable to establish the copper toxicity in plants.

MATERIALS AND METHODS

Plant and treatment

Micropropagated shoots of banana (*M. acuminata* cv. Bantala) cultured were collected form tissue culture laboratory of Regional Plant Resource Centre, Bhubaneswar, Odisha, India. The micropropagated shoots were then inserted into rooting medium comprising of Murashige and Skoog (1962) (MS) medium supplemented with 1 mg/L indoleacetic acid (IAA). The medium was then supplied with Cu in the form of copper sulphate to obtain a final concentration of 1, 10, 50 and 100 µM for each of the treatments. The pH of the medium was adjusted to 5.8 ± 01 using 0.1 N HCl or 0.1 N NaOH after addition of Cu before autoclaving.

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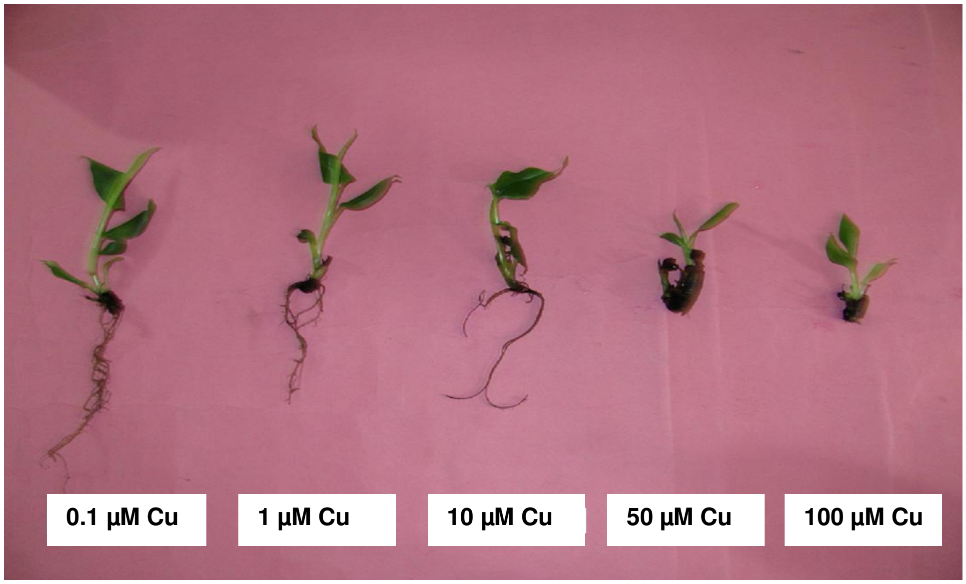

Figure 1. Tissue cultured plants grown at different concentration of Cu.

Table 1. Effect of different concentration of copper on shoot growth of *M. acuminata* cv. Bantala grown on MS medium after four weeks of culture.

Copper level (µM)	Root induction	Dry matter (%)	Ratio of shoot and root length (cm)
0	6.6 ± 0.3	6.61	2.45 ± 0.6
0.1	8.6 ± 0.5	7.20	2.77 ± 0.7
1	9.5 ± 0.7	7.31	3.91 ± 0.8
10	4.8 ± 0.3	5.84	4.90 ± 0.3
50	3.0 ± 0.7	4.33	2.13 ± 0.4
100	1.1 ± 0.2	2.93	1.80 ± 0.5

Means of 10 replications per treatments ± SE repeated thrice.

The medium was gelled with 0.8% (w/v) agar (Qualigen, India) and 20 ml of molten medium was dispersed into the culture tubes. The medium was then autoclaved at 121°C and 104 kPa for 15 min. The cultures were incubated at 25 ± 2°C in cool white fluorescent light (Phillips, India), with a photons flux density of 55 µM photons $mol^{-2}s^{-1}$ under a 16 h photoperiod. The morphological changes were recorded on the basis of visual observation Figure 1. Plantlets were then assorted and used for biochemical analysis.

Growth determination

Plant growth and development are essential processes of life and propagation of species. They are continuous and mainly depend upon external resources present in soil and air. The presence of Cu in excesses in media leads to changes in the growth and development pattern of the plant (*M. acuminata* cv. Bantala). These effects are summarized in Table 1.

Photosynthetic pigment content

Leaf samples (500 ± 20 mg fresh weight basis) from each treatment were collected at 15 days intervals for estimation of photosynthetic pigments (chlorophylls and caroteinoids). According to Porra et al. (1989), the amount of chlorophyll and caroteinoids were estimated. Pigment content was expressed as mg/g fresh weight of sample.

Total protein content

Leaf sample (500 ± 20 mg fresh weight basis) from each treatments were homogenized with 30 ml of 2% polyvinyl pyrolidon (pvp) and 0.5 ml extraction buffer (25 mM Tris-HCl, pH 8) in a mortar and pestle. The homogenate was centrifuged at 10000 rpm for 20 min and the supernatant was taken. 20 µl of supernatant was transferred to a test tube and the volume was made up to 1 ml with distilled water. Afterwards, 5 ml of Bradford reagent was added, and absorbance was taken at 595 nm. The amount of the total protein was estimated by the Bradford (1976) methods using Bovine Serum Albumin as a standard.

Estimation of carbohydrate content

Leaf sample (100 ± 20 mg dry weight basis) form each treatment were incubated in water bath with 80% alcohol for 10 min and then homogenized with mortar and pestle. The homogenate was centrifuged at 5000 rpm for 10 min and the supernatant was taken. Collected supernatant was diluted to 20 times with distilled water.

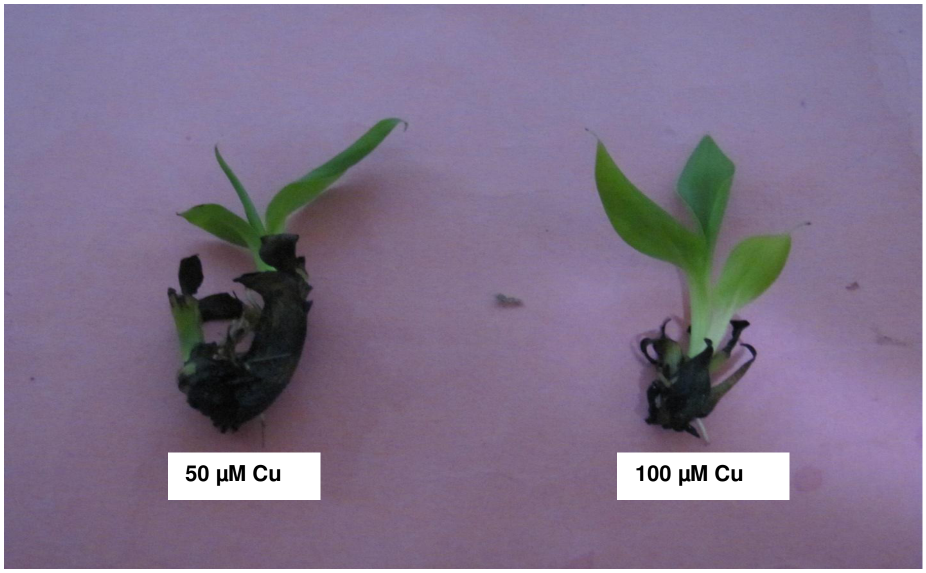

Figure 2. Tissue cultured plants grown in higher concentration of Cu.

Afterwards, 1 ml of diluted sample was transferred to a test tube and 4 ml of Anthrone reagent was added to it. It was kept in incubation for 10 min followed by rapid cooling. The absorbance was taken at 630 nm and total carbohydrate content was determined according to McCready et al. (1950) using sucrose as a standard. The result was expressed in terms of mg/g fresh weight.

Statistical analysis

The data were statistically analyzed by analysis of variance. Between the treatment, the average figures followed by the same letter within a column in the table were not significantly different at $P < 0.05$ level.

RESULTS AND DISCUSSION

Effect of copper toxicity on morphogenesis

Frequency of root induction, dry matter percentage, growth value as well as mean ratio of root and shoot length of *M. acuminata* cv. Bantala were recorded in different concentration of Cu in the culture medium (Table 1). Results indicated that incorporation of different concentration of Cu in the rooting medium enhanced the root formation and subsequently the *in vitro* regeneration. The highest frequency of root induction was observed with medium having 1.0 µM Cu. However, the highest percentage of dry matter as well as the growth of the plantlets (length) was scored with medium containing 1 µM Cu followed by 0.1 µM Cu containing medium. The present results are in agreement with those obtained by Bipasha et al. (2000) in their work. They reported that Cu is the most important element determining micro molecule for root induction and later plant regeneration in *Citrus reticulata*. Moreover, Nassar (2004) in his study on banana mentioned that Cu stimulated root induction, elongation and shoot growth. Higher level of Cu had toxic effect on banana leaves and completely inhibited root formation. In addition, Sanjeev et al. (2003) reported that the *Tinospora cordifolia* performed better growth subjected to different concentrations of $CuSO^4$ (25 to 125 µM) when compared with the controls on MS medium having copper level (0.1 µM). Yurekli and Porgali (2006) reported that there is an improved shoot and root formation of bean plants, when Cu concentration increased in the medium Figure 2. However, higher concentration of Cu was reported to be ineffective. On the basis of the previous findings, the present study suggested that elevated copper concentration can inhibit the normal growth and development of *M. acuminata* cv. Bantala.

Photosynthetic pigment content

The photosynthetic pigments of *M. acuminata* cv. Bantala were decreased with increase in Cu concentration in the medium Table 2. It may be due to the Cu effect on Photosystem II (PS II), which has been associated with the destruction of inner structure of chloroplast and modifications of the lipid protein composition of thylakoid membranes. Photosystem I (PS I) was the coupling factor or the thylakoid membrane through copper mediated lipid peroxidation, in addition to copper inhibition of enzymatic reaction in the photosynthetic carbon reduction cycle (Quzounidou et al., 1995; Porgali and Yurekli, 2005).

Protein content

The concentration of soluble proteins in *M. acuminate* cv.

Table 2. Total photosynthetic pigment content in the leaves of *M. acuminata* cv. Bantala grown on MS medium supplemented with different concentration of Cu after four weeks of culture.

Copper level (µM)	Chlorophyll A (mg/g)	Chlorophyll B (mg/g)	Total chlorophyll (mg/g)	Caroteinoids (mg/g)
0	0.222 ± 0.3	0.252 ± 0.6	0.474 ± 0.3	0.183 ± 0.3
0.1	0.236 ± 0.6	0.265 ± 0.8	0.501 ± 0.6	0.182 ± 0.6
1	0.323 ± 0.6	0.367 ± 0.3	0.690 ± 0.8	0.183 ± 0.8
10	0.323 ± 0.8	0.345 ± 0.8	0.668 ± 0.8	0.161 ± 0.3
50	0.210 ± 0.6	0.252 ± 0.8	0.462 ± 0.5	0.155 ± 0.6
100	0.102 ± 0.3	0.040 ± 0.6	0.142 ± 0.7	0.096 ± 0.6

Means of 10 replications per treatments ± SE; repeated thrice.

Table 3. Total protein (µg/g) and carbohydrate (mg/ml) content in the shoots of *M. acuminata* cv. Bantala cultured on MS medium supplemented with different concentration of Cu.

Biochemical parameter	Copper level (µM)					
	0	0.1	1	10	50	100
Total protein content (µg/g)	1110 ± 14.6	1132 ± 11.3	1206 ± 12.6	1422 ± 10.8	1876 ± 12.5	2033 ± 14.4
Total carbohydrate content (mg/mL)	5.6 ± 0.6	5.6 ± 0.4	4.8 ± 0.8	4.3 ± 0.6	3.6 ± 0.7	2.3 ± 0.6

Means of 10 replications per treatments ± SE; repeated thrice.

Bantala grown in different concentration of Cu was shown in Table 3. With increase in concentration of Cu, the protein content increases. This study suggests that plants are getting tolerance towards metal stress. The increase in protein content may be due to increase in metal binding protein. Similar observation has been reported by Lidon et al. (1993) in rice plants. On the other hand, some researchers reported that, under stress conditions, only proteins that specifically respond to stress (stress-induced proteins) are induced in many plants (Ben-Hayyim et al., 1989; Ferguson et al., 1994).

Carbohydrate content

The total carbohydrate content was less in the shoots growth in high Cu concentration as compared to control as shown in Table 3. The carbohydrate content decreases with increase in Cu concentration in the culture medium. Shoot grown in the medium with out Cu showed highest carbohydrate content and less content in 50 and 100 µM Copper containing medium. This suggests that copper counteract metal stress (Alba, 2004). However, Romeum-Moreno and Mas (1999) reported that Cu exposed to plants had lower levels of sugar and no change in starch.

Induction of rooting and field acclimatization

The shoots regenerated from MS medium supplemented with different concentration of Cu were excised and cultured. A high percentage of rooting (95%) was obtained on 1 µM Cu concentrated MS medium. The rooting plantlets were transferred to green-house condition for acclimatization. About 90% of tolerant plants survived in the greenhouse condition.

Conclusion

The present investigation concluded that 1 µM Cu concentration in the MS medium is suitable for the growth of this particular variety of banana *M. acuminata* cv. Bantala. The result obtained in the present investigation demonstrated that root induction, dry matter, shoot and root growth of banana were affected by higher Cu concentration in the rooting medium. The photosynthetic pigments are decreased with increase of Cu concentration in the medium. However protein content was increased due to the development of stress protein. The low cupric sulphate of MS medium (0.1 µM) was not optimum for banana shoots grown *in vitro*. Ten-fold increase of this concentration proved more suitable. From these results, suggestion given for other macro- micronutrients levels used in MS medium need to be tested and optimized for values that can be recommended for plant species cultured *in vitro*.

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