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Effect of Some Copper Compounds on Rhizogenesis of Micropropagated Banana Shoots

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ABSTRACT

The effect of different concentrations of cupric sulfate, cupric chloride or cupric acetate on rhizogenesis of banana micropropagated shoots was investigated. The results showed that 1 µM $CuSO_4$ stimulated root induction, elongation as well as shoot growth compared with the control (0.1 µM $CuSO_4$ present in Murashige & Skoog medium). Higher level of $CuSO_4$ (100 µM) had toxic effect on banana leaves and completely inhibited root formation. Cupric chloride proved more convenient in the culture medium than $CuSO_4$. Cupric chloride stimulated good quality of roots, enhanced shoot growth and showed no toxicity symptoms at higher concentrations. Cupric acetate was very toxic even at low concentration. Copper exposure increased chlorophyll and carotenoid contents, decreased sugars and proteins at 10 µM of both $CuSO_4$ and $CuCl_2$. Exposure to this concentration also accumulated more Cu, Zn and Mn in both shoots and roots. Fe concentration decreased in shoots and increased in roots in response to 10 µM Cu. The effect of Cu on rhizogenesis of micropropagated banana shoots was discussed.

Key Words: Copper; Banana micropropagated shoots; Rhizogenesis

INTRODUCTION

Copper (Cu) is an essential micronutrient for normal plant growth. It is a constituent of the protein component of several enzymes in plants, mainly those participating in electron flow, catalyzing redox reactions in mitochondria, chloroplasts, cell wall and cytoplasm of plant cells (Lolkema, 1985). Since copper is a plant micronutrient, exposure to high concentration of Cu can cause a broad range of deleterious effects such as inhibition of photosynthesis and pigment synthesis, damage to plasma membrane permeability as well as other metabolic disturbances, either in field plants (Lanaras *et al*., 1993; Ouzounidou *et al*., 1993) or *in vitro* grown plants (Gori *et al*., 1998; Romeu–Moreno & Mas, 1999).

Inorganic macronutrient and micronutrient levels used in most plant tissue culture media are based on levels established in the medium developed by Murashige and Skoog (1962) for tobacco tissue culture "MS medium". For several micronutrients, however, no clear optimal levels were apparent and the effect of alternative formulations of these nutrients in tissue culture media was scarcely studied. Micronutrient levels adequate for tobacco tissue culture may not be optimum for culture of other plant species. Neales (1959) obtained normal growth of excised flax (*Linum usitatissimum* L.) roots on a medium containing 0.005 mg/L Cu. Stiles (1961) reported normal vegetative growth of *Avena sativa* L. in solution cultures containing 0.02 mg/L Cu. These concentrations were much lower than in MS medium (0.1 µM = 0.25 mg/L). On the other hand, Purnhauser (1991) examined the effect of six copper levels on regeneration from callus cultures in hexaploid wheat. Regeneration rates were eight times higher on medium containing 100 times $CuSO_4$ than on the original MS copper level. Similar results were reported by Purnhauser and Gyulai (1993) when wheat anther cultures and triticale immature embryo–derived callus were used. Ghaemi *et al*. (1994) reported that the addition of 40 µM $CuSO_4$ to the medium significantly increased the embryoid production from wheat anther cultures.

Excessive copper, however, becomes toxic to many plant species (Wu & Lin, 1990). One of the most rapid responses to toxic Cu levels is inhibition of root growth (Eleftheriou & Karataglis, 1989). Root growth has also been considered as a very sensitive indicator to heavy metal exposure (Wilkins, 1978). Bipasha *et al*. (2000) has also reported that high Cu content affected root growth than shoot growth.

The aim of the present study was to investigate the effect of various copper compounds and concentrations on rhizogenesis (root induction & elongation) of banana shoots cultured *in vitro*. The study was also undertaken to evaluate the optimum copper concentration and formulation needed for banana cultures. The toxic effect of supra-optimal concentration of Cu in rooting medium on biochemical parameters (chlorophyll, carotenoid, sugars & total proteins) in *in vitro* plantlets was also studied. The effect of cupper exposure on the accumulation of Cu, Fe, Mn and Zn in shoots and roots of the produced plantlets was also determined.

MATERIALS AND METHODS

Micropropagated shoots of banana (*Musa* spp. Cv. Williams) grown *in vitro* were subcultured regularly every four weeks on MS medium supplemented with 5 mg/L BAP (benzoyl aminopurine) and 30 g/L sucrose. The pH of the medium was adjusted to 5.8±0.1 prior to the addition of 8 g/L bacteriological agar (Meron, Marine Chemicals, Cochin, India). Medium was autoclaved for 15 minutes at 121°C and 15 psi.

Effect of different formulations and concentrations of copper. Micropropagated shoots of banana with no visible signs of root development were inserted into rooting medium consisting of MS medium supplemented with 1 mg/L NAA. Medium was supplied with Cu as cupric sulfate, cupric chloride or cupric acetate to make a final concentration of 1, 10, 50 or 100 µM Cu for each of the three formulations. The pH of the medium was adjusted to 5.8±0.1 after the addition of copper compounds. The control was 0.1 µM Cu as cupric sulfate normally present in MS medium. Each treatment was represented by four shoots. Jars containing micropropagated shoots were incubated at 27°C±1 and 16 h photoperiod. Six weeks later, banana micropropagated shoots were harvested. The number of roots developed per shoot, the length of roots and the length of shoots were recorded. Morphological characteristics of shoots and roots were described. Plantlets were then assorted into shoots and roots and used for biochemical analysis.

Determination of biochemical parameters. Chlorophylls were determined according to Arnon (1949) and measured at 663 and 645 nm. The method of Davies (1976) was used for carotenoid determination at 480 nm. The concentrations of chlorophylls and carotenoids were determined as follows:

$$\text{Chlorophyll a (mg/L)} = 12.7\ abs_{663} - 2.60\ abs_{645}$$
$$\text{Chlorophyll b (mg/L)} = 22.9\ abs_{645} - 4.68\ abs_{663}$$
$$\text{Total chlorophyll} = \text{chlorophyll a} + \text{chlorophyll b}$$
$$\text{Carotenoids (mg/L)} = 20\ abs_{480} + 2.28\ abs_{663} - 12.76\ abs_{645}$$

Soluble sugars were determined according to Buysse and Merckx (1993). Proteins were estimated by the Bradford method (1976) using Bovine serum albumin as a standard. Microelements were measured spectro-photometrically (AAS, Hitachi Z–8200) after wet nitric/hydrochloric acid (3:1) digestion.

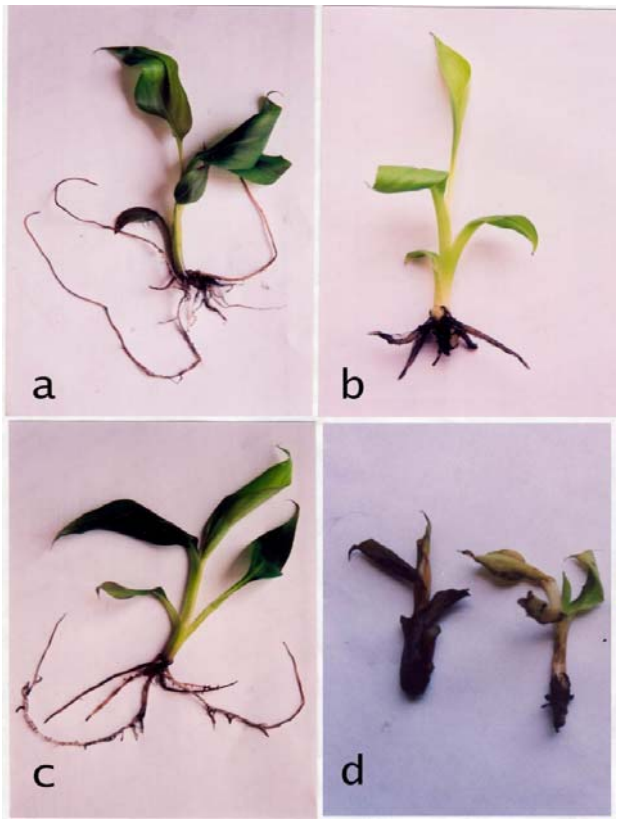
Statistical analysis. Data were statistically tested using Student's t–test for comparison between means of treatments.

RESULTS AND DISCUSSION

The addition of low concentration of copper (1 µM) to the rooting medium in the form of cupric sulfate had the same effect as control (0.1 µM) on the rate of root induction (about 10 roots/shoot). Root induction was measured by the number of roots regenerated per shoot (Table I). However, root elongation, as measured by the mean root length per shoot, was greater when 1 µM $CuSO_4$ was used compared with the control (Table I). Roots of both concentrations were long, thin with fine root hairs, which were condensed at the base of the shoot (Fig. 1a). A decline in both root induction and elongation was observed as the concentration of $CuSO_4$ was increased up to 50 µM (Table I). Roots at 50 µM appeared thick and stunted with inhibition of root hairs formation (Fig. 1b). A complete inhibition of rhizogenesis was recorded when 100 µM $CuSO_4$ were added to the rooting medium. The shoot length was stimulated with exposure to 1 µM $CuSO_4$ relative to the control (Table I). A gradual decline in shoot length was then noticed with increasing $CuSO_4$ concentrations (up to 50 µM). No growth was recorded at 100 µM $CuSO_4$ in the culture medium (starting materials were 4 cm long). At 50 µM, the leaves showed chlorosis whereas at 100 µM, leaf browning and senescence were recorded.

Similar results have been obtained when Arnold *et al.* (1994) studied the effect of Woody Plant Medium (Lloyd & McCown, 1980) supplemented with different copper compounds and concentrations on *in vitro* rhizogenesis and subsequent shoot growth of microcuttings of birch (*Bitula pubescens*) clone. They reported a reduction in root

Fig. 1. *In vitro* rhizogenesis of banana shoots after six weeks of copper exposure. (a) 1.0 µM copper sulfate, (b) 50 µM copper chloride, (c) 1.0 µM copper sulfate and (d) 50 µM "right" and 100 µM "left" copper acetate



formation in the presence of 79 to 118 µM $CuSO_4$ with near zero root regeneration and shoot growth inhibition at 157 µM. In their study, Cu toxicity symptoms were observed at concentrations higher than 79 µM in the culture medium. In addition, Sanjeev *et al.* (2003) reported that the medicinal plant *Tinospora cordifolia* subjected to different concentrations of $CuSO_4$ (25–125 µM) performed better growth compared with the controls on MS copper level (0.1 µM).

Purnhauser and Gyulai (1993) reported an improved shoot and root regeneration, from calli of wheat, triticale and tobacco plants, when $CuSO_4$ increased in the culture medium from 0.1 to 50 µM. However, higher concentration of $CuSO_4$ was reported by the same authors to be ineffective or inhibitory with rape callus culture. Dahleen (1995) studied the effect of different concentrations of $CuSO_4$ on callus culture of two cultivars of barley and found that medium containing 50 µM copper regenerated significantly more plants with an average of 17 plants per embryo of the cultivar "Hector", in comparison with normal MS level which regenerated only 5 plants per embryo. For the other cultivar "Excel", medium containing 5 µM $CuSO_4$ regenerated 14 plants per embryo while no regenerants were obtained on medium with MS copper level. Sahrwat *et al.* (1999) reported that somatic embryogenesis and number of plantlets regenerated in rice immature embryos were enhanced at 10 and 50 µM when a range of 0.1–100 µM $CuSO_4$ was tested. In addition, Kintzios *et al.* (2000, 2001) reported that a ten–fold increase in the concentration of $CuSO_4$ than the standard MS medium favored the production of somatic embryos from pepper and rose calli without reducing embryo maturation or germination.

Contradictory results were obtained by Gori *et al.* (1998) who reported that 50 µM $CuSO_4$ significantly inhibited callus growth and shoot regeneration in *Nicotiana tabacum* callus culture. In addition, Romeu–Moreno and Mas (1999) found that copper level over 50 µM inhibited root and plantlet development in *Vitis vinifera* cultured in a closed system (*in vitro* explants). El-Aref and Hamada (1998) found that Cu was toxic to tomato explants at 100 µM cupric sulfate as reflected by reduced callus growth and shoot regeneration. This was interpreted by them to a reduction in the expression of specific enzymes (dehydrogenases and esterases).

Banana micropropagated shoots, in the present investigation, responded differently when cupric chloride was added to the rooting medium. Although strong, thick roots with well developed hairs were initiated at 1 and 10 µM $CuCl_2$ (Fig. 1c), a stimulation in shoot growth was recorded in relation to controls (Table I). A noticeable decline was recorded in rhizogenesis and shoot growth as the concentration of $CuCl_2$ was increased to 50 and 100 µM. However, such inhibition was not severe as that observed with $CuSO_4$. Copper toxicity symptoms observed at higher concentrations of $CuSO_4$ were not detected with $CuCl_2$. Although shoots showed no pronounced elongation, they remained green with no signs of chlorosis at 100 µM of $CuCl_2$.

Root and shoot growth was more inhibited by cupric acetate to the extent that they cannot be measured. Negligible growth was recorded at 1 µM cupric acetate. Necrotic lesion, wilting, browning and death of plantlets exposed to over 10 µM of copper acetate were observed showing a remarkable toxicity of this compound in the culture medium (Fig. 1d).

In agreement with the present study, Arnold *et al.*

Table I. Effect of copper (1, 10, 50 and 100 µM) as $CuSO_4$ or $CuCl_2$ (control, 0.1 $CuSO_4$ of MS medium) on number of roots per shoot, length of roots (cm) and length of shoots (cm) after six weeks of copper exposure

	Control	$CuSO_4$ (µM)				$CuCl_2$ (µM)			
	(0.1 µM $CuSO_4$)	1	10	50	100	1	10	50	100
No. of roots / shoot	10.2±0.4	10.0±0.7	6.4±0.3	5.0± 0.6	_	8.1± 0.1	7.3± 0.1	6.0±0.3	4.0±0.3
Length of roots (cm)	9.6±0.3	10.3±0.6	7.2±0.1	4.3±0.5	_	9.0±0.6	9.3±0.2	6.5±0.4	3.1±0.2
Length of shoots (cm)	9.7±0.5	10.6±0.4	9.0±0.4	8.4±0.1	4.0±0.0	11.3±0.5	10.9±0.4	9.3±0.2	5.8±0.1

Data are mean ± SD of four replicates

Table II. Effect of copper (10 µM) as $CuSO_4$ or $CuCl_2$ on total chlorophyll, carotenoids, sugars and proteins (mg/g fresh weight) and Cu, Zn, Mn and Fe (µg/g dry weight) of shoots and roots after six weeks of Cu exposure

	Control		$CuSO_4$		$CuCl_2$	
	Shoot	Root	Shoot	Root	Shoot	Root
Chlorophyll	3.5±0.3	__	4.4±0.2	__	4.9±0.4	__
Carotenoids	2.6±0.2	__	3.5±0.6	__	3.7±0.4	__
Sugars	19.9±0.1.9	13.2±0.9	16.4±2.1	8.6±1.1	17.9±0.8	9.1±0.9
Proteins	11.3±0.8	8.4±0.7	9.1±1.2	7.3±0.4	10.8±0.7	7.9±0.4
Cu	3.5±0.1	11.2±0.7	14.9±0.3	61.3±2.2	11.7±0.5	52.3±1.4
Zn	56.8±2.1	137.2±3.3	72.3±1.8	152±3.2	69.6±1.4	161.5±1.9
Mn	174.1± 4.2	44.9±1.5	218.4±3.4	61.5±1.8	192.2±2.7	51.9±0.9
Fe	181.1±2.8	452.5±2.9	116.6±2.4	480.3±4.3	148.6±2.9	469.0±3.6

Data are mean ± SD of four replicates.

(1994) reported an increase in root and shoot growth of *Betula pubescens* by 79 µM $CuCl_2$ in the culture medium with no significant increase in Cu-toxicity symptoms at concentration up to 157 µM. They also indicated that root and shoot growth were virtually eliminated and Cu-toxicity symptoms drastically increased, even at low concentrations, when cupric carbonate or acetate was used.

The root growth was more sensitive than shoot when Cu was added to the medium (Table I). Same result was earlier reported by Wilkins (1978), Eleftheriou and Karataglis (1989), Arduini *et al*. (1994) and Bipasha (2000). The reduction in root growth was explained by the previous authors by an inhibition either in cell division or cell elongation or both. In addition, Mengel and Kirkby (1982) demonstrated that root growth inhibition is often the first expression of Cu toxicity symptoms, probably because roots tend to bind Cu, while leaves and stems usually exhibit Cu toxicity symptoms at higher concentrations.

Cu, when used as $CuSO_4$ or $CuCl_2$, increased the total chlorophyll content and carotenoids in banana micro-propagated shoots. However, a decrease in sugars and proteins by Cu were obtained (Table II).

Romeu–Moreno and Mas (1999) reported that copper–exposed plants (0.07 to 10 µM Cu/g medium) had higher levels of chlorophyll and carotenoids, lower level of sugars and no change in starch or sucrose. However, Ouzounidou *et al*. (1992) indicated that Cu decreased chlorophyll and carotenoid contents in *Thlaspi ochroleum* and oat. *Gori et al*. (1998) found no difference in chlorophyll content or chloroplast structure among *Nicotiana tabacum* shoots, which survived 100 µM copper.

The increase in photosynthetic pigments and the decrease in photosynthates, observed in the present investigation, were more pronounced in $CuSO_4$ than $CuCl_2$. Moya *et al*. (1993) reported that when the photosynthetic pigments were increased while the photosynthates were not, this might be due to a reduction in CO_2 fixation, through Rubisco inhibition as well as other metabolic enzymes. Lack of sugar utilization for growth due to excessive heavy metals may be stronger than inhibition of CO_2 fixation (Moya *et al.*, 1993). Both possibilities may be the case in the present study.

Llorens *et al.* (2000) reported that copper exposure upon tissue cultured *Vitis vinifera* resulted in a dramatic changes in nitrogen metabolism, which was reflected in reduced total nitrogen, amino acids and protein contents in both roots and leaves. This may explain the protein changes observed in this study. A reduction in wheat protein and sugar concentrations was reported by Laranas *et al.* (1993) in response to Cu.

Studying the effect of 10 µM of both formulations of copper, as a moderate concentration, on the accumulation of Cu in banana plantlets showed an increase in Cu levels in both shoots and roots (Table II). This result is in agreement with the finding of Lidon *et al.* (1993) and Moya *et al.* (1993). However, the increase was more pronounced in roots suggesting an immobilization of Cu from root to shoot (Romeu-Moreno & Mas, 1999). This immobilizing mechanism resulted in high content of Cu in the root, which has a stronger effect on this organ than on others (Llorens *et al*., 2000). Cu (10 µM) increased the concentration of Mn and Zn in both shoots and roots of treated micropropagated plantlets compared with the controls (Table II). However, the concentration of iron decreased in shoots and increased in roots. Punz and Sieghardt (1993) and Reboredo (1994) stated that the immobilizing mechanism might be effective with other elements, and thus roots can act as storage organs for trace elements.

Ouzounidou *et al.* (1992), however, reported that increasing Cu concentration in nutrient medium reduced the uptake of nutrient elements such as Ca, Mg, K and Fe. Reboredo (1994) also indicated that Cu exposure induced changes in mineral metabolism, especially Fe and Zn. An increase in leaf Cu, Fe and Zn levels due to Cu exposure of wheat was demonstrated by Laranas *et al.* (1993). Moreover, Kintzios *et al.* (2003) reported that callus cultures of mistletoe (*Viscum album*) derived from stem explants accumulated more Fe, Mn, Zn and Cu than calli derived from leaf explants.

The results obtained in the present investigation demonstrated that root induction, elongation and subsequent shoot growth of banana micropropagated shoots were affected by both copper concentration and formulation in the rooting medium. The low cupric sulfate of Murashige and Skoog medium (0.1 µM) was not optimum for banana shoots grown *in vitro*. Ten-fold increase of this concentration proved more suitable. Results of rhizogenesis, shoot growth and other parameters tested indicated that banana plantlets adapted better to copper exposure in the form of cupric chloride than cupric sulfate, suggesting that this formulation of copper compounds might be more convenient in the culture medium of banana. The results also suggest that other macro- or micronutrient levels in MS medium need to be tested and optimized for every plant species cultivated *in vitro*.

REFERENCES

Arduini, I., D.L. Godbold and A. Onnis, 1994. Cadmium and copper change root gtowth and morphology of *Pinus pinea* and *Pinus pinaster* seedlings. *Physiol. Plant.*, 92: 675–80

Arnold, M.A., R.D. Lineberger and D.K. Struve, 1994. Copper compounds influence *in vitro* rooting of birch microcuttings. *J. American Soc. Hort. Sci.*, 119: 74–9

Arnon, D.I., 1949. Copper enzymes in isolated chloroplasts. Polyphenol oxidase in *Beta vulgaris. Plant Physiol.*, 24: 1–15

Bipasha, C., B.C. Goswami and B. Chakravarty, 2000. Effect of zinc and copper on growth and regenerating cultures of *Citrus reticulata* Blanco. *Indian J. Plant Physiol.*, 5: 52–8

Bradford, M.M.A., 1976. A rapid method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. *Anal. Biochem.*, 72: 248–54

Buysse, J. and R. Merckx, 1993. An improved colorimetric method to quantity sugar content of plant tissue. *J. Expt. Bot.*, 44: 1627–9

Dahleen, L.S., 1995. Improved plant regeneration from barley callus cultures by increased copper levels. *Plant Cell Tiss. Org. Cult.,* 43: 267–9

Davies, D.H., 1976. Carotenoids..*In*: Goodwin, T.W. (ed.), *Chemistry and Biochemistry of Algal pigments*. Vol. 2, pp: 38–165. Academic Press, New York, USA

El-Aref, H.M. and A.M. Hamada, 1998. Genotypic differences and alterations of protein patterns of tomato explants under copper stress. *Biol. Plant.,* 41: 555–64

Eleftheriou, E.P. and S. Karataglis, 1989. Ultrastructural and morphological characteristics of cultivated wheat growing on copper–polluted fields. *Bot. Acta.,* 102: 134–40

Ghaemi, M., A. Sarrafi and G. Alibert, 1994. The effects of silver nitrate, colchicine, cupric sulfate and genotype on the production of embryoids from anthers of tetraploid wheat (*Triticum turgidum*). *Plant Cell Tiss. Org. Cult.,* 36: 355–9

Gori, P., S. Schiff, G. Santandrea and A. Bennici, 1998. Response of *in vitro* cultures of *Nicotiana tabacum* L. to copper stress and selection of plants from Cu–tolerant callus. *Plant Cell Tiss. Org. Cult.,* 53: 161–9

Kintzios, S., M. Barberaki, J. Drossopoulos, P. Turgelis and J. Konstas, 2003. Effect of medium composition and explant source on the distribution profiles selected micronutrients in mistletoe tissue cultures. *J. Plant Nutr.,* 26: 369–97

Kintzios, S., J.B. Drossopoulos and C. Lymperopoulos, 2000. Effect of vitamins and inorganic micronutrients on callus growth and somatic embryogenesis from young leaves of rose. *J. Plant Nutr.,* 23: 1407–1420

Kintzios, S., J.B. Drossopoulos and C. Lymperopoulos, 2001. Effect of vitamins and inorganic micronutrients on callus growth and somatic embryogenesis from leaves of chilli pepper. *Plant Cell Tiss. Org. Cult.,* 67: 55–62

Lanaras, T., M. Moustakas, L. Symeonidis, S. Diamantoglou and S. Karataglis, 1993. Plant metal content, growth responses and some photosynthetic measurements on field–cultivated wheat growing on ore bodies enriched in Cu. *Physiol. Plant.,* 88: 307–14

Lidon, F.C., J. Ramalho and F.S. Henriques, 1993. Copper inhibition of rice photosynthesis. *J. Plant Physiol.,* 142: 12–7

Llorens, N., L. Arola, C. Blade and A. Mas, 2000. Effects of copper exposure upon nitrogen metabolism in tissue cultured *Vitis vinifera*. *Plant Sci.,* 160: 159–63

Lloyd, G. and B. McCown, 1980. Commercially–feasible micropropagation of mountain laurel, *Kalmia latifolia*, by use of shoot–tip culture. *Int. Plant Propag. Soc. Proc.,* 30: 421–7

Lolkema, P.C., 1985. Copper resistance in higher plants. *Ph.D. Thesis*, Free Univ. Press, Amesterdam

Mengel, K. and E.A. Kirkby, 1982. *Principles of Plant Nutrition*. 3rd Ed. p. 655. Int. Potash Inst.

Moya, J.L., R. Ros and I. Picazo, 1993. Influence of cadmium and nickel on growth, net photosynthesis and carbohydrate distribution in rice plants. *Photosynth. Res.*, 36: 75–80

Murashige, T. and F. Skoog, 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.,* 15: 473–97

Neales, T.F., 1959. The boron requirement of flax roots grown in sterile culture. *J. Expt. Bot.,* 10: 426–36

Ouzounidou, G., E.P. Eleftheriou and S. Karataglis, 1992. Ecological and ultrastructural effects of copper in *Thlaspi ochroleucum* (Cruciferae). *Canadian J. Bot.,* 70: 947–57

Ouzounidou, G., R. Lannoye and S. Karataglis, 1993. Photoacoustic measurements of photosynthetic activities in intact leaves under copper stress. *Plant Sci.,* 89: 221–6

Punz, W.F. and H. Sieghardt, 1993. The response of roots of herbaceous plant species to heavy metals. *Environ. Exp. Bot.,* 33: 85–98

Purnhauser, L., 1991. Stimulation of shoot and root regeneration in wheat (*Triticum eastivium*) callus cultures by copper. *Cereal Res. Comm.,* 19: 419–23

Purnhauser, L. and G. Gyulai, 1993. Effect of copper on shoot and root regeneration in wheat, triticale, rape and tobacco tissue cultures. *Plant Cell Tiss. Org. Cult.,* 35: 131–9

Reboredo, F., 1994. Interaction between copper and zinc and their uptake by *Halimione portulacoides* L. Aellen. *Bull. Environ. Contam. Toxicol.,* 52: 598–605

Romeu–Moreno, A. and A. Mas, 1999. Effect of copper exposure in tissue cultured *Vitis vinifera*. *J. Agric. Chem.,* 47: 2519–22

Sahrawat, A.K., C. Suresh and S. Chand, 1999. Stimulatory effect of copper on plant regeneration in indica rice (*Oryza sativa* L.). *J. Plant Physiol.,* 154: 517–22

Sanjeev, K., A. Narula, M.P. Sharma, P.S. Srivastava, S. Kumar and A. Narula, 2003. Effect of copper and zinc on growth, secondary metabolite content and micropropagation of *Tinospora cordifolia*: a medicinal plant. *Phytomrph*., 53: 79–91

Stiles, W., 1961. *Trace Elements in Plants*. p. 249. Cambridge Univ. Press, Cambridge, UK

Wilkins, D.A., 1978. The measurement of tolerance to edaphic factors by means of root growth. *New Phytol.,* 80: 623–33

Wu, L. and S.L. Lin, 1990. Copper tolerance and copper uptake of *Lotus purshianus* (Benth.) Clem & Clem and its symbiotic *Rhizobium loti* derived from a copper mine waste population. *New Phytol.,* 116: 531–9

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