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Effect of copper and zinc on the *in vitro* regeneration of *Rauvolfia serpentina*

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Abstract

The present study exemplifies morphogenic roles played by copper and zinc during micropropagation of *Rauvolfia serpentina*, an important medicinal shrub. Incorporation of 20 μM $CuSO_4$ or 25 μM $ZnSO_4$ to a Murashige and Skoog (MS) medium with optimized concentrations of auxins and cytokinins induced a maximum number of shoots per explant (40.67 ± 1.76 and 45.47 ± 0.24, respectively). However, higher concentrations of both the micronutrients negatively affected the morphogenic potential. The pigment content of the regenerants increased up to the optimal concentrations of both metals and thereafter decreased, whereas the maximum proline content was at the highest concentrations used. *In vitro* rooting of healthy shoots was accomplished using 0.5 μM IBA in a half strength liquid MS medium with 8.20 ± 0.37 roots, and root length of 5.50 ± 0.14 cm per microshoot. The plants survived a hardening procedure and were successfully acclimatized to field conditions with 95 % survival.

Additional key words: auxin, clonal propagation, cytokinin, *ex vitro* transfer, micronutrients, morphogenesis, nodal segments.

Introduction

Rauvolfia serpentina of the family *Apocynaceae* (commonly known as sarpgandha) is an erect, evergreen perrenating undershrub. The plant is a rich source of several pharmaceutically important alkaloids. Apart from this, the plant also contains flavanoids, phenols, tannins, and vitamins like ascorbic acid, riboflavin, thiamine, and niacin.

The *in vitro* cultivation of medicinal plants is still not very common. Mostly they are collected from wild population and their increasing use imposed a great threat to the resources. Also *Rauvolfia serpentina* is seriously threatened with extinction, and propagation through seeds or vegetative parts cannot meet the increasing demand. Therefore, some alternatives, such as plant tissue culture have been adopted (Mathur *et al*. 1987, Mukhopadhyay *et al*. 1991, Roy *et al*., 1995, Roja and Heble 1996, Tiwari *et al*. 2003, Kataria and Shekhawat 2005, Baksha *et al*. 2007, Bhatt *et al*. 2008, Salma *et al*. 2008, Mishra *et al*. 2010). Surprisingly, these available reports are based strictly on the optimization of different plant growth regulators (PGRs), especially cytokinins. None of them have stressed upon the significance of the inorganic nutrient composition of basal media. They have considered the nutrient composition of the Murashige and Skoog (MS) medium as standard, however, inorganic nutrients apart from PGRs are very crucial for plant growth and development, a fact well established by Preece (1995) and Ramage and Williams (2002).

Copper is a vital component of electron transfer reactions mediated by cytochrome *c* oxidase and plastocyanin (Yruela 2005), whereas zinc increases the biosynthesis of chlorophyll and carotenoids (Broadley *et al*. 2007). Therefore, optimum Cu and Zn concentrations in the medium positively affect development of the membrane system of chloroplasts and chlorophyll content. Proline accumulation is reported to occur in response to heavy metal toxicity (Sharma and Dietz 2009).

In the present manuscript, we report an improved protocol for rapid clonal propagation of *R. serpentina* through high frequency axillary shoot proliferation from nodal explants by enhanced concentrations of copper and zinc.

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Abbreviations: BA - 6-benzyladenine; Car - carotenoids; Chl - chlorophyll; IBA - indole-3-butyric acid; MS medium - Murashige and Skoog medium; NAA - α-naphtha-lene acetic acid; PGRs - plant growth regulators.
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Materials and methods

Nodal segments of healthy *Rauvolfia serpentina* (L.) Benth. *ex* Kurz plants were collected from the Botanical garden, the Aligarh Muslim University, during September - October 2009. These segments were washed under running tap water, treated with a 5 % (v/v) detergent *Labolene* (*Qualigens*, Mumbai, India) for 5 min followed by 3 - 4 washing with distilled water. Then the material was surface sterilized with 0.1 % (m/v) $HgCl_2$ for 4 min following repeated washes with sterile distilled water under aseptic conditions. Nodal explants were excised aseptically and cultured on a shoot induction medium consisting of a Murashige and Skoog (1962; MS) medium with 30 g dm^3 sucrose (*Qualigens*) supplemented with 10.0 μM 6-benzyladenine (BA) and 0.5 μM α-naphthalene acetic acid (NAA) (an already optimized medium, Ahmad *et al*. 2002) and micronutrients in different concentrations. All the salts used were of analytical grade. The pH of the medium was adjusted to 5.8 and the medium was solidified with 8 g dm^{-3} bacteriological grade agar (*Qualigens*) before autoclaving at 121 °C for 15 min. All the culture vials were incubated in a culture room at a temperature of 25 ± 2 °C, a 16-h photoperiod, an irradiance of 50 μmol m^{-2} s^{-1} (supplied by cool white fluorescent lamps, 40 Watt, *Philips*, Kolkata, India), and a 60 - 65 % relative humidity. MS media without or with different concentrations of $CuSO_4 . 5\ H_2O$ (5, 10, 20, 25, 50, 100, 200 μM) and $ZnSO_4 . 7\ H_2O$ (5, 10, 20, 25, 50, 100, 200, 500 μM) were used for comparison of morphogenic responses. The cultures were subcultured onto fresh media after every three weeks and the frequency of explants producing shoots, the number of shoots per explant, and the shoot length were recorded after eight weeks of culture.

Leaves from eight-week-old *in vitro* derived shoots were used for chlorophyll (Chl) and carotenoids (Car) estimations. The pigments were determined using the method described by Arnon (1949). Fresh leaves (200 g) were grounded in small volumes of 80 % (v/v) acetone solutions and filtered through *Whatman No. 1* filter paper. The extract obtained was made to a final volume of 10 cm^3 using 80 % acetone. The absorbances of the samples were read at 663 and 645 nm for Chl and at 480 and 510 nm for Car on a UV-VIS spectrophotometer (*UV-1700 Pharma Spec, Shimadzu*, Kyoto, Japan).

The proline content of the regenerants was determined by the Bates *et al*. (1973) method using eight-week-old cultures. Fresh leaves (300 g) were homogenized in 10 cm^3 of 30 % (m/v) aqueous sulphosalicylic acid. The homogenate was centrifuged at 9 000 *g* for 15 min. The supernatant (2.0 cm^3) was mixed with 2 cm^3 of acid ninhydrin and 2 cm^3 of glacial acetic acid and incubated at 100 °C for 1 h. After termination of reaction in ice bath, the reaction mixture was extracted with 4 cm^3 of toluene. The chromatophore phase was aspirated from the aqueous phases and its absorbance was determined at 520 nm using the *UV-1700* spectrophotometer.

Healthy and well elongated shoots (4 - 5 cm) were excised from the culture and transferred to liquid rooting media composed of a half-strength MS medium supplemented with different concentrations (0.0, 0.1, 0.5, 1.5 μM) of IBA on a filter paper bridge. Data on the percentage of rooting, the mean number of roots, and the root length per shoot were recorded at four weeks after the transfer to the rooting media.

Plantlets with well-developed shoots and roots were removed from the culture medium, washed gently under running tap water and transferred to *Thermocol* cups containing sterile *Soilrite*™ (*Keltech*, *Energies*, Bangalore, India). Potted plantlets were covered with transparent polythene bags to ensure high humidity, maintained in a culture room and watered with a ½ MS solution every alternate day for two weeks. Thereafter, the bags were removed in order to harden the plants to field conditions. After four weeks, the acclimatized plants were transferred to pots containing normal garden soil and maintained in a greenhouse followed by shifting to field under full sun.

The data were analysed with *ANOVA* using the *SPSS v. 16* (*SPSS Inc*., Chicago, USA) software. The experiments were repeated thrice for morphogenesis and twice for biochemical analyses. The significance of differences among means was carried out using the Duncan's multiple range test at $\alpha = 0.05$.

Results

In the control MS medium supplemented with 10 μM BA and 0.5 μM NAA, development of 9.00 ± 0.57 shoots with a mean shoot length of 3.13 ± 0.13 cm in 85 % cultures was induced after 4 weeks. The shoots subsequently multiplied onto the same medium to form 33.33 ± 0.67 shoots with a mean shoot length of 6.26 ± 0.14 cm after 8 weeks. The addition of $CuSO_4$ at the concentration from 5.0 to 25.0 μM was beneficial for production of microshoots and their growth as compared to the control. An optimum regeneration response was recorded with a mean shoot number of 40.67 ± 1.76 and an average shoot length of 7.00 ± 0.06 cm after 8 weeks when the media were supplemented with 20 or 10 μM $CuSO_4$ (Fig. 1). However, the higher $CuSO_4$ concentrations (25, 50, 100, and 200 μM) drastically reduced all the evaluated parameters (Fig. 1). Shoots grown on these concentrations turned necrotic and visible symptoms of toxicity were observed especially at the 200 μM concentration.

The most effective concentration of $ZnSO_4$ was

25 µM, inducing 45.47 ± 0.24 shoots with 7.33 ± 0.07 cm of a mean shoot length after 8 weeks of culture (Fig. 2). Higher concentrations beyond 25 µM yielded lower regeneration frequencies with a lesser number of shoots and a lower shoot length (Fig. 2). The treatments with $ZnSO_4$ below the optimal concentration also yielded favorable results and a better response compared to the control.

The Chl *a*, Chl *b*, and total Chl content in the regenerants increased with the increasing copper and zinc concentrations up to the optimal level (20 µM $CuSO_4$ and 25 µM $ZnSO_4$). Higher concentrations of $CuSO_4$ and $ZnSO_4$ caused a decline in the photosynthetic pigments (Figs. 3 and 4). As in the case of Chl, the Car content increased up to an optimal level of $CuSO_4$ (20 µM) and $ZnSO_4$ (25 µM) and declined thereafter (Figs. 3, 4).

The proline accumulation increased with the increasing Cu and Zn concentrations and the maximum proline content of 0.45 or 0.55 µg g^{-1}(f.m.) was on the MS medium containing either 200 µM $CuSO_4$ or 500 µM $ZnSO_4$, respectively (Fig. 5).

The best treatment for *in vitro* rooting was the half strength MS medium supplemented with 0.5 µM IBA, inducing a 91.3 % rooting response, with 8.20 ± 0.37

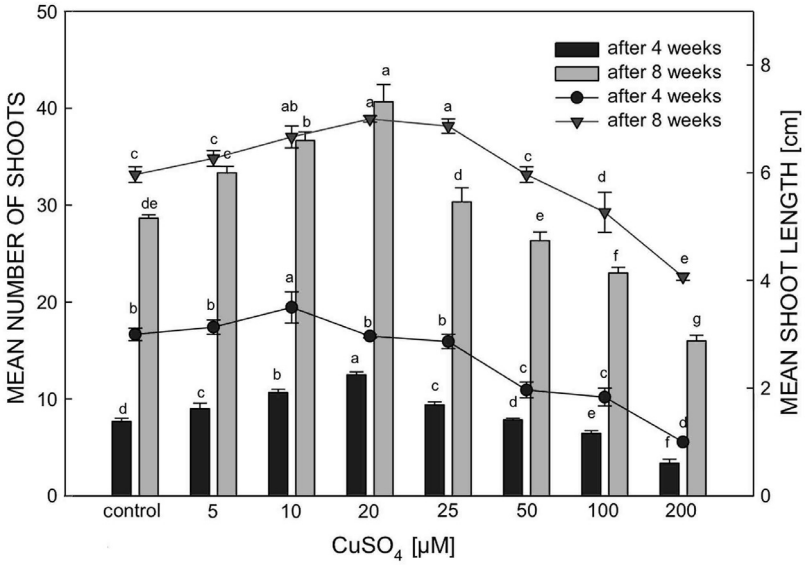

Fig. 1. Effects of various concentrations of copper sulphate on shoot number (the *columns*) and shoot length (the *lines*) developed from nodal segments of *Rauvolfia serpentina* after four and eight weeks of culture. Means ± SE, $n = 20$. The *bars* denoted by different letters are significantly different ($P \leq 0.05$) using DMRT.

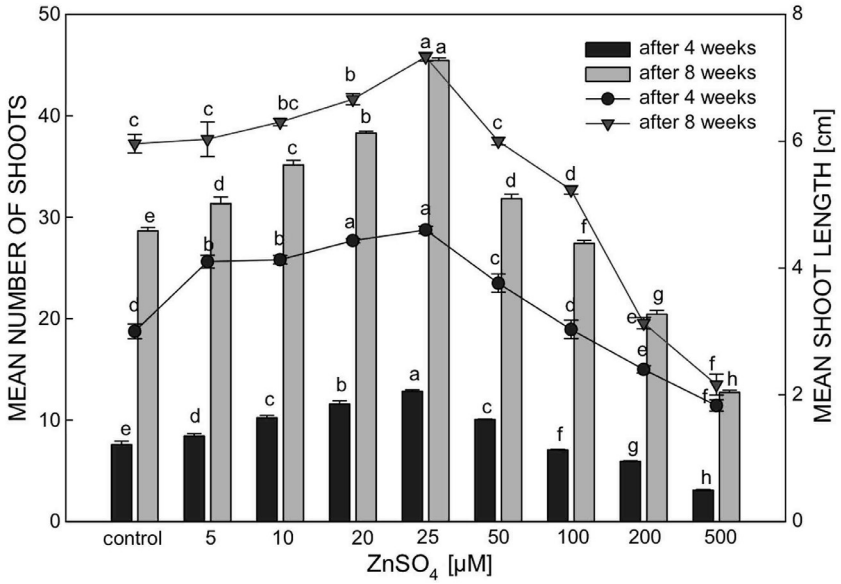

Fig. 2. Effects of various concentrations of zinc sulphate on shoot number (the *columns*) and shoot length (the *lines*) developed from nodal segments of *Rauvolfia serpentina* after four and eight weeks of culture. Means ± SE, $n = 20$. The *bars* denoted by different letters are significantly different ($P \leq 0.05$) using DMRT.

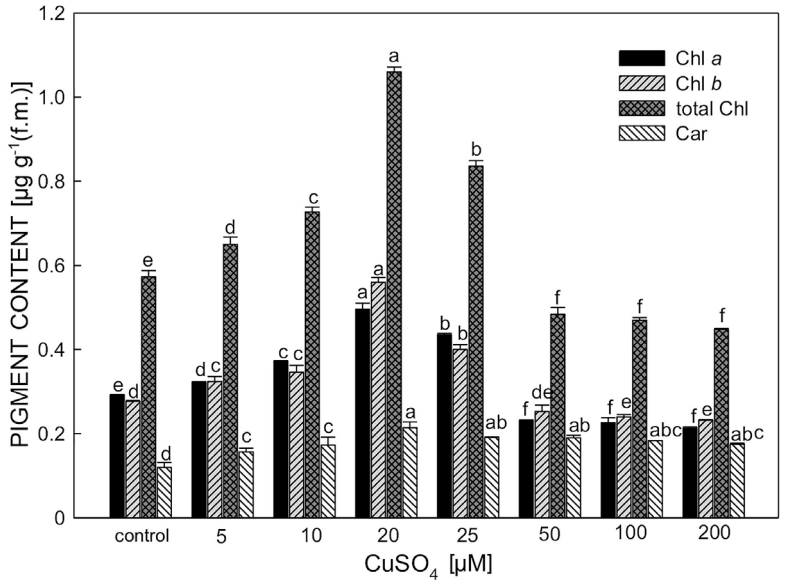

Fig. 3. Effects of $CuSO_4$ on Chl *a*, Chl *b*, total Chl, and Car content in *Rauvolfia serpentina* regenerants. Means ± SE, $n = 3$. The *bars* denoted by different letters are significantly different ($P \leq 0.05$) using DMRT.

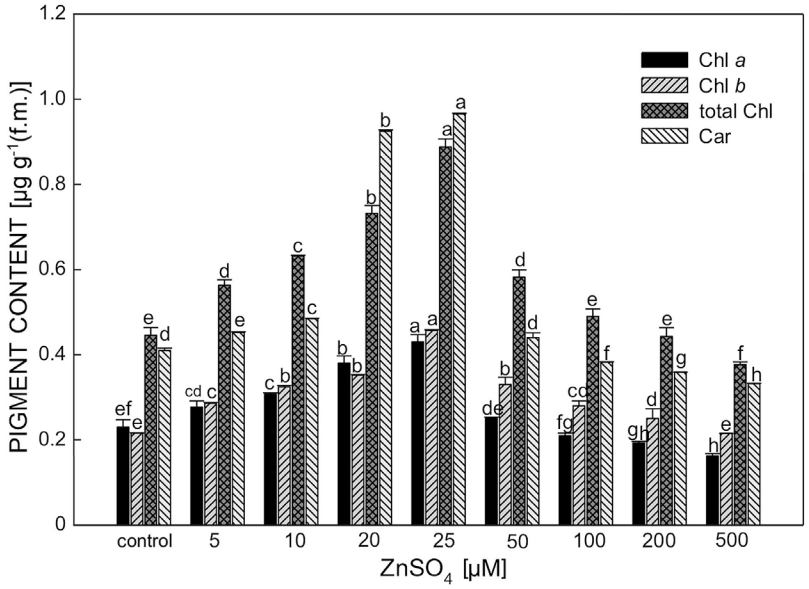

Fig. 4. Effects of $ZnSO_4$ on Chl *a*, Chl *b*, total Chl, and Car content in *Rauvolfia serpentina* regenerants. Means ± SE, $n = 3$. The *bars* denoted by different letters are significantly different ($P \leq 0.05$) using DMRT.

roots of an average root length of 5.50 ± 0.14 cm at 4 weeks after transfer to the rooting medium (Table 1).

All rooted plantlets transferred to *Soilrite* containing *Thermocol* cups survived the initial phases of hardening inside the growth room. Subsequent transfer to earthen pots containing garden soil showed a 95 % survival rate. Finally, all these potted plantlets were shifted to a greenhouse (for 2 weeks) and to full sun without any undesirable damage to the plants.

Discussion

The MS medium supplemented with 10 µM BA and 0.5 µM NAA was used as the control medium because it was most effective in affecting maximum shoot production. We show here that manipulating the salt strength might also alter the growth and regeneration of plantlets since an appropriate salt strength may work as

important elicitor of *in vitro* morphogenesis. Likewise, cell growth and morphogenesis of some species may be promoted by increasing concentrations of mineral salts above those recommended by Murashige and Skoog (1962). Welander (1977) suggested that plant cells are more demanding for microelements when undergoing morphogenesis than in other stages.

Copper is an important micronutrient and culture

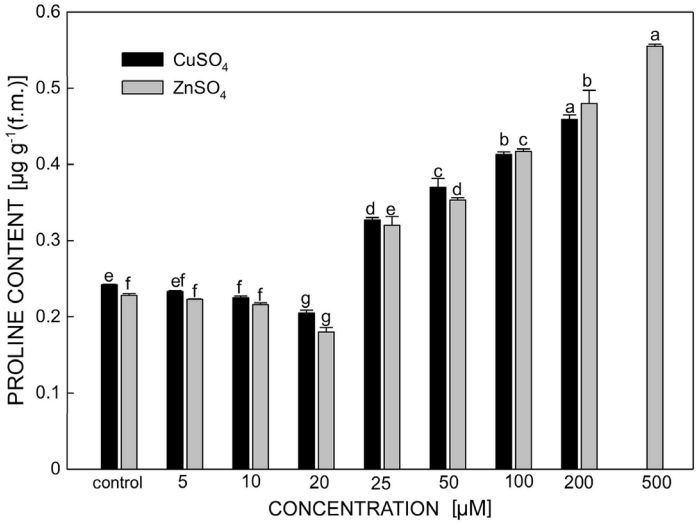

Fig. 5. Effects of $CuSO_4$ and $ZnSO_4$ on proline content in *Rauvolfia serpentina* regenerants. Means ± SE, $n = 3$. The *bars* denoted by different letters are significantly different ($P \leq 0.05$) using DMRT.

Table 1. Effects of different concentrations of IBA and of strengths of MS on *in vitro* root induction in *Rauvolfia serpentina* after four weeks of culture. Means ± SE, $n = 20$. Means followed by the same letter are not significantly different ($P \leq 0.05$) using DMRT.

IBA [µM] MS	½ MS	Response [%]	Number of roots	Root length [cm]
0.0	0.0	0.0 ± 0.00^{h}	0.0 ± 0.00^{d}	0.0 ± 0.00^{g}
0.1	-	29.6 ± 1.45^{g}	3.0 ± 0.31^{c}	3.4 ± 0.13^{c}
0.5	-	66.0 ± 1.00^{b}	5.8 ± 0.37^{b}	4.4 ± 0.14^{b}
1.0	-	60.6 ± 1.76^{d}	5.4 ± 0.67^{b}	2.7 ± 0.22^{de}
1.5	-	50.0 ± 1.15^{e}	3.8 ± 0.37^{c}	1.9 ± 0.12^{f}
	0.1	45.0 ± 1.73^{f}	5.0 ± 0.31^{b}	3.5 ± 0.21^{c}
	0.5	91.3 ± 1.33^{a}	8.2 ± 0.37^{a}	5.5 ± 0.14^{a}
	1.0	74.0 ± 1.00^{b}	5.6 ± 0.24^{b}	3.1 ± 0.18^{cd}
	1.5	61.0 ± 2.08^{d}	5.2 ± 0.37^{b}	2.5 ± 0.22^{e}

media usually contain 0.1 - 1.0 µM Cu^{+2} supplied in the form of $CuSO_4$, although occasionally $CuCl_2$ or $CuNO_3$ have been employed (George and De Klerk 2008). The concentration of Cu in culture media is much smaller than in plants. Therefore, it is not surprising that we obtained a better morphogenic response when the Cu concentration was raised. In our experiments, the optimum concentration was 20 µM, a concentration slightly higher than mentioned in many earlier reports (Dahleen 1995, Kintzios *et al*. 2001, Nirwan and Kothari 2003, Nas and Read 2004, Bouman and Tiekstra 2005). All these authors reported a strong increase in growth when Cu is added at concentrations of 1.0 - 5.0 µM. However, Purnhauser (1991) reported that the regeneration of wheat callus is eight times higher on a medium containing a 100 times higher $CuSO_4$ concentration than in the original MS medium. Similar findings were also reported by Purnhauser and Gyulai (1993) in anther or immature embryo cultures. As already stated, Cu is involved in many biochemical and enzymatic reactions in plants. Many crucial enzymes like cytochrome oxidase and superoxide dismutase, as well as plastocyanin, a pigment participating in electron transfer, contain copper. The optimum Cu concentration in medium positively affects development of membrane system of chloroplasts and Chl content. In our study, the Chl content was enhanced at the increased Cu concentrations up to the optimal concentration, which is in accordance with earlier reports in other species like *Stevia rebaudiana* (Jain *et al*. 2009), *Lupinus luteus* (Mourato *et al*. 2009), *Jatropha curcas* (Khurana-Kaul *et al*. 2010, and *Withania somnifera* (Fatima *et al*. 2011). However, the excess of Cu is known to cause damage to chloroplasts (Romeo-Puertas *et al*. 2004), destroys its structure and function and inhibits Chl biosynthesis. It also inhibits the uptake and transport of other elements like Mn, Zn, and Fe (Lin and Wu 1994, Gardea-Torresday *et al*. 2004), causes leaf necrosis (Liu *et al*. 2004), and decreases water content (Marschner 1995). These might be the reasons for the decrease in the Chl content found at the high concentrations of Cu in the MS medium. Similarly, an increase in the Car content was seen up to 20 µM $CuSO_4$ and a decline thereafter. Car are important quenchers of the singlet state of Chl and of singlet oxygen (Candan and Tarhan 2003). Several Cu dependent enzymes are involved in the oxidation and hydroxylation of phenolic compounds (Lerch 1981).

Excessive Cu is toxic to many plant species (Wu and Lin 1990) and one of the most rapid responses is inhibition of root growth (Eleftheriou and Karataglis 1989). In the present study, the production of proline at higher Cu concentrations correlated with a lower regeneration frequency and a decrease in biomass and pigment content. Such toxic responses of Cu have recently been reported (Jain *et al*. 2009).

Zinc is a component of many enzymes with different functions, including alcohol dehydrogenase, carbonic anhydrase, superoxide dismutase, RNA polymerase, *etc*. The MS medium normally contains 30 μM Zn^{2+} although the amounts added to culture media vary widely from 0.1 to 70 μM. For example, in our study with nodal segments of *R. serpentina*, the optimum response was observed when using 25 μM $ZnSO_4$ in the MS medium. Such enhanced responses of explants under elevated concentrations of $ZnSO_4$ have been reported earlier in *Bacopa monniera* (Ali *et al*. 1997) and in *Lepidium sativum* (Saba *et al*. 2000). In accordance to an earlier report by Candan and Tarhan (2003) on *Mentha pulegium*, the concentrations of $ZnSO_4$ above the optimum prove to be inhibitory for shoot formation. The increase in Chl and Car content up to the optimal Zn concentration and their decrease beyond it suggest the stimulatory and inhibitory roles played by Zn. The variations in growth, physiological, and biochemical responses of *Artemisia annua* to varied concentrations of Zn at different stages of plant growth and development have been discussed by Khudsar *et al*. (2004).

The proline content increased at the higher concentrations of $ZnSO_4$. This suggests that the regenerants were undergoing a stress at these Zn concentrations. Proline is produced under stress and is involved in an adaptive mechanism (Aspinall and Paleg 1981, Ali *et al*. 1998). Proline is believed to preserve plant tissues against stress by acting as osmoregulator, N-storage compound, and protectant of cellular structures and hydrophobic enzymes (Greenway and Munns 1980). Excess of Zn generates reactive oxygen species, displaces other ions from active sites in proteins, induces chlorosis, decreases water content, and brings changes in phosphorus and magnesium content in plant tissues (Marschner 1995). The present results corroborates with the earlier findings in *Bacopa monniera* (Ali *et al*. 1998, 1999).

In vitro rooting the regenerated shoots was carried out most efficiently in the liquid half-strength MS medium containing 0.5 μM IBA on the filter paper bridge. The effectiveness of IBA for efficient *in vitro* rooting the regenerants have been reported by Faisal *et al*. (2005) in *Rauvolfia tetraphylla*, Khan *et al*. (2011) in *Salix tetrasperma*, and also by Ahmad and Anis (2011) in *Vitex negundo*. Successful hardening and acclimatization of the regenerated plantlets was achieved first on *Soilrite* followed by transfer to normal garden soil, a procedure found suitable for many micropropagation protocols.

In conclusion, the enhanced multiplication and better growth of the regenerants was achieved using the elevated concentrations of $CuSO_4$ and $ZnSO_4$. The results obtained herein could be used for a large scale multiplication and conservation of this endangered medicinal plant.

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