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Morphogenic responses of *Rauvolfia tetraphylla* L. cultures to Cu, Zn and Cd ions

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Abstract The present work was undertaken to study the effect of heavy metals on morphogenic response from nodal explants of *Rauvolfia tetraphylla* L. The approach has been found to be significant for enhanced multiple shoot induction on Murashige and Skoog (MS) medium containing various concentrations of copper (Cu), zinc (Zn) and cadmium (Cd). Among various concentrations used, the optimum regeneration was recorded on MS medium supplemented with 10 μM 6-benzyladenine, 0.5 μM α-naphthalene acetic acid, and 100 μM zinc sulphate which exhibited 12.2 shoots per explant with 2.5 cm shoot length after 12 weeks of culture. In case of copper sulphate, the optimum concentration was found to be 20 μM with 11.6 shoots per explant and 3.3 cm shoot length. Cadmium chloride was found to be most toxic as it showed negative effect on shoot regeneration. Healthy growing in vitro microshoots (3 cm) rooted efficiently on ½ MS medium supplemented with indole-3-butyric acid (0.5 μM) which induced 4.8 roots with 1.8 cm root length after 4 weeks. Regenerated plantlets were successfully hardened off in Soilrite followed by their transfer to garden soil under normal sunlight.

Keywords Acclimatization · Heavy metals · Shoot regeneration · Micropropagation · Micronutrients

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Abbreviations

BA	6-Benzyladenine
NAA	α-Naphthalene acetic acid
IBA	Indole-3-butyric acid
MS	Murashige and Skoog (1962) medium
$CuSO_4$	Copper sulphate
$ZnSO_4$	Zinc sulphate
$CdCl_2$	Cadmium chloride

1 Introduction

Heavy metals are abundant and unavoidable environmental contents. The rapid increase in population coupled with fast industrialization causes serious environmental problems including the production and release of considerable amount of toxic waste materials, like heavy metals, into environment (Zhuang et al. 2007). Most of these heavy metals (e.g. Cu, Zn, B, Fe, Mn, Co, etc.) act as micronutrients and are essential for proper growth and development of plants. Micronutrient deficiency cause yellowing of leaves (Zn), browning of shoot tips and chlorosis (Cu, Fe), stunted growth (B, Mn) and in severe cases sometimes death of plants. But these micronutrients also exhibit cytotoxic effects when accumulated in larger quantities. Heavy metal toxicity is a problem of increasing significance for ecological, evolutionary, nutritional, and environmental reasons (Benavides et al. 2005). In recent years, public concerns relating to ecological threats caused by heavy metals have led to intensive research of new economical plant-based remediation technologies (Sarma 2011).

Indian subcontinent is a vast repository of medicinal plants that are used in traditional treatments, which also forms a rich source of knowledge. Medicinal and aromatic

plants appear to be good choice for heavy metal accumulation as these species are mainly grown for secondary products (essential oils), thus, the contamination of food chain with heavy metals is eliminated (Jeliazkova and Craker 2000). Researchers have shown that heavy metals accumulated by aromatic and medicinal plants do not appear in the essential oils or alkaloids (Zheljazkova and Nielsel 1996; Scora and Chang 1997) and that some of these species are able to grow in metal contaminated sites without significant yield reduction (Jeliazkova and Craker 2000).

Mostly medicinal plants are not cultivated but usually harvested from the wild. It is not possible for the pharmaceutical industries to sustain only on wild plants as a regular supply of uniform material. Growing population and contaminated environmental conditions pose a great threat to the available natural stock of valuable medicinal plants. As a better alternative of vegetative propagation, micropropagation has great potential for the conservation of medicinal plants as it greatly enhances the multiplication rate. Tissue culture techniques have helped not only raising metal tolerant plants but have also demonstrated that subjecting the cultures of medicinal plants to abiotic stresses (chemical and physical) can be crucial in increasing the yield of secondary metabolites (Saba et al. 2000).

Rauvolfia tetraphylla L. (Apocynaceae) is a woody shrub of high medicinal value. Roots are used for the commercial extraction of *reserpine* which has a depressant action on central nervous system and produces sedation and lowering of blood pressure, also considered valuable in the treatment of chronic schizophrenic patients and exerts bacteriostatic action against Gram-positive bacteria (Anonymous 2003). It is reported to occur in most of the moist and hotter parts of India, difficult to propagate through seeds and as for vegetative propagation survival rate of hardwood cuttings without proper hormonal treatment is almost zero (Anonymous 2003). Albeit, there are reports available on in vitro propagation of *Rauvolfia tetraphylla* (Sarma et al. 1999; Faisal and Anis 2002; Faisal et al. 2005, 2012; Harisaranjan et al. 2009), but no attempts have been made to evaluate the effect of metal ions in the culture medium on multiple shoot induction. Hence, in the present work an attempt has been made to study the effect of heavy metals (Cu, Zn and Cd) on in vitro regeneration, multiplication and subsequently rehabilitation of in vitro raised plantlets in natural environment.

2 Materials and methods

2.1 Plant material and explants preparation

Young stem segments with first two nodes from freshly sprouted shoots of *Rauvolfia tetraphylla* were collected from the plants maintained in Botanical Garden of the University, Aligarh. As it is taken from natural conditions (ex vitro) the possibilities of contamination are much more pronounced. To avoid the contamination a proper procedure for sterilization was followed before inoculation. The freshly excised nodal segments were washed thoroughly under running tap water, 5 % (v/v) teepol, mild liquid detergent, 5 % (v/v) Savlon (antiseptic solution) followed by surface sterilization with 0.1 % (w/v) $HgCl_2$ solution under laminar air flow by continuous shaking for 4–5 min. To remove the remnants of sterilant solution washing with sterile single distilled water for 5–6 times was sufficient. This procedure was found to be very effective to stop fungal growth and contamination in the cultures.

2.2 Culture media and conditions

MS medium (1962) was used as the basic nutrient medium supplemented with growth regulators, 6-benzyl adenine (10 μM), α-naphthalene acetic acid (0.5 μM) and considered as control (established protocol by Faisal and Anis 2002), and indole-3-butyric acid was used in rooting experiments. 3 % (w/v) sucrose as a carbon source was added in all the experiments. For evaluation of the effect of different heavy metals, different concentrations of $CuSO_4$ (10, 20, 40, 60, 80 or 100 μM), $ZnSO_4$ (50,100, 200, 300, 400 or 500 μM) and $CdCl_2$ (5, 10, 15, 20, 40 or 80 μM) were used in the optimized medium. The pH of the medium was adjusted to 5.8 by 1 N NaOH using pH meter (pH 510, Eutech Instruments, Cyberscan). The semisolid medium base was prepared with 0.8 % (w/v) bacteriological grade agar (Qualigens, India Ltd.) in all experiments (except rooting experiments). To provide proper aeration to the cultures without causing contamination culture tubes and flasks were plugged with cotton plugs made up of non-absorbent cotton wrapped in a single layer of muslin cloth. All the cultures were maintained in culture room at 25 ± 1 °C with 60–65 % relative humidity and a photoperiod of 16 h, light provided by cool fluorescent lamps (Phillips India Ltd. Kolkata, India).

2.3 Maintenance of cultures

Responding cultures were maintained in the culture room under controlled conditions as described above. Subculturing was carried out after every 4 weeks on the same respective medium and the morphogenic responses (number of shoots and shoot length) were observed at 4, 8 and 12 weeks of incubation.

2.4 In vitro rooting and acclimatization

Healthy microshoots (3–4 cm) were cut and transferred to freshly prepared liquid medium. The microshoots were

supported by filter paper bridges made up of filter paper, Whatmann No. 1. For hardening, rooted microshoots were removed from the culture tubes, washed with tap water and transplanted into thermocol cups filled with sterilized Soilrite™ (Keltech Energies Ltd. Bangalore) and covered with glass bottles such that a slight gap was left for ventilation. The cups were then kept in a culture room at 25 ± 2 °C under 16:8 h light/dark period. The plantlets were irrigated every 2 days with ½ MS lacking organic supplements for a period of 2 weeks and later normal tap water was used for the purpose. After 2 weeks the glass bottles were gradually removed and the plantlets were kept in the growth room for 1 week and then transferred to pots containing normal garden soil and maintained in the green house.

Table 1 Effect of different concentration of $CuSO_4$ on shoot bud induction and proliferation from nodal explants

Concentration of $CuSO_4$ (μM)	After 4 weeks		After 8 weeks		After 12 weeks	
	No. of shoots	Shoot length (cm)	No. of shoots	Shoot length (cm)	No. of shoots	Shoot length (cm)
Control	2.40 ± 0.24^{b}	0.52 ± 0.024^{b}	5.40 ± 0.24^{ab}	0.66 ± 0.014^{b}	11.40 ± 0.60^{ab}	1.04 ± 0.053^{b}
10	3.60 ± 0.24^{ab}	0.54 ± 0.004^{b}	4.20 ± 0.48^{b}	1.23 ± 0.074^{a}	9.00 ± 0.54^{c}	3.12 ± 0.205^{a}
20	5.20 ± 1.01^{a}	0.68 ± 0.036^{a}	6.80 ± 1.15^{a}	1.21 ± 0.143^{a}	11.60 ± 0.24^{a}	3.31 ± 0.422^{a}
40	4.00 ± 0.0^{ab}	0.35 ± 0.012^{cd}	4.00 ± 0.00^{b}	0.44 ± 0.011^{b}	11.00 ± 0.31^{ab}	0.69 ± 0.007^{b}
60	4.80 ± 0.48^{a}	0.35 ± 0.018^{c}	5.40 ± 0.24^{ab}	0.70 ± 0.010^{b}	10.20 ± 1.20^{abc}	1.08 ± 0.096^{b}
80	4.40 ± 0.67^{a}	0.29 ± 0.008^{cd}	5.20 ± 0.58^{ab}	0.57 ± 0.012^{ab}	10.20 ± 0.37^{abc}	0.95 ± 0.048^{b}
100	3.60 ± 0.24^{ab}	0.29 ± 0.017^{d}	4.00 ± 0.00^{b}	0.46 ± 0.017^{b}	9.60 ± 0.24^{bc}	0.79 ± 0.048^{b}

Values represent mean ± standard error of 10 replicates per treatment in three repeated experiments. Means followed by the same letter are not significantly different by the DMRT at 0.05 % probability level

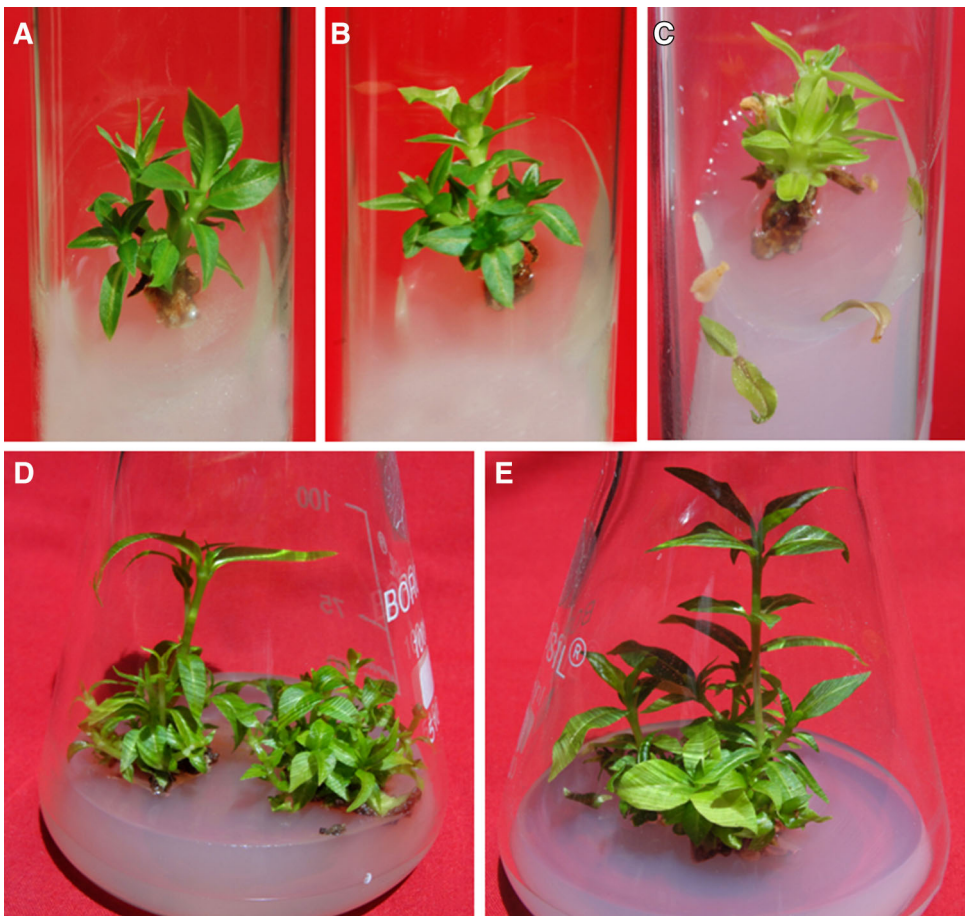

Fig. 1 **a** Multiple shoot bud induction from nodal explant on MS + BA (10 μM) + NAA (0.5 μM) + $CuSO_4$ (20 μM), **b** $ZnSO_4$ (100 μM), **c** $CdCl_2$ (20 μM) after 4 weeks of culture. **d** Multiplication and elongation of shoots on MS + BA (10 μM) + NAA (0.5 μM) + $CuSO_4$ (20 μM), **e** $ZnSO_4$ (100 μM) after 8 weeks of culture

3 Results and discussion

3.1 Effect of $CuSO_4$

Copper (Cu) is an important part of enzymes and protein involved in plant metabolic processes such as photosynthesis and mitochondrial electron transport. It is an essential micronutrient required for proper plant growth and development. In the light of this factor different concentrations of copper sulphate (in increasing order) were tested for nodal explant culture of *R. tetraphylla*. The cultures maintained on different concentrations of copper sulphate exhibited better growth and multiplication in comparison to control (Table 1) after 4 weeks of culture. Of the various concentrations of $CuSO_4$ (10–100 μM) tested, 20 μM was found optimum to induce highest number of shoots (5.2 ± 1.01) and shoot length (0.68 ± 0.03 cm) per nodal explant after 4 weeks of culture (Fig. 1a). The stimulatory effect of copper in the basal medium has also been reported in many plants including *Elucine coracana* (Kothari et al. 2004) *Stevia rebaudiana* (Jain et al. 2009), *Withania somnifera* (Fatima et al. 2011) and *Bacopa monniera* (Ali et al. 1998). Further increase in concentration of copper beyond optimal level showed adverse effects on number of shoot bud formation and also on the shoot length. Same pattern of growth and multiplication was obtained after maintaining the cultures on their respective medium after 8 (Fig. 1d) and 12 weeks (Fig. 2a). In the initial culture stage (4 weeks) the higher concentrations of copper sulfate (beyond optimal level) were better than the control in producing relatively large number of shoots per nodal explant but with subsequent culture passages higher concentrations showed negative effect on shoots as well as shoot length in comparison to control. This is in accordance with the earlier reports on plants like *Capsicum annum* (Joshi and Kothari 2007), *Lepidium sativum* (Saba et al. 2000) *Stevia rebaudiana (*Jain et al. 2009) and *Withania somnifera* (Fatima et al. 2011).

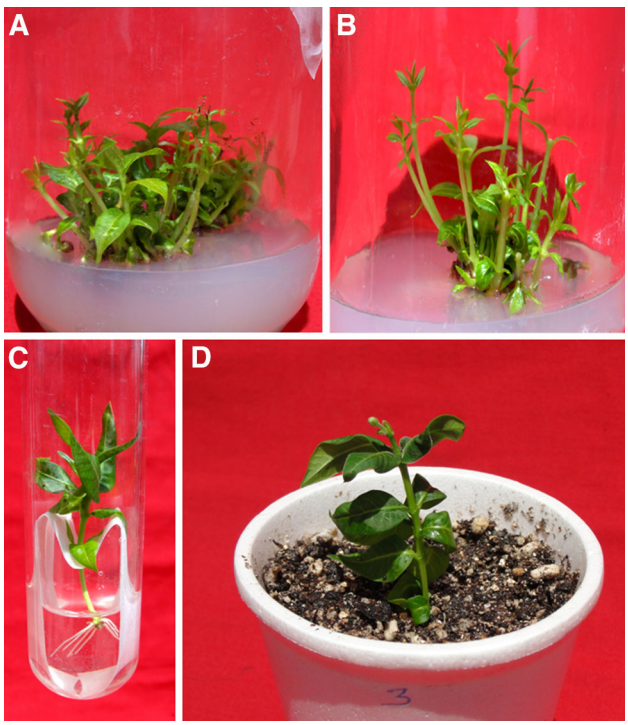

Fig. 2 **a** Multiplication of shoots on MS + BA (10 μM) + NAA (0.5 μM) + $CuSO_4$ (20 μM) and **b** $ZnSO_4$ (100 μM) after 12 weeks of culture. **c** In vitro rooted plantlet on ½ MS + IBA (0.5 μM) after 4 weeks. **d** acclimatized plantlet

3.2 Effect of $ZnSO_4$

Zinc is also an essential micronutrient and plays an important role in protein synthesis, enzyme activation and growth regulation (Welch 1995). The biochemical function of zinc in the maintenance of membrane structure and functions have been reported by several investigators (Bettger and O'Dell 1981). In *Rauvolfia tetraphylla* cultures, multiple shoots were induced from the nodal explants on an optimized MS medium supplemented with different concentrations of $ZnSO_4$ (50–500 μM) (Table 2). Optimized MS

Table 2 Effect of different concentration of $ZnSO_4$ on shoot bud induction and proliferation from nodal explants

Concentration of $ZnSO_4$ (μM)	After 4 weeks		After 8 weeks		After 12 weeks	
	No. of shoots	Shoot length (cm)	No. of shoots	Shoot length (cm)	No. of shoots	Shoot length (cm)
Control	2.40 ± 0.24^{c}	0.52 ± 0.024^{b}	5.40 ± 0.24^{a}	0.66 ± 0.014^{cd}	11.40 ± 0.60^{ab}	1.04 ± 0.053^{b}
50	3.80 ± 0.80^{abc}	0.50 ± 0.040^{b}	4.00 ± 0.63^{bc}	0.97 ± 0.059^{b}	11.20 ± 1.01^{ab}	1.09 ± 0.045^{b}
100	5.20 ± 0.37^{a}	0.80 ± 0.027^{a}	5.60 ± 0.24^{a}	1.41 ± 0.148^{a}	12.20 ± 0.20^{a}	2.56 ± 0.072^{a}
200	4.60 ± 0.24^{ab}	0.45 ± 0.009^{b}	5.20 ± 0.20^{a}	0.74 ± 0.011^{c}	10.40 ± 0.24^{b}	1.06 ± 0.028^{b}
300	3.80 ± 0.48^{abc}	0.34 ± 0.024^{c}	4.60 ± 0.40^{ab}	0.56 ± 0.002^{cd}	7.00 ± 0.44^{c0}	0.91 ± 0.022^{c}
400	3.40 ± 0.24^{bc}	0.35 ± 0.033^{c}	4.00 ± 0.31^{bc}	0.53 ± 0.009^{d}	6.40 ± 0.24^{c}	0.79 ± 0.034^{c}
500	3.40 ± 0.50^{bc}	0.30 ± 0.016^{c}	3.40 ± 0.24^{c}	0.49 ± 0.014^{d}	4.80 ± 0.48^{d}	0.51 ± 0.013^{d}

Values represent mean ± standard error of 10 replicates per treatment in three repeated experiments. Means followed by the same letter are not significantly different by the DMRT at 0.05 % probability level

medium supplemented with different concentration of $ZnSO_4$ was better than control with respect to multiple shoot induction and also length of shoots. Medium containing $ZnSO_4$ (100 μM) induced maximum number (5.20 ± 0.37) of shoots with shoot length (0.80 ± 0.027 cm) after 4 weeks of culture (Fig. 1b). The positive role of $ZnSO_4$ has been documented earlier in various genera like *Bacopa monniera* (Ali et al. 1999), *Lepidium sativum* (Saba et al. 2000) and *Withania somnifera* (Fatima et al. 2011). The higher concentrations and also prolonged exposure of $ZnSO_4$ inhibit cell division (Davies et al. 1991) and cell elongation (Goldbold et al. 1983) and also cause chlorosis of young leaves. Small leaf size and yellowing of leaves was observed after 12 weeks of incubation on the optimum concentration of $ZnSO_4$ (Fig. 2b).

3.3 Effect of $CdCl_2$

Cadmium is one of the most important metals in terms of food chain contamination, because it is readily taken up by the cells of different plant species (Gomes-junior et al. 2006). Cadmium has been shown to cause many morphological, physiological, biochemical, and structural changes in plants (Benavides et al. 2005; Mishra et al. 2006). Varied morphogenic responses were obtained when nodal explants were exposed to different concentrations of $CdCl_2$ (5–80 μM). The optimum concentration for maximum number (3.80 ± 0.20) of shoots and length (0.53 ± 0.01 cm) of shoots was found to be 10 μM (Fig. 1c), with an increase in cadmium concentration, there was a gradual decrease in shoot regeneration and shoot length (Table 3). The toxic effect of cadmium on biological systems has been reported by several authors (Bingham et al. 1976; Mukherjee et al. 1984; Das et al. 1997). Most of the cultures showed pale yellowish bud formation and severe browning of cultures. The toxic effects of cadmium on in vitro cultures have been reported in *Vigna radiata* and *Vigna unguiculata* (Chandra et al. 2010) and *Bacopa monniera* (Ali et al. 1998, 2001).

3.4 Rooting and acclimatization

In vitro multiplication is often associated with difficulties in rooting of microshoots. For successful micropropagation, the ability to produce roots is very important and the survival of plantlets after transferring to soil and field conditions depends on the healthy growth of roots. In vitro raised shoots were able to form roots when transferred to half strength liquid MS medium augmented with 0.5 μM IBA (Fig. 2c; Table 4). To provide minimal stress and optimal growth conditions the rooted microshoots that have grown in vitro have been continuously exposed to a unique microenvironment which causes problems during the transplantation stage. To overcome this problem, the plantlets were acclimatized for 3 weeks in the growth room condition to increase the survival rate. These acclimatized and hardened plantlets were then transferred to the net house under natural sunlight.

4 Conclusion

It is concluded that optimization of metal ions (Cu or Zn except Cd) in the medium plays a significant role in obtaining multiple shoot regeneration form nodal explants

Table 3 Effect of different concentration of $CdCl_2$ on shoot bud induction and proliferation from nodal explants

Concentration of $CdCl_2$ (μM)	After 4 weeks		After 8 weeks		After 12 weeks	
	No. of shoots	Shoot length (cm)	No. of shoots	Shoot length (cm)	No. of shoots	Shoot length (cm)
Control	2.40 ± 0.24^{c}	0.52 ± 0.024^{a}	5.40 ± 0.24^{a}	0.66 ± 0.014^{b}	11.40 ± 0.60^{a}	1.04 ± 0.053^{b}
5	3.40 ± 0.24^{b}	0.43 ± 0.029^{b}	4.40 ± 0.24^{bc}	0.64 ± 0.006^{b}	10.60 ± 0.40^{a}	0.93 ± 0.027^{bc}
10	3.80 ± 0.20^{ab}	0.53 ± 0.010^{a}	5.00 ± 0.31^{ab}	0.76 ± 0.025^{a}	11.20 ± 0.48^{a}	1.38 ± 0.076^{a}
15	4.00 ± 0.31^{ab}	0.33 ± 0.013^{c}	4.60 ± 0.24^{abc}	0.54 ± 0.012^{c}	10.40 ± 0.67^{a}	0.85 ± 0.010^{c}
20	4.20 ± 0.20^{a}	0.23 ± 0.025^{d}	4.60 ± 0.40^{abc}	0.40 ± 0.015^{d}	10.00 ± 0.63^{a}	0.82 ± 0.022^{c}
40	4.40 ± 0.24^{a}	0.21 ± 0.007^{de}	4.40 ± 0.24^{bc}	0.35 ± 0.023^{de}	0.00 ± 0.0^{b}	0.00 ± 0.0^{d}
80	3.80 ± 0.20^{ab}	0.17 ± 0.008^{e}	4.00 ± 0.00^{c}	0.31 ± 0.011^{e}	0.00 ± 0.0^{b}	0.00 ± 0.0^{d}

Values represent mean ± standard error of 10 replicates per treatment in three repeated experiments. Means followed by the same letter are not significantly different by the DMRT at 0.05 % probability level

Table 4 Effect of different strength of MS medium on root induction in microshoots after 4 weeks

MS strength	IBA (μM)	Sucrose (%)	No. of roots	Root length (cm)
1	0.5	2	1.60 ± 0.24	0.38 ± 0.21
½	0.5	2	4.80 ± 0.37	1.85 ± 0.18

Values represent mean ± standard error of 10 replicates per treatment in three repeated experiments

of *Rauvolfia tetraphylla* and provide an efficient and reproducible protocol for mass multiplication of this medicinally important plant. The present study could also be used to investigate the mechanism of heavy metal hyperaccumulation, phytoremediation and in other crop improvement programmes.

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