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Virginia Sarropoulou
Hellenic Agricultural Organization - 'Demeter', Institute of Plant Breeding and Genetic Resources, Laboratory of Conservation and Evaluation of Native and Floricultural Species, Thermi, Thessaloniki, Greece

Eleni Maloupa
Hellenic Agricultural Organization - 'Demeter', Institute of Plant Breeding and Genetic Resources, Laboratory of Conservation and Evaluation of Native and Floricultural Species, Thermi, Thessaloniki, Greece

Correspondence
Virginia Sarropoulou
Hellenic Agricultural Organization - 'Demeter', Institute of Plant Breeding and Genetic Resources, Laboratory of Conservation and Evaluation of Native and Floricultural Species, Thermi, Thessaloniki, Greece

Elevated levels of 3 different MS medium micronutrients $CuSO_4$, $MnSO_4$ and $ZnSO_4$ on *in vitro* shoot proliferation of *Sideritis raeseri* Boiss & Heldr – Hellenic Mountain tea of Velouchi or Parnassus

Virginia Sarropoulou and Eleni Maloupa

Abstract
This study highlights the effect of different micronutrients including copper sulfate ($CuSO_45H_2O$) manganese sulphate ($MnSO_4H_2O$) and zinc sulphate ($ZnSO_47H_2O$) combined with 2.22 µM benzyladenine (BA) in MS medium on *in vitro* shoot proliferation of *Sideritis raeseri* Boiss & Heldr. The results showed that 0.5-100 µM $CuSO_4$ increased shoot multiplication from 57.14% (control: 0.1 µM) to 80-100% and 200-1600 µM $MnSO_4$ from 55.55% (control: 100 µM) to 90-100%. A 2-fold increase in shoot number was recorded with 2.5 or 50 µM $CuSO_4$ (3 to 5.9-6) or 200 µM $MnSO_4$ (2.67 to 5.56). Shoot multiplication 100% and 20.98 mm shoot length were achieved with 120 and 240 µM $ZnSO_4$, accordingly, compared to the control (30 µM). Therefore, 1-2.5 µM $CuSO_4$ (x10-25 the normal in MS medium, 0.1 µM), 200 µM $MnSO_4$ (x2, 100 µM) or 240-480 µM $ZnSO_4$ (x8-16, 30 µM) were proved to be effective for better *in vitro* shoot regeneration of *S. raeseri.*

Keywords: Copper sulphate, Heavy metals, Manganese sulphate, Micronutrients, Shoot multiplication, Zinc sulphate.

Introduction
Propagation through seeds is an inadequate solution due to low viability, poor seed germination rate and scanty and delayed rooting of the seedling on account of presence of germinating inhibitors in *Sideritis* genus [1, 2]. Clonal propagation or micro propagation is an invaluable aid in rapid clonal multiplication of superior genotypes having desirable traits using tissue culture technology. Thus, selection and rapid multiplication of superior genotypes would help in development of medicinal products. In the present communication, an efficient protocol for regeneration of plants from apical shoot explants is reported. This protocol may be useful in conservation of this plant species, which is currently extensively exploited from the nature.

Plant culture *in vitro* has enabled selection of plants tolerant to ions of some metals, such as copper (Cu), manganese (Mn), nickel (Ni) or aluminium (Al) [3, 4, 5]. The metals like cobalt, iron, Mn, Cu and Zn are essential for plant life but are required in a very small or trace amounts and become toxic at higher concentrations [6].

Cu is an essential micronutrient for normal plant growth. In plant organisms it performs very important physiological and biochemical functions. It takes part in the processes of photosynthesis, respiration, conversion of nitrogen compounds, transport of carbohydrates and also regulates the process of DNA formation [7]. Also, 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 [8]. Since Cu 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 [9, 10] or *in vitro* grown plants [4, 11]. Excessive Cu, however, becomes toxic to many plant species [12]. One of the most rapid responses to toxic Cu levels is inhibition of root growth [13]. Root growth has also been considered as a very sensitive indicator to heavy metal exposure [14]. Bipasha *et al.* (2000) [15] has also reported that high Cu content affected root

growth than shoot growth.

Mn is a micronutrient essential for all stages of plant development. It is involved in photosynthesis, respiration, and lignin and amino acid biosynthesis, in addition to performing a key function in the activation of several enzymes, including decarboxylating malate dehydrogenase, malic enzyme, isocitrate dehydrogenase, or nitrate reductase [16]. On the other hand, Mn can be detrimental when available in excess in the surroundings. Symptoms of Mn toxicity are quite diverse among plant species and include marginal chlorosis and necrosis of leaves in *Medicago sativa* L., *Brassica napus* L., *Lactuca sativa* L., and *Nicotiana tabacum* [17], brown root discoloration in rapeseed [18] and wheat [19]; and loss of apical dominance and enhanced formation of auxiliary shoots – "witches' broom" [20]. Toxic Mn effects have also been described in different *in vitro* developmental processes, including callus induction and growth and shoot regeneration from callus [17, 21, 22, 23]. Moreover, high Mn levels have direct cytotoxic effects, causing extensive cytoplasmic injures, mitochondrial modification, and plasma membrane ruptures in the outer root cap and meristematic cells [23].

Zn is a trace element, essential for plants because of its presence in the enzymes composition catalyzing important life processes [14]. However, in a very high concentration, it can cause toxic effects and decline of a plants growth. In this respect, it is important to investigate which concentration of Zn is toxic to different plant species and how essential metal ions in high concentrations affect plant growth at different developmental stages [25].

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) [26] for tobacco tissue culture MS medium. However, no clear optimal levels were apparent and the effect of alternative formulations of Cu, Mn and Zn nutrients in tissue culture media was scarcely studied or it have not proved to be significant in spite of added to certain media, but their requirements for cell growth has not been precisely established for *S. raeseri* plant species. Therefore, the aim of the present study was to investigate the effect of various concentrations of copper sulfate ($CuSO_45H_2O$) manganese sulphate ($MnSO_4H_2O$) and zinc sulphate ($ZnSO_47H_2O$) on shoot proliferation of *S. raeseri* shoot-tip explants cultured *in vitro*. The study was also undertaken to evaluate the optimum $CuSO_4$, $MnSO_4$ and $ZnSO_4$ concentration needed for the Hellenic mountain tea of Velouchi or Parnassus cultures enabling mass multiplication, *ex situ* conservation and genetic transformation.

Materials and Methods

Plant material and culture conditions

The experimental material was shoot tip explants from previous *S. raeseri in vitro* cultures. For the initial establishment of the plant material *in vitro* apex meristems were cut and removed from the mother plants maintained in a peat:perline (1:1) substrate in pots under unheated-greenhouse conditions. For the disinfection of the collected plant material, shoot tips were soaked in 70% ethanol for 1 min followed by 2% NaOCl solution for 15 min under continuous stirring. The successfully established explants were sub-cultured every 4 weeks until a sufficient amount of plant material to be concentrated.

Three experiments were conducted. In the first experiment, $CuSO_45H_2O$ (Sigma-Aldrich) was applied exogenously at 9 concentrations (0.1-CONTROL, 0.5, 1, 2.5, 5, 10, 25, 50, 100 μM) in combination with 2.22 μM BA (Sigma-Aldrich). In the second experiment, $MnSO_4$ H_2O (Sigma-Aldrich) was tested at 5 concentrations (100-CONTROL, 200, 400, 800, 1600 μM) combined with 2.22 μM BA. In the third experiment, $ZnSO_4$ $7H_2O$ (Sigma-Aldrich) was added at 6 concentrations (30-CONTROL, 60, 90, 120, 240 μM) in conjunction with 2.22 μM BA. All 3 experiments aimed at stimulating shoot regeneration of *S. raeseri* shoot tip explants under *in vitro* conditions.

The nutrient medium used was the MS (Murashige and Skoog, 1962) supplemented with all the essential macronutrients, micronutrients, vitamins and amino acids. The culture medium was also supplemented for all 3 experiments with 30 g/l sucrose (Duchefa Biochemie) as a carbon source and 3 g/l Phytagel (Sigma-Aldrich) as a gelling agent. The pH of the media in all 3 experiments was adjusted to 5.8 before adding the gelling agent and afterwards the medium was sterilized at 121 ºC for 20 min. The initial experimental plant material for all 3 experiments was shoot tip explants, 1.5 cm long, which were transferred into flat-base glass test tubes of 25 x 100 mm containing 10 ml of MS medium and covered with aluminium foil. All cultures were maintained in a growth chamber. The chamber was programmed to maintain 16-h light duration (40 μmol/m^2/s) supplied by cool white fluorescent lamps and a constant temperature of 22 ± 2 ºC.

The first experiment with $CuSO_45H_2O$ included 9 treatments with 10 replications (explants)/treatment, the second one with $MnSO_4H_2O$ was comprised of 5 treatments with 10 replications/treatment and the third one with $ZnSO_47H_2O$ consisted of 5 treatments with 10 replications/treatment and one shoot tip explant in each test tube. After 6 weeks of culture for the first experiment with $CuSO_45H_2O$ and 4 ½ weeks for the other 2 experiments with $MnSO_4$ H_2O and $ZnSO_47H_2O$, respectively, measurements were taken regarding shoot proliferation attributes such as shoot number/explant, shoot length (mm) and percentages (%) of shoot multiplication, callus formation, vitrification, necrosis and chlorosis.

Statistical Analysis

All 3 experiments, each separately were completely randomized and analyzed by ANOVA (Analysis of Variance) using the statistical program SPSS 17.0 (SPSS Inc., Illinois, New York, USA) at $P \leq 0.05$, according to Duncan's multiple range test ± S.E. in order significant differences among the treatments to be established.

Results

Experiment 1: Effect of $CuSO_45H_2O$ on *in vitro* shoot proliferation of S. raeseri

$CuSO_4$ applied at 2.5, 50 or 100 μM significantly increased the number of shoots/explant, from 5.25 to 6, compared to the control (3 shoots/explant). In contrast, shoot length did not change significantly under the influence of 0.5-100 μM $CuSO_4$ (13.03-19.2 mm) (Fig. 1a-1h). $CuSO_4$ (0.5-100 μM) significantly raised shoot proliferation percentage to 80-100% with respect to the control group (57.14% - 0.1 μM $CuSO_4$). Callogenesis percentage was higher (90.91%) when explants were exposed to the highest $CuSO_4$ concentration (100 μM) and lower (30%) in the 0.5 μM $CuSO_4$ treatment. Hyperhydricity symptoms were less extensive, ranging from 18.18 to 40%, compared to the control (57.14%) when $CuSO_4$ was applied at 0.5-2.5 μM concentrations. In contrast, 5 μM $CuSO_4$ increased vitrification percentage to 20.64%.

Vitrification was observed to the 40% of explants treated with 2.5 μM $CuSO_4$ making-derived plant material commercially useless, while the 2 highest applied $CuSO_4$ concentrations (50 and 100 μM) decreased vitrification percentage to 18.18-20%. Low $CuSO_4$ concentrations (0.5-2.5 μM) significantly reduced or completely inhibited the occurrence of necrotic symptoms (0-20%), while higher $CuSO_4$ concentrations (5-25 μM) exacerbated the incidence of necrosis in explants (30 to 55.56 %) due to toxicity. In the 3 higher applied $CuSO_4$ concentrations (25, 50, 100 μM), a slight chlorosis of explants was observed. The explants with chlorosis did not exhibit symptoms of hyperhydricity and browning/necrosis while the non-chlorotic explants performed simultaneously vitrification and browning/necrosis problems. The explants with chlorosis after the end of the experiment were transferred for a subsequent culture into a plant growth regulators-free MS medium in devoid of BA + $CuSO_4$ and in due time, approximately 4 weeks re-obtained their normal color. In addition to this positive response, there was a stimulating effect in their vegetative growth and development including height, stem thickness and leaf size (Table 1).

Table 1: Effect of $CuSO_45H_2O$ (0.1-100 μM) concentration combined with 2.22 μM BA on shoot number/explant, shoot length (mm) and percentages (%) of shoot multiplication, callus induction, vitrification, chlorosis and necrosis in *Sideritis raeseri* Boiss & Heldr.

Treatments (μM)	Shoot number /explant	Shoot length (mm)	Shoot multiplication (%)	Callus induction (%)	Vitrification (%)	Chlorosis (%)	Necrosis (%)
0.1 $CuSO_4$ (Control)	3.00 ± 0.52 a	15.65 ± 1.56 ab	57.14 a	71.43 d	57.14 d	0 a	22.22 b
0.5 $CuSO_4$	4.30 ± 0.82 ab	17.70 ± 1.97 ab	80 b	30 a	30 b	0 a	20 b
1 $CuSO_4$	4.60 ± 0.90 ab	19.20 ± 1.49 b	80 b	70 d	20 a	0 a	0 a
2.5 $CuSO_4$	6.00 ± 0.68 b	16.66 ± 1.56 ab	100 d	90 e	40 c	0 a	0 a
5 $CuSO_4$	4.00 ± 0.45 ab	13.03 ± 0.90 a	100 d	44.44 b	77.78 e	0 a	55.56 d
10 $CuSO_4$	4.20 ± 0.92 ab	15.17 ± 1.89 ab	80 b	40 d	40 c	0 a	30 c
25 $CuSO_4$	4.20 ± 0.57 ab	16.09 ± 1.32 ab	100 d	40 d	40 c	30 b	30 c
50 $CuSO_4$	5.90 ± 0.82 b	15.71 ± 1.11 ab	90 c	60 c	20 a	30 b	20 b
100 $CuSO_4$	5.25 ± 0.71 ab	16.43 ± 1.56 ab	90.91 c	90.91 e	18.18 a	27.27 b	18.18 b
P-values	0.099 ns	0.279 ns	0.000***	0.000***	0.000***	0.000***	0.000***

Means ± S.E, n=10. Those denoted by the same letter in each column are not statistically significant different from each other according to the Duncan's multiple range test at $P \leq 0.05$, ns $P \geq 0.05$; ***$P \leq 0.001$

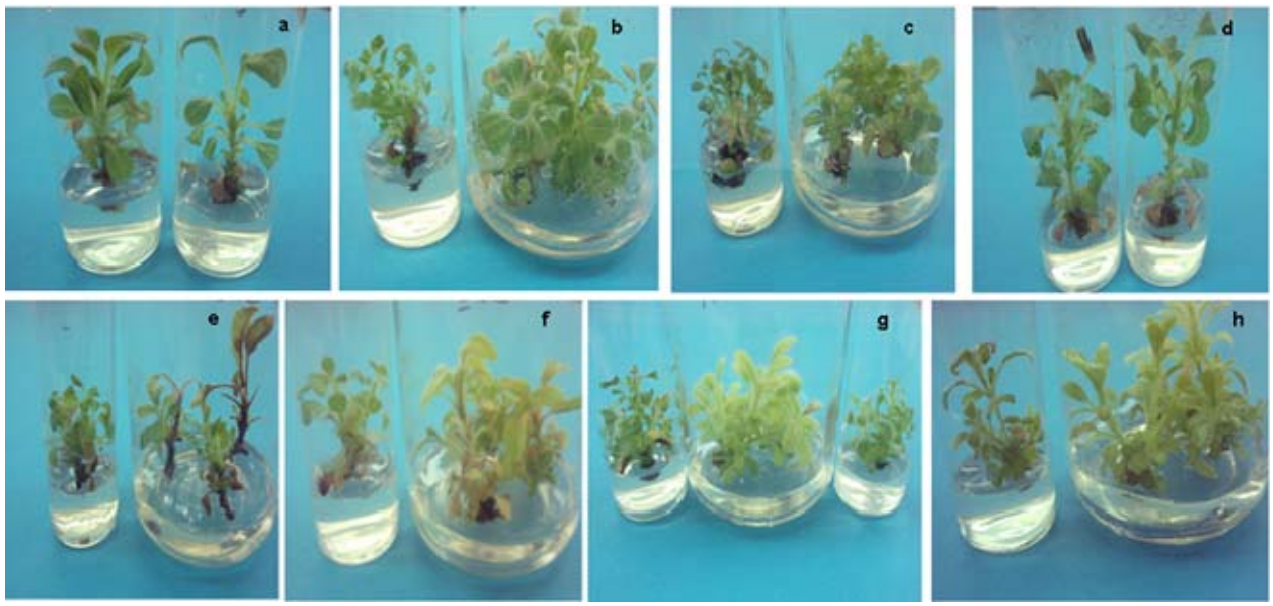

Fig 1: Effect of $CuSO_4$ (0.5-100 μM) concentration combined with 2.22 μM BA on *in vitro* shoot proliferation of *Sideritis raeseri* Boiss & Heldr.: **(a)** 0.5 μM, **(b)** 1 μM, **(c)** 2.5 μM, **(d)** 5 μM, **(e)** 10 μM, **(f)** 25 μM, **(g)** 50 μM, **(h)** 100 μM.

Experiment 2: Effect of $MnSO_4H_2O$ on *in vitro* shoot proliferation of S. raeseri

$MnSO_4$ at 200 μM doubled the number of shoots/explant (5.56) compared to the control (2.67 shoots/explant). Shoot length did not change significantly when the substrate was fortified with higher $MnSO_4$ concentrations (200-1600 μM) (14.99-18.54 mm) in relation to the effect of 100 μM $MnSO_4$ (14.26 mm). $MnSO_4$ (200-1600 μM) led to a significant increase in percentages of shoot multiplication (90-100%) and callus formation (90-100%) (Fig 2b-2e) with respect to the control (Fig. 2a), being 55.55% and 66.67%, accordingly. In all treatments with $MnSO_4$ including control problem of hyperhydricity in explants tissues was evident. The increase in the concentration of $MnSO_4$ did not diminish nor exacerbated this abnormality as evidenced by the vitrification percentages ranged between 30 and 40% among treatments (Table 2).

Table 2 Effect of $MnSO_4H_2O$ (100-1600 μM) concentration combined with 2.22 μM BA on shoot number/explant, shoot length (mm) and percentages (%) of shoot multiplication, callus induction, vitrification, chlorosis and necrosis in *Sideritis raeseri* Boiss & Heldr.

Treatments (μM)	Shoot number /explant	Shoot length (mm)	Shoot multiplication (%)	Callus induction (%)	Vitrification (%)	Chlorosis (%)	Necrosis (%)
100 $MnSO_4$ (Control)	2.67 ± 0.68 a	14.26 ± 0.82 a	55.55 a	66.67 a	33.33 b	0	0
200 $MnSO_4$	5.56 ± 0.62 b	14.99 ± 1.29 a	100 c	100 c	33.33 b	0	0
400 $MnSO_4$	4.30 ± 0.40 ab	16.50 ± 1.05 a	100 c	90 b	30 a	0	0
800 $MnSO_4$	3.60 ± 0.52 a	18.38 ± 1.53 a	90 b	100 c	40 c	0	0
1600 $MnSO_4$	2.90 ± 0.43 a	18.54 ± 2.27 a	100 c	90 b	30 a	0	0
P-values	0.03*	0.161 ns	0.000***	0.000***	0.000***	-	-

Means ± S.E, n=10. Those denoted by the same letter in each column are not statistically significant different from each other according to the Duncan's multiple range test at $P \leq 0.05$, ns $P \geq 0.05$; *$P \leq 0.05$; ***$P \leq 0.001$

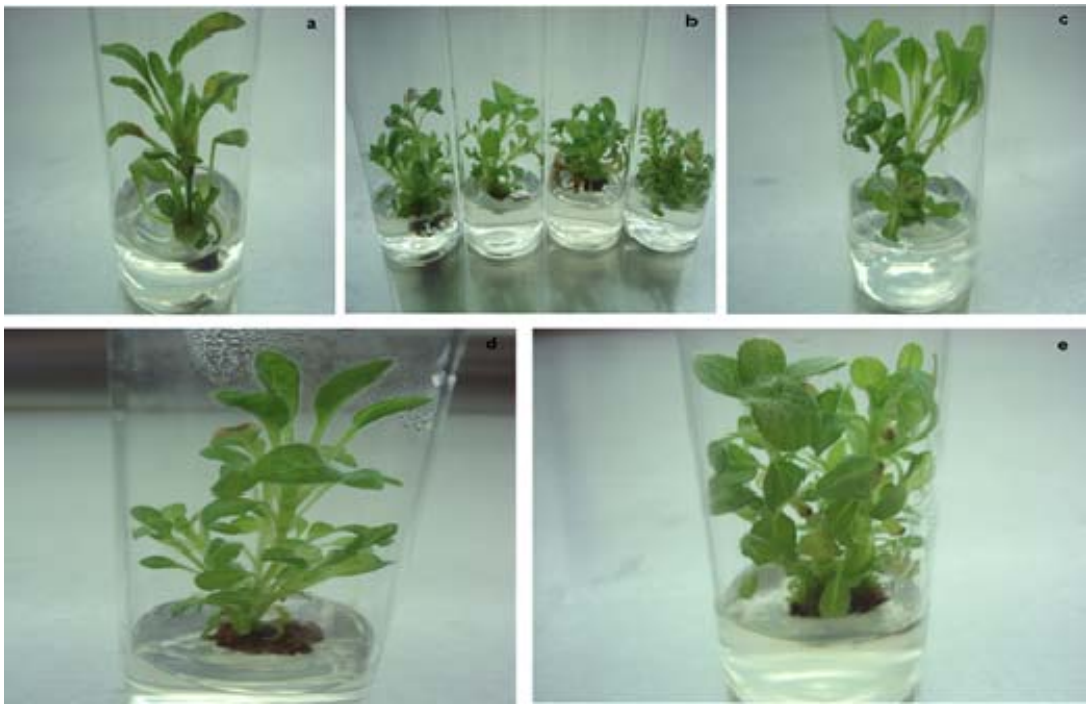

Fig 2: Effect of $MnSO_4H_2O$ (100-1600 μM) concentration combined with 2.22 μM BA on *in vitro* shoot proliferation of *Sideritis raeseri* Boiss & Heldr.: **(a)** 100 μM (Control), **(b)** 200 μM, **(c)** 400 μM, **(d)** 800 μM, **(e)** 1600 μM.

Experiment 3: Effect of $ZnSO_47H_2O$ on *in vitro* shoot proliferation of S. raeseri

$ZnSO_4$ (60-480 μM) did not modify shoot number/explant (2.8-3.3) significantly (Fig. 3b-3f), compared to the control (30 μM $ZnSO_4$ - 2.67 shoots/explant) (Fig. 3a). Shoot length was increased by 0.67 cm, from 14.26 to 20.98 cm, when explants were exposed to 240 μM $ZnSO_4$ while lower $ZnSO_4$ concentrations (60, 90, 120 μM) and the highest applied one (480 μM) did not bring any substantial change. Shoot multiplication (70-100%) and callus induction percentages (88.89-100%) were increased to a significant extent in relation to the control (55.55% and 66.67%, respectively) when MS medium supplemented with $ZnSO_4$ concentrations (60-480 μM) beyond the normal one (30 μM). In specific, both 100% shoot multiplication and callus induction percentages were obtained with 120 μM $ZnSO_4$. Hyperhydricity in explants was apparent in all treatments including control. $ZnSO_4$ at lower concentrations (60, 90, 120 μM) intensified the problem of vitrification (44.44-50%) whereas at higher ones (240 and 480 μM) slightly reduced the relative percentage (20-30%), compared to control (33.33% vitrification) (Table 3).

Table 3 Effect of $ZnSO_47H_2O$ (30-480 μM) concentration combined with 2.22 μM BA on shoot number/explant, shoot length (mm) and percentages (%) of shoot multiplication, callus induction, vitrification, chlorosis and necrosis in *Sideritis raeseri* Boiss & Heldr.

Treatments (μM)	Shoot number /explant	Shoot length (mm)	Shoot multiplication (%)	Callus induction (%)	Vitrification (%)	Chlorosis (%)	Necrosis (%)
30 $ZnSO_4$ (Control)	2,67 ± 0,68a	14,26 ± 0,82 a	55,55 a	66,67 a	33,33 c	0	0
60 $ZnSO_4$	2,89 ± 0,28a	15,32 ± 1,01 a	88,89 d	88,89 b	44,44 d	0	0
90 $ZnSO_4$	2,80 ± 0,51a	15,58 ± 2,00 a	70 b	90 b	50 e	0	0
120 $ZnSO_4$	3,10 ± 0,28a	13,53 ± 1,83 a	100 e	100 c	50 e	0	0
240 $ZnSO_4$	3,30 ± 0,63a	20,98 ± 2,88 b	80 c	90 b	30 b	0	0
480 $ZnSO_4$	3,10 ± 0,48a	14,90 ± 1,15 a	90 d	100 c	20 a	0	0
P-values	0,059 ns	0,955 ns	0,000***	0,000***	0,000***	-	-

Means ± S.E, n=10. Those denoted by the same letter in each column are not statistically significant different from each other according to the Duncan's multiple range test at $P \leq 0.05$, ns: $P \geq 0.05$; ***$P \leq 0.001$

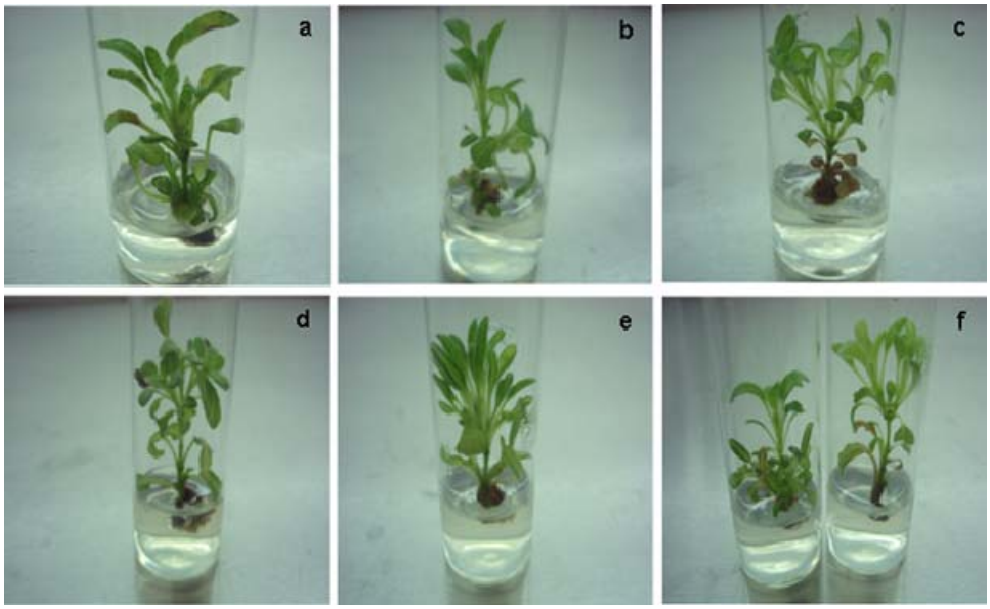

Fig 3: Effect of $ZnSO_47H_2O$ (30-480 µM) concentration combined with 2.22 µM BA on *in vitro* shoot proliferation of *Sideritis raeseri* Boiss & Heldr.: (**a)** 30 µM (Control), **(b)** 60 µM, **(c)** 90 µM, **(d)** 120 µM, **(e)** 240 µM**, (f)** 480 µM.

Discussion

Numerous *in vitro* studies have been concerned with plant sensitivity to toxic metal ions [27, 28, 5, 29]. A lower sensitivity of plants to toxic ions in media can be reflected in their limited uptake, or synthesis of compounds (enzymes, lipids or chelates) that cause their detoxification inside cells [30, 31, 32]. Plant sensitivity to toxic metal ions depends on the kinds of compounds present in the substrate and on their concentrations [33]. The stimulating effect of some metal ions on plant development may be due to their impact on solubility and hence availability of some nutrients to plants [34, 35].

In the current research employing *S. raeseri*, shoot length was not influenced substantially due to $CuSO_4$ (0.5-100 µM) application. However, in *Ailanthus altissima* Swingle [36] and poplar (*P. tremula* L. × *P. alba* L.) [37] *vitro* cultures, supplementing the culture medium with 50-200 µM and 250-1000 µM $CuSO_4$, respectively, led to a decrease in shoot number and shoot length. Our findings are also not in line with those presented by Sanjeev *et al.* (2003) [38] where $CuSO_4$ (25-125 µM) exhibited better results in terms of shoot growth in relation to the controls on MS copper level (0.1 µM) in the medicinal plant *Tinospora cordifolia*. In *S. raeseri*, shoot number was positively influenced by incorporating 2.5 or 50 µM $CuSO_4$ into the culture medium. Similarly, in *in vitro* shoot tips and nodal explants of the annual herbaceous plant *Eclipta alba* (L.) Hassk., 1 µM $CuSO_4$ gave the maximum number of shoot buds [39]. $CuSO_4$ concentration in the medium significantly enhanced multiple shoot number in several other plants like wheat [40], sorghum [41], barley [42] and capsicum [43]. In *S. raeseri*, all tested $CuSO_4$ concentrations enhanced explants' shoot multiplication ability in a considerable degree. On the contrary, in *Holarrena antidysenterica* [44] and aspen (*Populus tremula tremuloides*) [45] *vitro* cultures, $CuSO_4$ adversely affected shoot proliferation. In *in vitro* shoot-tip explants of *S. raeseri*, 100% shoot proliferation was noted by adding 2.5, 5 or 25 µM $CuSO_4$ to the culture medium. In accordance with our results, in *Withania somnifera* L., 25-200 µM $CuSO_4$ stimulated shoot bud formation and subsequent elongation of the *in vitro* nodal explants where 100 µM $CuSO_4$ gave 95% shoot regeneration percentage [46].

In the present study with *S. raeseri*, symptoms of chlorosis to the 27.27-30% of the explants due to high $CuSO_4$ concentrations (25-100 µM) as well as necrotic/total browning symptoms to the 30-55.56% of the microcuttings were evident when 5-25 µM $CuSO_4$ were applied, showing that exogenous $CuSO_4$ concentration above a level is being toxic causing stress to microshootlets. These phenomena were also evident in poplar (*P. tremula* L. × *P. alba* L.) explants cultured *in vitro* where 250-1000 µM $CuSO_4$ intensified the degree of chlorosis and browning in microcuttings [37]. In this research with *S. raeseri*, callus induction percentage was meaningfully decreased when the explants were subjected to 0.5 or 5-50 µM $CuSO_4$. El-Aref and Hamada (1998) [47] found that Cu was toxic to tomato explants at 100 µM $CuSO_4$ as reflected by reduced callus growth and this result was interpreted by them to a reduction in the expression of specific enzymes (dehydrogenases and esterases).

In the current study employing *S. raeseri*, a 2-fold increase was observed in shoot number (2.67 to 5.56) with the presence of 200 µM $MnSO_4$ in the MS medium. This finding is in agreement with those reported by Kothari *et al.* (2008) [48] where the 2x Mn concentration in the MS medium (2x100=200 µM) was the best for regeneration in case of *Paspalum*. Shoot multiplication of *S. raeseri* shoot-tip explants was remarkably promoted due to $MnSO_4$ application (200-1600 µM), reaching to 90-100% for 55.55% in the case of the control treatment (100 µM $MnSO_4$). Similarly, in *Eclipta alba* (L.) Hassk. an improvement of *in vitro* response has been discerned when $MnSO_4$ concentration was increased to 300 µM, which was 3-fold higher than that included in MS medium (100 µM) [40]. Positive was also the effect of increased $MnSO_4$ levels (300-500 µM) in MS medium on shoot number and shoot multiplication percentage in *Stevia rebaudiana* (Bert.) Bertoni under *in vitro* conditions [49]. In *in vitro* culture of *S. raeseri*, $MnSO_4$ regardless its concentration (200-1600 µM) did not have a significant effect on shoot elongation. On the other hand, Gatti (2008) [37] reported that 400-1600 µM $MnSO_4$ led to a decrease in shoot length of *Ailanthus altissima* Swingle *in vitro* explants.

Elevated levels of $MnSO_4$ (200-1600 µM) in the MS medium resulted in increased callus induction percentages *in S.*

raeseri, being 90-100% (2 times higher compared to the 66.67% of the control's). According to Rout *et al.* (1999) [50], $MnSO_4$ showed significant reduction of growth in callus lines of *Brassica campestris* and *B. juncea.* In *S. raeseri*, no symptoms of chlorosis nor necrosis were observed to the explants due to $MnSO_4$ (100-1600 μM). In contrast, Todorović *et al.* (2009) [51] reported that both excess and absence of Mn in media (<100 μM and > 100 μM up to 10 mM) caused appearance of necrotic plants in lesser centaury [*Centaurium pulchellum* (Sw.) Druce] under *in vitro* conditions. The observed necrosis is likely a consequence of oxidative stress imposed by either deficiency or excess of Mn. To be specific, when Mn is deficient, the activity of antioxidative enzymes is reduced, and so is the ROS-scavenging capacity of the plants. On the other hand, excessive uptake of Mn can cause direct generation of ROS through Fenton-like reactions [52].

Zinc is essential for protein synthesis, IAA synthesis and nitrogen metabolism but it is toxic at high levels and produces adverse effects on plant growth and development [53]. In the present study with *S. raeseri*, 60-480 μM $ZnSO_4$ did not differentiate substantially the number of produced shoots, exhibiting similar results to the control (30 μM $ZnSO_4$). Similar results were obtained by Gatti (2008) [36] in *Ailanthus altissima* where shoot number and shoot length were not modified substantially due to 60-120 μM $ZnSO_4$ application whereas higher $ZnSO_4$ concentrations (240-480 μM) had an inhibitory effect. Different results were reported by Bardar *et al.* (2014) [39] demonstrating that the number of shoot buds formed *in vitro* were maximum when *Eclipta alba* (L.) Hassk. nodal segments were cultured on basal MS concentration of Zn in the medium (30 μM) whereas higher $ZnSO_4$ concentrations (>30 μM) had an inhibitory effect. Shoot number and shoot length of *Paulownia tomentosa* (Thunb.) Steud. microcuttings were negatively affected due to $ZnSO_4$ application at high concentrations (600-1000 μM) while $ZnSO_4$ lower concentrations (200-400 μM) did not alter these 2 macroscopic attributes in a considerable degree [54]. However, stimulatory effects of $ZnSO_4$ on shoot number have been reported by Jain *et al.* (2012) [49] in *Stevia rebaudiana* (Bert.) Bertoni, where 90 μM $ZnSO_4$ (3-fold higher than the normal concentration in MS medium) increased the number of shoots/microexplant. Shoot length of *S. raeseri* explants was enhanced by 0.6 cm when the culture medium was fortified with 240 μM $ZnSO_4$ (x8 the normal concentration of Zn in MS medium being 30 μM) while lower (60-120 μM) and higher $ZnSO_4$ concentrations (480 μM) gave similar results to the control. Fatima *et al.* (2011) [46] found that 300 μM $ZnSO_4$ gave the greatest shoot length in *in vitro* cultures of *Withania somnifera* L. In addition, according to Gatti (2008) [36], *Ailanthus altissima* microshoots cultured on MS medium containing 30 μM $ZnSO_4$ when treated with 60-120 μM $ZnSO_4$ shoot length were not modified substantially whereas the application of higher $ZnSO_4$ concentrations (240-480 μM) had an inhibitory effect. *S. raeseri* explants treated with 60-480 μM $ZnSO_4$ performed significantly higher shoot multiplication percentages (70-100%) compared to the control containing 30 μM $ZnSO_4$ (55.55%). Our findings are in line with those performed in nodal explants of *Withania somnifera* L., where the addition of 50-500 μM $ZnSO_4$ in the optimized MS improved shoot proliferation attributes [46]. In *S. raeseri*, 100% shoot multiplication was recorded with 120 μM $ZnSO_4$ while in *Withania somnifera* L. with 300 μM $ZnSO_4$ [46].

Stress-tolerant plants produced *in vitro* preserve a greater tolerance to toxic metal ions in the next few generations, also after transfer to natural conditions [3, 55]. 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*.

Conclusion

In vitro selection of plants tolerant to toxic ions contained in the soil may lead to production of plants that are better adapted to environmental pollution and can enable better management of degraded soil (e.g. industrial areas and highways). Cultures of *S. raeseri* exposed to heavy metals (Cu, Zn, Mn) demonstrated a tolerance comparable to species already utilized in phytoremediation. This is the first report on plant regeneration of *S. raeseri* utilizing shoot tip segments using $CuSO_4$, $MnSO_4$ and $ZnSO_4$. As *S. raeseri* endangered species is medicinally important, the present protocol is valuable for conservation and restoration purposes.

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References

1. Ferreira AG, Cassol B, Rosa SGT, Silveira TS, Stival AL, Silva AA. Germination of seeds of Asteraceae native of Rio Grande do sul. Brazil. Acta Botanica Brasilica. 2001; 15(2):231-242.
2. Shahnaz S, Agrawa V. Efficient *in vitro* micropropogation of purple fleabane (*Vernonia anthelmintica* Willd.): a multipurpose under-exploted oil-yielding herb. International Journal of Pharma and Bio Sciences. 2011; 2(4):231-241.
3. Van Sint Jan V, Costa de Macedo C, Kinet JM, Bauharmont J. Selection of Al–resistant plants from a sensitive rice cultivar, using somaclonal variation, *in vitro* and hydroponic cultures. Euphytica. 1997; 97:303-310.
4. Gori P, Schiff S, Santandrea G, Bennici A. Response of *in vitro* cultures of *Nicotiana tabacum* L. to cooper stress and selection of plants from Cu-tolerant callus. Plant Cell, Tissue and Organ Culture. 1998; 53(3):161-169.
5. Rout GR, Samantary S, Das P. *In vitro* selection and characterization of Ni-tolerant callus lines of *Setaria italica* L. Acta Physiologiae Plantarum. 1998; 20(3):269-275.
6. Hussein K, Sahadevan KK, Salim N. Bioaccumulation and release of mercury in *Vigna mungo* (L.) hepper seedlings. Journal of Stress Physiology and Biochememistry. 2010; 6(3):56-63.
7. Podlesna A, Wojcieska-Wyskupajtys U. Collection and use of copper by cereals. Zesz. Nauk. Komit. Człow. i Środ. 1996; 14:129-133.
8. Lolkema PC. Copper resistance in higher plants. Free University Press, Amsterdam, Ph. D Thesis, 1985.
9. Lanaras T, Moustakas M, Symeonidis L, Diamantoglou S, Karataglis S. Plant metal content, growth responses and some photosynthetic measurements on field–

cultivated wheat growing on ore bodies enriched in Cu. Physiologia Plantarum. 1993; 88(2):307-314.
10. Ouzounidou G, Lannoye R, Karataglis S. Photoacoustic measurements of photosynthetic activities in intact leaves under copper stress. Plant Science. 1993; 89(2):221-226.
11. Romeu–Moreno A, Mas A. Effect of copper exposure in tissue cultured *Vitis vinifera.* Journal of Agricultural and Food Chemistry. 1999; 47(7):2519-2522.
12. Wu L, Lin SL. Copper tolerance and copper uptake of *Lotus purshianus* (Benth.) Clem & Clem and its symbiotic *Rhizobium loti* derived from a copper mine waste population. New Phytology. 1990; 116(3):531-539.
13. Eleftheriou EP, Karataglis S. Ultrastructural and morphological characteristics of cultivated wheat growing on copper–polluted fields. Botanica Acta. 1989; 102(2):134-140.
14. Wilkins DA. The measurement of tolerance to edaphic factors by means of root growth. New Phytology. 1978; 80(3):623-633.
15. Bipasha C, Goswami BC, Chakravarty B. Effect of zinc and copper on growth and regenerating cultures of *Citrus reticulate* Blanco. Indian Journal of Plant Physiology. 2000; 5(1):52-58.
16. Mukhopadhay MJ, Sharma A. Manganese in cell metabolism of higher plants. The Botanical Review. 1991; 57(2):117-149.
17. Petolino JF, Collins GB. Manganese toxicity in tobacco (*Nicotiana tabacum* L.) callus and seedlings. Journal of Plant Physiology. 1985; 118(2):139-144.
18. Moroni JS, Scott BJ, Wratten N. Differential tolerance of high manganese among rapeseed genotypes. Plant and Soil. 2003; 253(2):507-519.
19. Moroni JS, Briggs KG, Taylor GJ. Chlorophyll content and leaf elongation rate in wheat seedlings as a measure of manganese tolerance. Plant and Soil. 1991; 136(1):1-9.
20. Kang BT, Fox RL. A methodology for evaluating the manganese tolerance of cowpea and some preliminary results of field trials. Field Crops Res. 1980; 3:199-210.
21. Santandrea G, Schiff S, Bennici A. Manganese toxicity to different growth processes *in vitro* in Nicotiana. Plant Cell, Tissue and Organ Culture. 1997; 50(2):125-129.
22. Santandrea G, Schiff S, Bennici A. Effects of manganese on *Nicotiana* species cultivated *in vitro* and characterization of regenerated Mn-tolerant tobacco plants. Plant Science. 1998a; 132(1):71-82.
23. Santandrea G, Tani C, Bennici A. Cytological and ultrastructural response of *Nicotiana tabacum* L. roots to manganese stress. Plant Biosystems. 1998b; 132(3):197-206.
24. Drab M, Greinert A, Kostecki J, Grzechnik M. Seed germination of selected plants under the influence of heavy metals. Civil and Environmental Engineering Reports. 2011; 7:47-57.
25. Li W, Khan M, Yamaguchi S, Kamiya Y. Effects of heavy metals on seed germination and early seedling growth of *Arabidopsis thaliana*. Plant Growth Regulation. 2005; 46(1):45-50.
26. Murashige T, Skoog F. A revised medium for rapid growth and bioassay with tobacco tissue cultures. Physiologia Plantarum. 1962; 15(3):473-497.
27. Bharti N, Singh RP, Sinha SK. Effect of calcium chloride on heavy metal induced alternation in growth and nitrate assimilation of *Sesamum indicum* seedlings. Phytochemistry. 1996; 41(1):105-109.
28. Burzyński M, Buczek J. Uptake and assimilation of ammonium ions by cucumber seedlings from solutions with different pH and addition of heavy metals. Acta Societatis Botanicorum Poloniae. 1998; 67(2):197-200.
29. Samantaray S, Rout GR, Das P. *In vitro* selection and regeneration of zinc tolerant calli from *Setaria italica* L. Plant Science. 1999; 143(2):201-209.
30. Knecht De JA, Dillen Van M, Koevoets PLM, Schat H, Verkleij JAC, Ernst WHO. Phytochelatins in cadmium-sensitive and cadmium-tolerant *Silene vulgaris*. Plant Physiology. 1994; 104(1):255-261.
31. Leopold I, Gunther D. Investigation of the binding properties of heavy-metal-peptide complexes in plant cell cultures using HPLC-ICP-MS. Journal of Analytical Chemistry. 1997; 359(4):364-370.
32. Silva IR, Smyth TJ, Raper CD, Carter TE, Rufty TW. Differential aluminium tolerance in soybean: An evaluation of the role of organic acids. Physiologia Plantarum. 2001; 112(2):200-210.
33. Arnold MA, Lineberger RD, Struve DK. Copper compounds influence *in vitro* rooting of birch microcuttings. Journal of the American Society for Horticultural Science. 1994; 119(1):74-79.
34. Keltjens WG, Van loenen E. Effects of aluminium and mineral nutrition on growth and chemical composition of hydroponically grown seedlings of five different forest species. Plant and Soil. 1989; 119(1):39-50.
35. Jerzykiewicz J. Aluminium effect on nitrate assimilation in cucumber (*Cucumis sativus* L.) roots. Acta Physiologiae Plantarum. 2001; 23(2):213-219.
36. Gatti E. Micropropagation of *Ailanthus altissima* and *in vitro* heavy metal tolerance. Biologia Plantarum. 2008; 52(1):146-148.
37. Bojarczuk K. Effect of toxic metals on the development of poplar (*Populus tremula* L. × *P. alba* L.) cultured *in vitro*. Polish Journal of Environmental Studies. 2004; 13(2):115-120.
38. Sanjeev K, Narula A, Sharma MP, Srivastava PS, Kumar S, Narula A. Effect of copper and zinc on growth, secondary metabolite content and micropropagation of *Tinospora cordifolia*: a medicinal plant. Phytomorphology. 2003; 53:79-91.
39. Bardar S, Khurana-Kaul V, Kachhwaha S, Kothari SL. Nutrient optimization for improved *in vitro* plant regeneration in *Eclipta alba* (L.) Hassk. and assessment of genetic fidelity using RAPD analysis. Plant Tissue Culture & Biotechnology. 2014; 24(2):223-234.
40. Tahiliani S, Kothari SL. Increased copper content of the medium improves plant regeneration from immature embryo derived callus of wheat (*Triticum aestivum*). Journal of Plant Biochemistry and Biotechnology. 2004; 13(1):85-88.
41. Nirwan RS, Kothari SL. High copper levels improve callus induction and plant regeneration in *Sorghum bicolor* (L.) Moench. *In vitro* Cellular and Developmental Biology-Plant. 2003; 39(2):161-164.
42. Dahleen LS. Improved plant regeneration from barley callus cultures by increased copper levels. Plant Cell, Tissue and Organ Culture. 1995; 43(3):267-269.
43. Joshi A, Kothari SL. High copper levels in the medium improves shoot bud differentiation and elongation from the cultured cotyledons of *Capsicum annum* L. Plant Cell, Tissue and Organ Culture. 2007; 88(2):127-133.
44. Agraval V, Sharma K. Phytotoxic effects of Cu, Zn, Cd and Pb on *in vitro* regeneration and concomitant protein changes in *Holarrhena antidysenterica.* Biologia

Plantarum. 2006; 50(2):307-310.
45. Kalisova-Spirochova I, Puncocharova J, Kafka Z. Accumulation of heavy metals by *in vitro* cultures of plants. Water, Air & Soil Pollution. 2003; 3(3):269-276.
46. Fatima N, Ahmad N, Anis M. Enhanced *in vitro* regeneration and change in photosynthetic pigments, biomass and proline content in *Withania somnifera* L. (Dunal) induced by copper and zinc ions. Plant Physiology and Biochemistry. 2011; 49(12):1465-1471.
47. El-Aref HM, Hamada AM. Genotypic differences and alterations of protein patterns of tomato explants under copper stress. Biologia Plantarum. 1998; 41(4):555-564.
48. Kothari-Chajer A, Sharma M, Kachhwaha S, Kothari SL. Micronutrient optimization results into highly improved *in vitro* plant regeneration in kodo (*Paspalum scrobiculatum* L.) and finger (*Eleusine coracana* (L.) Gaertn.) millets. Plant Cell, Tissue and Organ Culture. 2008; 94(2):105-112.
49. Jain P, Kachhwaha S, Kothari SL. Optimization of micronutrients for the improvement of *in vitro* plant regeneration of *Stevia rebaudiana* (Bert.) Bertoni. Indian Journal of Biotechnology. 2012; 11:486-490.
50. Rout GR, Samantaray S, Das P. *In vitro* selection and biochemical characterisation of zinc and manganese adapted callus lines in *Brassica* spp. Plant Science. 1999; 146(2):89-100.
51. Todorović S, Giba Z, Simonović A, Božić D, Banjanac T, Grubišić D. Manganese effects on *in vitro* development of lesser centaury [*Centaurium pulchellum* (Sw.) Druce]. Archives of Biological Sciences Belgrade. 2009; 61(2):279-283.
52. González A, Steffen KL, Lynch JP. Light and excess manganese. Plant Physiology. 1998; 118(2):493-504.
53. Wang H, Liu RL, Jin JY. Effect of zinc and soil moisture on photosynthetic rate and chlorophyll fluorescence parameters of maize. Biologia Plantarum. 2009; 53(1):191-194.
54. Bahri BN, Laribi B, Bettaieb T. Morphological effects of zinc and its accumulation in micropropagated plants of *Paulownia tomentosa* (Thunb.) Steud. International Journal of Medicinal and Aromatic Plants. 2013; 3(1):40-46.
55. Ali G, Srivastava PS, Iqbal M. Morphogenic and biochemical responses of *Bacopa monnieri* cultures to zinc toxicity. Plant Science. 1999; 143(2):187-193.