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Uptake and Accumulation of Aluminium, Copper and Cobalt in Tissue Cultured *Melastoma malabathricum* Linn. Plantlets

Wilson Thau Lym Yong[1*], Joan Li Jett Chang[2], Vun Yee Thien[1], Harry Lye Hin Chong[3] and Janna Ong Abdullah[4]

[1]Biotechnology Research Institute, Universiti Malaysia Sabah, Jalan UMS, 88400 Kota Kinabalu, Sabah, Malaysia.
[2]School of Science and Technology, Universiti Malaysia Sabah, Jalan UMS, 88400 Kota Kinabalu, Sabah, Malaysia.
[3]Water Research Unit, Universiti Malaysia Sabah, Jalan UMS, 88400 Kota Kinabalu, Sabah, Malaysia.
[4]Department of Microbiology, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia.

Authors' contributions

This work was carried out in collaboration between all authors. Author YWTL designed the study and wrote the protocol. Authors CJLJ and TVY performed the practical work and data acquisition. Authors CHLH and AJO supervised the work in all its aspects and performed manuscript editing and review. All authors read and approved the final manuscript.

Original Research Article

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ABSTRACT

Aims: This study was carried out to investigate the metal tolerance levels of *Melastoma malabathricum* Linn. plantlets.
Study Design: The metal tolerance levels of *M. malabathricum* L. were examined using an *in vitro* approach. The ability of the plant to survive in tissue culture medium containing aluminium (Al), copper (Cu) and cobalt (Co) were assessed.
Place and Duration of Study: The study was carried out in the Plant Culture Laboratory, Biotechnology Research Institute, Universiti Malaysia Sabah (UMS), between September 2011 and February 2013.
Methodology: *M. malabathricum* L. plantlets were cultured on half strength MS media

**Corresponding author: E-mail: wilsonyg@ums.edu.my;*

supplemented with Al, Cu and Co at concentrations ranging from 0, 0.5, 1.0, 1.5 to 2.0 mM. The growth and survival of the plantlets were observed at every 10 days of treatment and the metals accumulation levels in the leaves and roots were analyzed after 30 days of culture.
Results: The order of the survival rate for *M. malabathricum* L. plantlets subjected to these three metals was demonstrated to be Al > Cu > Co in the highest metal concentration tested. More accumulation of Al was observed in the roots, and Cu was found to be higher than Co in the leaves.
Conclusion: Data obtained from this study on the potential uptake and accumulation of toxic metals for *M. malabathricum* L. will be used in future for the development of this plant species as a phytoremediator.

Keywords: Heavy metal hyperaccumulation; Melastoma malabathricum; metal tolerance; phytoremediation; tissue culture.

1. INTRODUCTION

Melastoma malabathricum Linn, also known as *M. candidum* is one of the heavy metal accumulating plants [1]. This plant is commonly found in light forests, clearings and grasslands, and is native to Southeast Asia countries including Singapore, India, Thailand, and Malaysia. This species is an aluminum accumulator growing in tropical acid soils [2] which has been reported to be a good candidate for phytoextraction of lead, zinc, chromium, tin and arsenic [3,4,5] from the soils. However, it is unclear whether hyperaccumulation of copper (Cu) and cobalt (Co) is possible by this species. Cu contaminations in soils are normally from the source of poultry manures, pesticides, metal finishing and microelectronics by-products [6] while contaminations of Co are found in serpentine soils and rocks, and industrial wastes [7,8]. Soils contamination due to the presence of toxic metals can cause damage to ecosystems, reduction of agricultural productivity, deterioration of food chain, and serious health problems to animals and humans [9].

In metal biology, it was experimentally proven that some metals including Cu that are essential for the normal plants growth [10] may become toxic, depending on the oxidation state, complex form, dose and mode of exposure [11]. Study of [12] reported that 5 ppm of Cu was able to promote root growth by 155% in alfalfa plant. However, the Cu showed a concentration dependent inhibition of root growth at doses of 20 and 40 ppm. Excess amount of Cu can be toxic because of its participation in redox cycles producing hydroxyl radicals, which are extremely toxic to living cells [13], and induces stress and injury to plants leading to plant growth retardation and leaf chlorosis [14]. Cu concentrations in plant leaves are naturally controlled within a range (about 10 μg g^{-1}) even when they are grown on metalliferous soils with higher concentrations of Cu [15].

Co which is an essential element for animals and prokaryotes can be accumulated by plants in small amount from the soil, however, it causes toxicity to plants at higher concentration. Plants may exhibit symptoms such as leaf fall, inhibition of greening, discolored veins, premature leaf closure and reduced shoot weight when exposed to excess concentrations of the elements [16]. The heavy metal has been reported by [17] to have adverse effect on shoot growth and biomass in barley (*Hordeum vulgare* L.), oilseed rape (*Brassica napus* L.) and tomato (*Lycopersicon esculentum* L.). Besides, [18] reported that high level of Co affected the translocation of P, S, Mn, Zn and Cu from the roots to the tops in cauliflower and

also significantly decreased water potential and transpiration rate in the plant. Its concentrations are normally found in very low quantity in plant leaves (0.03-2.00 μg g^{-1}) and less than 20.0 μg g^{-1} in metalliferous soils [19].

The removal and recovery of heavy metals from contaminated media are greatly important in terms of protection of the environments as well as abatement of heavy metal toxicity. Depending on the plant species, heavy metal tolerance or adaptations that enable them to grow in heavy metal contaminated media are the results of two basic strategies: exclusion and accumulation [20]. The accumulation strategy involves physiological processes that require the cells to maintain the intra cellular heavy metal ions in non toxic forms [21] and the stored heavy metal ion complexes may be removed by leaf fall [22]. The criterion for Cu and Co hyperaccumulation is 300 μg g^{-1} [19,23]. The objectives of this study are to analyze the effect of Cu and Co on the growth performance of *M. malabathricum* L. *in vitro* and the capability of hyperaccumulation of these metals in their biomass with aluminium (Al) accumulation as a reference. The data compiled from this study may be of utility for further development of this crop plant in soil reclaimation.

2. MATERIALS AND METHODS

2.1 Preparation of Explants

In vitro *M. malabathricum* L. plantlets maintained in the Plant Culture Laboratory, Biotechnology Research Institute of Universiti Malaysia Sabah (UMS), Malaysia, were used. Nodal explants were cut and cultured on metal-free half strength MS medium [24] solidified with 0.4% gelrite (Duchefa, Netherlands). The pH of the medium was adjusted to 5.7. The cultures were placed in a culture room maintained at 25±2°C under 16 h light/ 8 h dark photoperiod. The light source was provided with cool white fluorescent tubes with a photon flux density of 30-40 μmol m^{-2} s^{-1}. The explants were pre-cultured for seven days to obtain the same age plantlets with uniform size (2.3±0.2 cm in height) for subsequent experiments.

2.2 Heavy Metals Treatment

The regenerated *M. malabathricum* L. plantlets were transferred to half strength MS media containing 1.5 mg L^{-1} 2,4-dichlorophenoxyacetic acid and 2.0 mg L^{-1}benzylaminopurine supplemented with different concentration of metals: 0 (control), 0.5, 1.0, 1.5 and 2.0 mM for 30 days. Three heavy metals were used: Al, Cu and Co, added as chloride salts to the medium. The pH of media was adjusted to 4.5 in the experiment and the media were solidified with 0.4% gelrite. The metal treatments were performed as a single one with four replicates in each concentration test and six plantlets in each replicate. The growth and survival of plantlets were observed every 10 days and the samples were collected after 30 days for heavy metals analyses. Any symptoms of metal toxicity (stunting, necrosis, yellowing, pigmentation, discoloration, leaf blister, rut, black spot) exhibited by the plantlets were visually noted and the severity of toxicity was recorded throughout the experimental period.

2.3 Heavy Metals Analyses by ICP-OES

The shoots and roots were excised from the treated plantlets and rinsed with deionized water to remove the adhering particles. Tissues were then washed with 0.001 M $CaCl_2$ to remove the weakly adsorbed cations followed by rinsing with deionized water. The plant

materials were dried in an oven at 80ºC for 72 hours. Roots and shoots were separated in order to obtain information about the ability to accumulate metals in different biomass. The dried tissues were weighed and ground into powder for metal concentration analyses. Metal contents (Al, Cu and Co) of the plant tissues were extracted by acid digestion (60% nitric acid) followed by determination of metals concentrations using an analytical instrument, Optima 5300 DV ICP-OES (Inductively Coupled Plasma-Optical Emission Spectrometry), purchased from Perkin Elmer, USA.

2.4 Statistical Analyses

Statistical analyses of data were carried out by one-way analysis of variance (ANOVA) followed by Turkey-HSD test using SPSS Statistic 17. Metal translocation from root to shoot was measured by translocation factor (TF) = C_{shoot} / C_{root}, where C_{shoot} and C_{root} are metal concentration (mg kg^{-1}) in the shoot and root of plant respectively. TF > 1 represent that translocation of metals was made effectively from root to shoot [25].

3. RESULTS AND DISCUSSION

3.1 Plant Growth and Survival under Heavy Metal Treatments

The mean percentage of *M. malabathricum* L. plantlets surviving under each heavy metal treatment was recorded every 10 days for 30 days. The Al-treated plantlets were observed to achieve the highest survival percentage (100%) under metal concentrations of 1.5 and 2.0 mM (Fig. 1). The survived plantlets on Al supplemented media did not show any morphological changes, the leaves remained greenish and no signs of deterioration were observed on culture quality. Higher shoot development with greater plant height was observed in Al-treated plantlets as compared to the control plantlets (Table 1). Besides, roots were developed from the Al-treated plantlets after 10 days of culture. Although roots were also developed in control plantlets, but Al-treated plantlets were observed to exhibit higher root development with greater number of roots (Table 2).

The declines in the survival percentage of *M. malabathricum* L. plantlets were 75% under 2.0 mM Cu treatment (Fig. 2) to 33% under 2.0 mM Co treatment (Fig. 3), all observed after 30 days of exposure. At lower metal concentration (0.5-1.0 mM), growth were still observed for Cu- and Co-treated plantlets. Exposures of plantlets to higher concentrations (1.5-2.0 mM) of Cu and Co were resulted in growth reductions (Table 1). Metal toxicity of Cu and Co was revealed by visible changes in the appearance of the leaves, where obvious yellow and brown spots were observed. No such damage was resulted in control and Al-treated plantlets. Cu and Co at all concentrations (0.5-2.0 mM) tested completely blocked root development in all the treated plantlets. The shortest shoot length, characteristic of unhealthy growth, and deteriorated culture quality (increased level of chlorosis and browning) were observed in plantlets subjected to higher concentration of Co (1.5-2.0 mM).

Plantlets grew on Al supplemented medium did not show noticeable morphological changes probably because the level of tested Al concentrations did not exceed the critical level of tolerance for *M. malabathricum* L. as the plant is known as an Al hyperaccumulator [26]. Moreover, we postulate that the improvement in the growth efficiency of Al-treated plantlets with higher shoot development may be due to the beneficial effects of Al on the physiological activities of the plants. Both Cu and Co are required by the plant system in low quantities as essential elements for metabolic reactions, but their presence become toxic at high

concentrations probably due to the wide range of their cytotoxic effects. Once the metals are taken up into plant system, they are concentrated in less sensitive locations such as vacuoles, cell walls, epidermal cells or trichomes [27]. In sensitive plants, high concentrations of these metals may cause inhibition of enzymes involved in the photosynthetic reactions [28]. The decrease in shoot length under heavy metal stress was reported earlier by [29] in runner bean plants as the result of preferential decrease of cell wall elasticity.

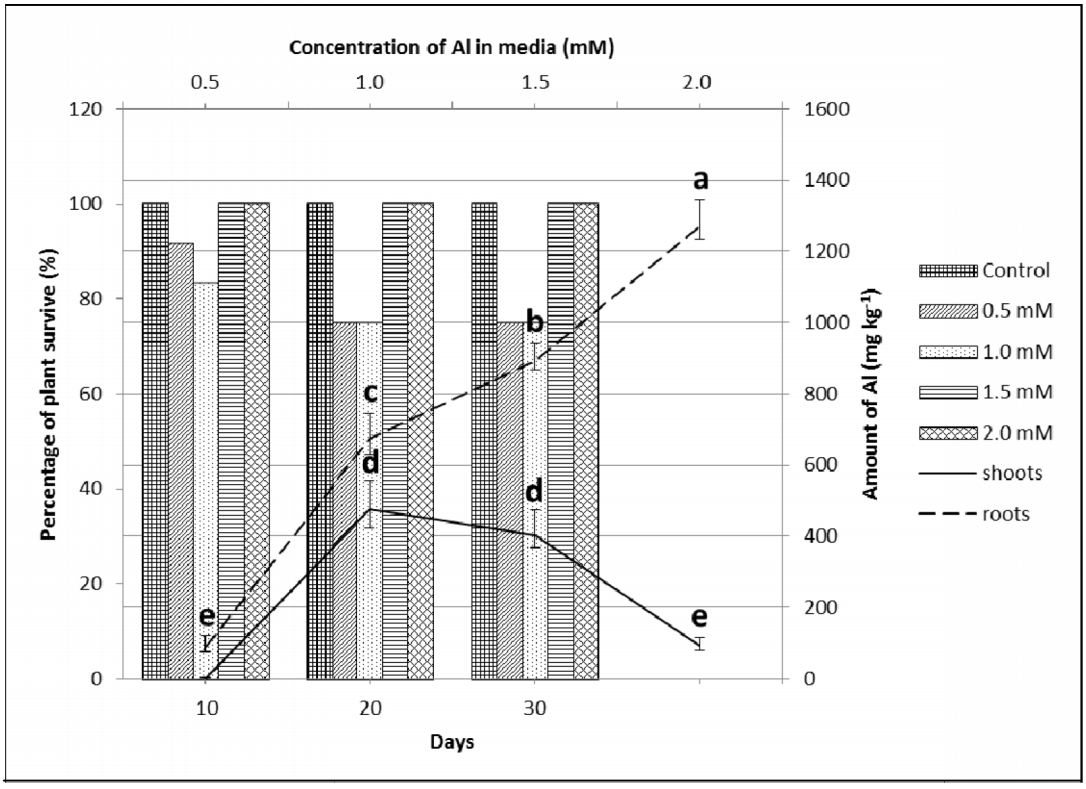

Fig. 1. Survival percentage and amount of Al accumulated in treated plantlets.

Results were obtained from four replicates with six plantlets in each. Bars data represent the survival rate of M. malabathricum L. plantlets under different concentrations of Al treatment for 30 days of culture. Line data represent the amount of Al accumulated in shoots and roots of M. malabathricum L. plantlets under different concentrations of Al treatment. Error bars correspond to the standard deviation. Different letters indicate the values are significantly different ($p \leq 0.05$).

Table 1. Effects of different concentrations of heavy metals on plant growth and toxicity symptoms

Days of exposure		0		10		20		30	
Metal	**Concentration (mM)**	**Plant height (cm)**	**Leaf symptom**	**Plant height (cm)**	**Leaf symptom**	**Plant height (cm)**	**Leaf symptom**	**Plant height (cm)**	**Leaf symptom**
Control		2.1±0.26	NA	3.0±0.26	V	3.8±0.22	V	4.5±0.37	V
Al	0.5	2.4±0.11	NA	3.3±0.14	V	4.2±0.34	V	5.1±0.44	V
	1.0	2.5±0.05	NA	3.4±0.21	V	4.2±0.42	V	5.2±0.41	V
	1.5	2.2±0.24	NA	3.4±0.19	V	4.3±0.15	V	5.2±0.33	IV
	2.0	2.2±0.22	NA	3.4±0.12	V	4.5±0.21	IV	5.5±0.56	IV
Cu	0.5	2.3±0.16	NA	2.5±0.13	III	2.6±0.18	II	2.7±0.17	I
	1.0	2.5±0.10	NA	2.7±0.08	III	2.8±0.11	II	2.8±0.21	I
	1.5	2.3±0.18	NA	2.2±0.11	II	2.0±0.21	II	1.8±0.12	I
	2.0	2.4±0.14	NA	2.4±0.06	II	2.2±0.17	II	1.9±0.15	I
Co	0.5	2.1±0.14	NA	2.2±0.18	III	2.3±0.12	II	2.4±0.06	I
	1.0	2.3±0.23	NA	2.4±0.12	III	2.5±0.18	II	2.5±0.15	I
	1.5	2.3±0.19	NA	2.3±0.09	II	2.2±0.12	II	2.0±0.17	I
	2.0	2.5±0.08	NA	2.3±0.05	II	2.1±0.16	II	1.8±0.21	I

Values correspond to the means of four replicate ± standard error (n=24). The level of metal toxicity on leaf were observed and evaluated (Level I – very severe; Level II – severe; Level III – mild; Level IV – slightly toxic; Level V – absent / very slightly toxic); NA – not applicable.

Table 2. Effect of different Al concentrations on number of root and root length

Days of exposure	0		10		20		30	
Al concentration (mM)	**number of root**	**Root length (cm)**	**Number of root**	**Root length (cm)**	**Number of root**	**Root length (cm)**	**Number of root**	**Root length (cm)**
0 (control)	0	NA	2	1.7±0.29	4	2.3±0.37	5	2.7±0.25
0.5	0	NA	4	1.5±0.70	7	2.0±0.46	7	2.5±0.88
1.0	0	NA	3	1.6±0.58	6	2.1±0.13	7	2.4±0.19
1.5	0	NA	3	1.7±0.27	5	2.4±0.39	6	2.5±0.33
2.0	0	NA	3	1.8±0.36	6	2.2±0.41	7	2.6±0.62

Values correspond to the means of four replicate ± standard error (n=24); NA – not applicable.

In the present study, the order of plant survival for all treatments after 30 days was recorded to be Al-treated followed by Cu- and Co-treated plantlets (Table 1). The reduction in plant growth during stress may be due to stomatal resistance and oxidative stress as revealed by [30,31]. Particularly in Al- and Cu-treated plantlets, the survival rates after 10 days were reported lower in the lower metal concentration than in the higher metal concentrations and remaining lower survival rate for all the treatment duration (Fig. 1 and Fig. 2). The phenomenon may be attributed to the important role of antioxidative defense system in Al and Cu tolerance. The increased survival rate with increasing metal concentration has suggested the activation of enzymatic antioxidants upon Al and Cu stress. The stimulation of antioxidative enzymes activity has also been reported in several plants subjected to higher concentration of Al and Cu treatment [32,33,34]. Decreasing survival rate with increasing experimental duration in Cu- and Co-treated plantlets may be attributed by the range of interactions at the cellular/molecular level due to the presence of excessive amounts of heavy metals [35].

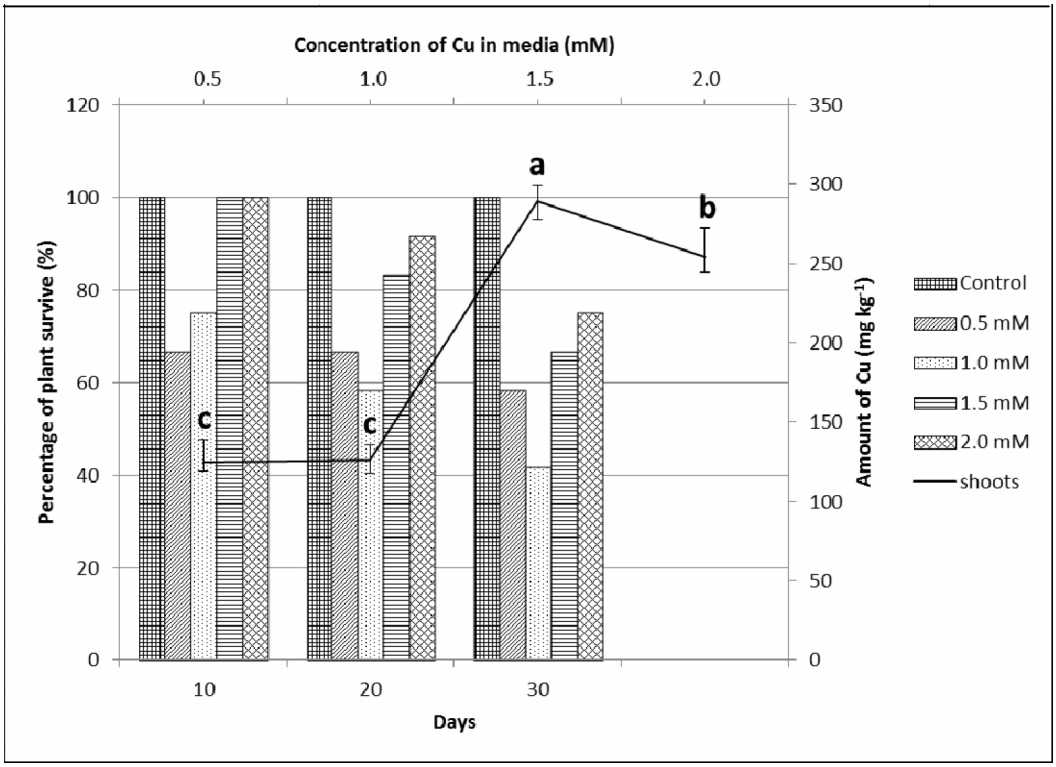

Fig. 2. Survival percentage and amount of Cu accumulated in treated plantlets.

Results were obtained from four replicates with six plantlets in each. Bars data represent the survival rate of M. malabathricum L. plantlets under different concentrations of Cu treatment for 30 days of culture. Line data represent the amount of Cu accumulated in shoots and roots of M. malabathricum L. plantlets under different concentrations of Cu treatment. Error bars correspond to the standard deviation. Different letters indicate the values are significantly different ($p \leq 0.05$).

3.2 Uptake and Accumulation of Heavy Metals in Treated Plantlets

The highest amount of Al accumulation (1270.83±12.26 mg kg^{-1}) was found in the roots that had been treated with 2.0 mM of Al (Fig. 1). The results showed that plantlets accumulated

higher amount of Al in roots rather than leaves. TF was observed to be less than 1.0 in *M. malabathricum* L. plantlets treated with all the concentrations of Al. Low translocation of Al indicates that *M. malabathricum* L. was reluctant to transfer Al from their roots to shoots. Highest translocation was noticed at 1.0 mM Al as illustrated in Fig. 1. Due to failure of root regeneration in Cu- and Co-treated plantlets, only their shoots were subjected to ICP-OES analysis. Maximum amounts of Cu (289.42±9.68 mg kg^{-1}) and Co (136.16±3.79 mg kg^{-1} accumulated in the shoots of *M. malabathricum* L. plantlets under the treatments of 1.5 mM of each respective metal were illustrated in Fig. 2 and Fig. 3 respectively.

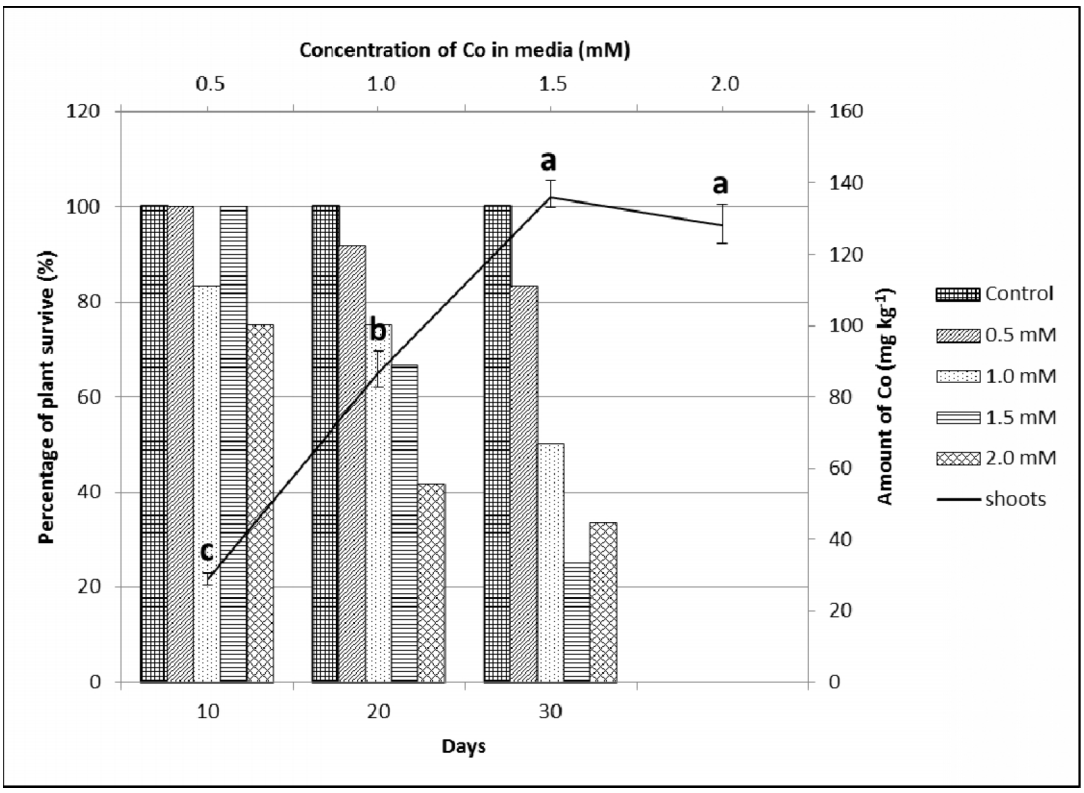

Fig. 3. Survival percentage and amount of Co accumulated in treated plantlets.

Results were obtained from four replicates with six plantlets in each. Bars data represent the survival rate of M. malabathricum L. plantlets under different concentrations of Co treatment for 30 days of culture. Line data represent the amount of Co accumulated in shoots and roots of M. malabathricum L. plantlets under different concentrations of Co treatment. Error bars correspond to the standard deviation. Different letters indicate the values are significantly different ($p \leq 0.05$).

In the present study, 124.92±9.50 mg kg^{-1} of Cu accumulated in the shoots under the treatment of 0.5 mM of Cu, and this significantly reduced new shoot development from the plantlets. The shoot growth showed gradual retardation as cumulative amount of Cu in the leaves increased due to increased supplementation of Cu in the medium. Plantlets treated with Coat all concentrations tested showed reduced shoot production and elongation and overall growth retardation, especially at higher concentration treatments. Efficiency of plantlets uptake of Co was first increased and later decreased with increased Co concentration in media. No heavy metal was detected in the control plantlets and their presence was negligible.

Cu may have toxic effects on the plant growth and development when exceeding 20 mg kg^{-1} of metal accumulated in their leaves [36]. The mean concentration of Cu in shoots of normal plants is 10 mg kg^{-1} as reported by [37]. Retarded shoot growth of several ruderal plant species due to excess Cu level in soil was reported by [38]. Cu was also reported to be able to inhibit photosynthesis and reproductive processes [39,40], induce ethylene synthesis [41] and subsequently increase the senescence process after extended exposure of treated plants to excess metal [29]. High levels of ethylene produced under stress conditions can increase the rigidity of cell walls through enhancement of lignifications process followed by growth inhibition [42]. Besides, [43] also reported that ethylene may be involved in the inhibitory action of Cu on roots and leaves of dicotyledons plants.

Although beneficial effect of leaf senescence retardation via inhibition of ethylene biosynthesis was reported for Co [44], our results indicated that Co had the highest inhibition effects on the plantlets growth and root development. Treatments with Cu and Co showed high inhibition of root development. No roots were observed in both Cu and Co supplemented media regardless of the total concentrations of heavy metals in media. Roots are usually shortened and thickened or poorly developed when exposed to high concentration of heavy metals [45]. It has earlier been reported that increasing Co supply to *Silene cucubalus* resulted in decreased root biomass indicating the alterations of plant physiology and metabolism [46]. Study of [47] found that 5 min of Cu action showed a rapid and long lasting inhibitory effect on root growth implying that a heavy metal, besides its direct effect, can induce a signalling cascade leading to changed processes connected with extension of cells.

In this study, Al was the only metal which could support the plantlets growth even at a concentration as high as 2.0 mM in the growth medium. There were no symptoms of metal toxicity observed on the Al-treated plantlets as the plantlets were found to be well adapted in acidic Al supplemented media. Furthermore, the treated plantlets were capable of regenerating roots under the tested Al concentrations. This observation suggested the status of *M. malabathricum* L. as potential Al accumulator, and the addition of Al to the culture medium may in fact be stimulatory to the plantlets. Our observations are in accordance with [48] where *M. malabathricum* L. has increased leaf, stem and roots growth when cultured on Al supplemented media.

Present study also showed that the roots of Al-treated plantlets accumulated significantly greater amount of Al than the shoots. This is consistent with previous observations on other Al accumulator plants [49,50]. Higher concentrations of Al were said to accumulate in the roots rather than shoots because roots are the first organ to take up the heavy metal from the media before distributing it to other parts of the plants and the root hairs contain higher surface area for adsorption and absorption. According to [51], hyperaccumulation of Al in *M. malabathricum* L. is caused by the higher capacity of the species to retain Al in root symplasts rather than the higher Al uptake rate into the symplast.

There was reduction in metal translocation from roots to shoots with increase in Al concentrations in media. It is possible that high levels of metal ions are transported into the cells at the lower metal concentration. According to [52], only a part from the total amount of ions associated with the roots is absorbed into the cells. A significant ion fraction is physically adsorbed at the extracellular negatively charged sites (COO^-) of the root cell walls and therefore cannot be translocated to the shoots. Besides, metals can also be complexed and sequestered in cellular structure (e.g., vacuole) becoming unavailable for translocation to the shoot [53].

4. CONCLUSION

The present study presents a screening process of *M. malabathricum* L. for potential uptake and accumulation of toxic metals that includes Cu, Co and Al in the microenvironmental condition, avoiding the interference posed by the complexity of the chemical and biological processes of the plant's rhizosphere. The addition of Al is observed to have positive influence on the shoot and root developments of *M. malabathricum* L. plantlets. The optimum accumulation of Cu and Co in this plant species did not exceed the hyperaccumulation criterion of 300 μg g^{-1}, indicating the plant is not feasible for large scale removal of Cu and Co metals. Inhibition of root regeneration and retardation of plant growth due to metal toxicity demonstrated that the plant was stressed under high concentrations of Cu and Co. There is need to assess the interaction of metals on the uptake of nutrients by this plant species as well as its remediation potential in the tolerance and accumulation of other untested metals.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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