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The potential use of *Epilobium hirsutum* L. in phytoremediation of zinc and an efficient method for *in vitro* propagation

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Abstract: This study aimed to evaluate the capacity of *Epilobium hirsutum* L. (Onagraceae), a wetland plant, to accumulate and tolerate zinc (Zn) and its *in vitro* propagation potential. Root-shoot length, fresh weight, pigment, and protein content were analyzed in the plants grown in different Zn concentrations, including 0, 10, 20, 30, 40, 50, 75, 100,150, and 200 mg Zn/L. In the seedlings grown at 50 and 75 mg Zn/L concentrations, a reduction in the relative root length, shoot length, and fresh weight was detected. It was found that there was a negative correlation between pigment and protein contents of *E. hirsutum* and increased Zn concentrations of solutions. On the other hand, it was determined that a considerable amount of Zn was accumulated by *E. hirsutum* in its roots (10 598 mg Zn/kg DW). In tissue culture experiments, it was found that MS medium was effective for the germination of the plant (97%). When the growth parameters of plants grown in different concentrations of Gibberellic acid were evaluated, the highest growth parameters were obtained at 50 mg/L. It has been concluded that the most successful mediums on shoot development were 1.0BAP/1.0NAA and 1.0BAP/1.0IBA. The highest number of shoots per explant was 1.0BAP/1.0NAA (3.96). The longest root length was also determined on medium with 1.0BAP/1.0IBA (0.28 cm). Regenerated shoots were transferred to different concentrations of root mediums. It was concluded that MS medium with 1.0IBA has been superior for root formation compared to other hormone concentrations.

Keywords: Phytoremediation, Purple loosestrife, Zinc

1. Introduction

Heavy metal pollution in freshwater is a global environmental problem (Sabreena et al. 2022; Ali and Khan 2018, Tiwari et al. 2015). Rapid population growth, unplanned use of natural resources, and pollution caused by industrial wastes have caused a reduction in freshwater resources recently (Phillips et al. 2015; Akpor et al. 2014). With scientific studies, effective remediation techniques have been improved, such as adsorption, biosorption, remediation, and phytoremediation, using plants to remove pollution or contaminants from the environment. However, in the last decade, one of these techniques, studies on phytoremediation, has gained speed worldwide (Azubuike et al. 2016; Phillips et al. 2015; Fu and Wang 2011; Hegazy et al. 2011). One of the heavy metals, Zn, is an essential micronutrient for plants, and it is the second most common element found in living organisms after iron. However, zinc deficiency and toxicity affect plant growth. There is a considerable difference in the tolerance of plants to zinc levels in soil. Zinc-tolerant plants have developed mechanisms to regulate excess zinc by sequestering Zn in vacuoles or forming complexes that reduce its harmful effects. Zn exists in five distinct forms in soils, including water-soluble, adsorbed, chelated, and Zn complexes (Noulas et al. 2018). The structure of more than 300 enzymes, such as superoxide dismutase, carbonic anhydrase, and polymerase, participates in carbohydrate, lipid, protein, phosphate, DNA, and RNA synthesis in plants (Nardis et al. 2018; Broadley et al. 2012). Besides, Zn promotes growth hormones and starch metabolism; it is also an essential microelement in a plant for nitrogen metabolism and seed maturation (Ackova 2018; Sharma et al. 2013; Tsonev and Lidon 2012; Cakmak 2000). However, increased anthropogenic activities and rapid industrialization caused the accumulation of Zn in the environment (Nardis et al. 2018). In this regard, the ability of plants to remove pollutants from the environment and

convert them to non-toxic forms for a sustainable environment has become increasingly important in recent years (Adki et al. 2014; Doran 2009). Plant tissue culture is a technique used to cultivate and maintain new plant tissues, cells, or organs under aseptic and controlled conditions on a nutrient medium (Sarasan et al. 2006). Compared to traditional methods, tissue culture methods appear as reliable and practical techniques for large-scale plant multiplication and conservation of plants. Plant tissue culture has broad application areas in various fields, such as environmental problems, phytoremediation, plant improvement, and secondary metabolite production. It is also a suitable tool for phytoremediation studies (Adki, Jadhav, and Bapat 2014; García-Gonzáles et al. 2010; Sarasan et al. 2006). There are many wetland plants for using phytoremediation studies (Eid et al. 2021; Rodrigues et al. 2020; Schück and Greger 2020). One of these wetland plants *Epilobium hirsutum* L. (Onagraceae), is a perennial medicinal plant popularly known as great hairy willowherb (Davis 1965). Epilobium species contain various flavonoids; therefore, the *E. hirsutum* plant has been used to treat benign prostatic hyperplasia (BPH) (Granica et al. 2014). Nowadays, remediation methods are frequently used to remove Zn pollution in aquatic environments. Thus, *E. hirsutum* plants accumulated very high concentrations of metals and were used to detect heavy metal pollution in wetlands (Adki, Jadhav, and Bapat 2014; Guittonny-Philippe et al. 2015). *E. hirsutum* plant is suitable for the remediation of especially Cu contaminated areas (Ghaderian and Ghotbi Ravandi 2012). Also, the biotechnological methods mentioned in our study are fundamental to reproducing and conserving the *E. hirsutum* genotype, a medicinal plant.

The objective of the present study is to reveal the usability of *E. hirsutum* in phytoremediation of Zn in hydroponic culture, to detect the response of *E. hirsutum* to Zn stress, and to develop an *in vitro* propagation protocol for the use of *in vitro* plantlets in the future phytoremediation studies. With this study, we target *E. hirsutum,* a native herbaceous plant in Turkey, and will take its place among the plants that can accumulate Zn in the literature. Considering this plant's high Zn accumulation capacity, this research will be a good sample for the possible use of wetland plants in phytoremediation studies.

2. Materials and Method

2.1. Plant Material

E. hirsutum capsules were collected from September to October 2016 from populations in Köprüören, Kütahya, Türkiye (39º 30 ' 27 " N, 29º 44 ' 59 " E). In phytoremediation experiments, the healthy seeds were sewn into pots filled with soil and placed in pools filled with water in the greenhouse until seedlings had 8-10 leaves. *E. hirsutum* seedlings were transplanted into 2.5-liter pots containing 10% Hoagland solution for acclimatization and kept in this solution for seven days.

2.2. Phytoremediation Experiments

To determine the maximum Zn concentration that seedlings could accumulate a high amount of Zn along with showing healthy development, *E. hirsutum* seedlings were kept in 10% Hoagland solutions (pH 6.2) containing ten different Zn concentrations (0 as a control group, 10, 20, 30, 40, 50, 75, 100, 150 and 200 mg Zn/L) for seven days. Two stock solutions, including 1000 ppm Zn solution and Hoagland solution without Zn, were prepared for the experiment. Calculated Zn stock solution amounts were added to 10% Hoagland solutions to adjust the concentrations mentioned above. At the end of the seven days, the roots of the harvested seedlings were washed with sodium-EDTA (1%) and ultra-pure water to eliminate heavy metal contamination. The relative growth parameters (RGP) were calculated as (1):

$$RGP(\%)=\frac{\text{Growth parameters in zinc solutions}}{\text{Growth parameters in control solution}} \text{ x } 100 \quad (1)$$

2.2.1. Zinc Analysis

Plant samples (root, shoot, and leaf parts) were dried at 70 ºC oven for 48 hours and then ground in a RETCH brand mortar grinder for Zn analysis. The dried plant parts were weighed and recorded as dry weight (DW). 0.1 g dried plant samples were digested with nitric acid and hydrogen peroxide in glass digestion tubes (Kaçar and İnal 2008). The heavy metal content of seedlings was analyzed by an Atomic Absorption Spectrometer (Analytikjena ContrAA 300, Dumlupinar University 2018) by Dumlupinar University Advanced Technologies in Design, Research, and Development, and Application Centre.

2.2.2. Measurement of Protein and Chlorophyll Contents of Seedlings

The chlorophyll contents of fresh leaves were determined by crushing them in a mortar with acetone. The supernatant absorbance was read at 450, 647, and 663 nm using an Optizen POP spectrophotometer (Arnon, 1949). 0.5 g of fresh leaves were extracted with 5 mL of phosphate buffer to determine protein content. The extracts were centrifuged at 20,000 rpm for 20 min, and the absorbance of 0.1 mL supernatant sample was read at 595 nm (Bradford 1976).

2.3. Tissue Culture Experiments

2.3.1. Sterilization of Seeds

The seeds were sterilized by adding a few drops of tween 20 to the 3% sodium hypochlorite for 10 minutes and then rinsing with sterile ultrapure water three times. Murashige and Skoog (Murashige and Skoog 1962) (MS) nutrient medium containing mineral salts, 3% sucrose, and 7 g agar were used in germination, regeneration, and rooting experiments. MS nutrient medium was sterilized in an autoclave at 121 °C and under 1.1 atm-pressure for 20 minutes. Sterilized seeds were sown in magenta vessels, left in a growth cabinet at 25 °C, and under a 16/8 hours photoperiod for 30 days.

2.3.2. In Vitro Germination

After 30 days of germination, *in vitro* germinated seedlings with approximately 0.3-0.5 cm shoot tips were transferred to an MS medium containing 25, 50, and 75 mg/L gibberellic acid (GA_3-filter-sterilized and added to the growth medium following to autoclaving) for three weeks. After three weeks, *E. hirsutum* seedlings were transferred to MS basal medium with different combinations of 6-benzyl amino purine (BAP)/naphthalene acetic acid (NAA) and BAP/indole-3-butyric acid (IBA) for four weeks. The study used these mediums as the initiation media for *in vitro* multiplication. The regenerated shoots (about 5 cm in length) of *E. hirsutum* were excised and individually transferred to an MS root medium with various concentrations of NAA and IBA to test the rooting potential. The number of roots per shoot, root lengths, and rooting percentages were determined after four weeks after the culture initiation. Plantlets grown in tissue culture were gradually adapted to hydroponic culture.

2.4. Statistical Analysis

JMP6 SAS Statistical Analysis Program was used to evaluate the data obtained from this study (JMP, 2005). The experiments were conducted in three replicates containing five explants in each culture vessel. F-test and Tukey HSD multiple comparisons test (at $p<0.05$ level) were used to reveal the differences between different Zn concentrations and average Zn accumulation, root lengths, shoot lengths, fresh weights, chlorophyll, and protein contents of *E. hirsutum* seedlings (Kocaçalışkan and Bingöl 2017). All calculated mean values are given together with their standard deviations.

3. Results and Discussion (Times New Roman 10 pt, Bold)

3.1. Phytoremediation Experiment

A plant's tolerance to heavy metal stress can be achieved through some changes in its growth (Ackova 2018). Similarly, our research determined a significant relationship between relative growth parameters and Zn concentrations. The mean relative root lengths, shoot lengths, and fresh weights of *E. hirsutum* seedlings grown in different Zn concentrations are given in Figure 1. In this study, increasing Zn concentrations have caused a decrease in relative root and shoot lengths and fresh weights of *E. hirsutum* according to an increase in Zn concentrations from 10 to 75 mg/L in solutions, except for 10 mg/L fresh weight (86.5 to 55.4%; 79.9 to 63%; 101.5 to 34.2%, respectively) (Fig. 1).

Our data revealed a statistically significant relation between increasing Zn concentration in the solutions and root-shoot lengths and fresh weight of *E. hirsutum* compared to the control ($p\leq 0.01$). While blackening and rupture were determined on the roots of the seedlings grown in solutions containing 40 mg/L and above Zn concentrations, drying and red-brown spots were also observed on the leaves. It was observed that the fresh weight of the seedlings decreased at 50 and 75 mg/L Zn concentrations. However, it was found that in all concentrations above 75 mg Zn/L (100, 150, and 200 mg Zn/L), seedlings died at the end of the experiment. In another study related to Zn heavy metal stress, the *Lythrum salicaria* plant was shown to decrease the root-shoot length and fresh weight (Bingöl et al. 2021). A similar result has been reported by Akin et al. (2022), and they also found that increasing Zn concentrations have produced a reduction in the root length, shoot length, and fresh weight of the *E. hirsutum* plant according to the control, which made it tolerant to the Zn heavy metal by salicylic acid. Malik et al. (1970) reported that the length of roots and shoots, fresh weight, and dry weight of *Amaranthus* sp. decreased depending on the increasing Zn concentration, while the root and shoot length of the rice plant increased due to the increasing Zn concentration. Ehsan et al. (2015) investigated the effect of Zn on the *Lupinus uncinatus* plant. They found that the plant showed healthy growth in a medium containing 30 µM Zn but showed toxic symptoms in 40 µM Zn solution, and the plant died at 50 µM Zn concentration. Arán et al. (2017) found that the root length and leaf number of the *Limnobium laevigatum* plant decreased in parallel with the increasing Zn concentration. In their review, Bolat and Kara (2017) emphasized that depending on the increase in the Zn concentration, the plant's root and leaf development and iron and phosphorus uptake decreased significantly. Zn toxicity caused significant changes, especially in the root system of plants. Several studies have revealed that plants exposed to high metal concentrations cause changes in root morphology and develop a higher percentage of branching, especially in the metal contact area of the roots. Zn stress also causes a reduction in primary root length (Balafrej et al. 2020). Thus, the results of the studies mentioned above had similar findings to this study. As a result, excess Zn causes growth inhibition, development of chlorosis, and necrosis in the plant and inhibits cell elongation and division (Tsonev and Lidon 2012).

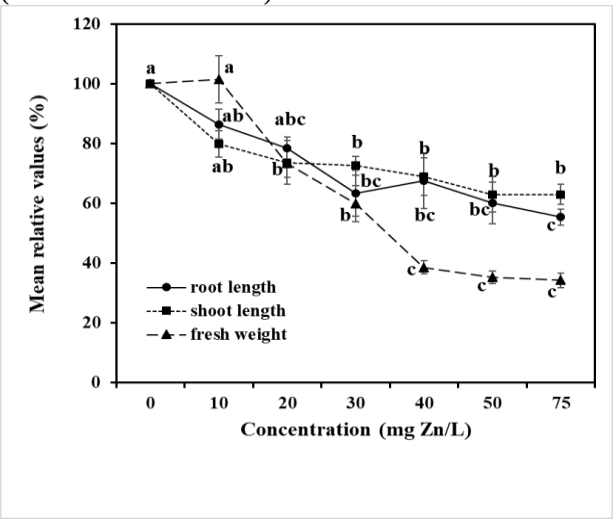

Fig. 1 Mean relative values of root length, shoot length, and fresh weight of *E. hirsutum* at different Zn concentrations

According to the data obtained from this study, *E. hirsutum* can accumulate Zn in the whole plant under hydroponic conditions. It was determined that there was a statistically significant relation between the amount of Zn accumulated by seedlings and Zn concentrations in solutions ($F = 11\,660.69$; $p<0.001$). The seedlings kept in 30 and 40 mg Zn/L

solutions accumulated a high amount of Zn. At the same time, the lowest Zn accumulation was calculated in solutions containing 75 mg/L Zn (Fig. 2). When all these data were evaluated, it was determined that the highest Zn concentration that the seedlings showed well-grown and accumulated more Zn was at 30 mg Zn/L (14 894.90 ± 17.11 mg Zn/kg DW) (Fig. 2). When the Zn accumulations in three organs were compared to each other, it was determined that the roots (10 598 mg Zn/kg DW) had higher Zn accumulation than shoots (3 503 mg Zn/kg DW) and leaves (793.8 mg Zn/kg DW). Zn phytotoxicity in plants can differ according to the plant type, genotype, age, environment state, and the concentration of heavy metals and other ions (Tsonev and Lidon 2012). The results obtained from phytoremediation studies with different plants show that plants can accumulate Zn in their vegetative organs, including roots, shoots, or leaves (Hesami et al. 2018; Mahdavian et al. 2017; Mazumdar and Das 2015). Doğan (2011) reported that some aquatic plants, *Alisma plantago-aquatica*, *Sagittaria sagittifolia*, *Juncus effu*sus, *Lythrum salicaria,* and *Phalaris arundinacea* accumulated Pb, Cd, Zn, and Cu in their roots. Matthews et al. (2004) investigated the growth rate, Zn intake, and metal tolerance of *Eriophorum angustifolium*, *Juncus effusu*s, and *Juncus articulatus* plants. Maximum Zn accumulation was 53±28 µmol/g in the roots and 43±2 µmol/g Zn in the leaves of the *J. effesus* plant. The plant showed some toxicity symptoms at 40 mg/L and above Zn concentrations in this research, such as necrosis and red-brown spots in leaves. Young leaves show chlorosis, the first symptom of Zn toxicity in most species (Reichman 2002). There are many studies on Zn toxicity (Al Chami et al. 2015; Mirshekali et al. 2012; Rout and Das 2003). Thus, when the accumulation results of this study were evaluated, it was understood that *E. hirsutum* has a substantial phytoremediation property.

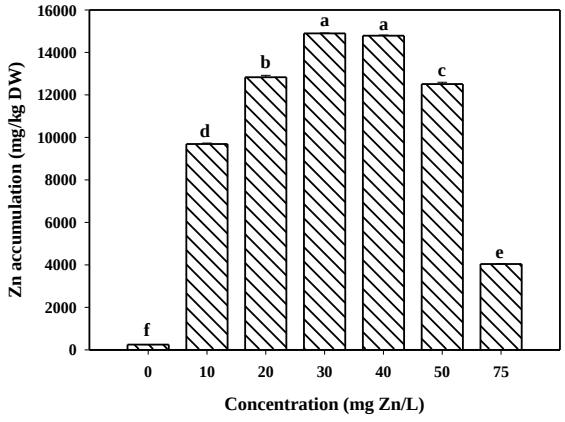

Fig. 2 Zn accumulation (mg/kg DW) of *E. hirsutum* at concentrations of 0, 10, 20, 30, 40, 50, and 75 mg/L Zn

In this research, chlorophyll a, b, the total chlorophyll, and carotenoid amounts in leaves were studied to put forward the plant's response against Zn heavy metal. Zn toxicity has caused reductions in chlorophyll synthesis because of the inhibition of pigment synthesis (Broadley et al. 2007). It was found that there was a statistically significant relation between the pigment contents of the seedlings and the increasing Zn concentrations (chlorophyll a F=3116.70; chlorophyll b F=536.77; total chlorophyll F=9204.30 and total carotenoid (F=105147.1). When photosynthetic pigments were compared in the *E. hirsutum* plant treated with Zn, it was determined that there was a significant decrease in pigment levels (Fig. 3). Consequently, total chlorophyll content decreased at excessive Zn concentrations (50 and 75 mg Zn/L). In plants, the functionality and efficiency of the photosynthetic system are hampered by Zn toxicity. Thus, the reason for reducing of chlorophyll at high Zn concentrations could be the inhibition of chlorophyll biosynthesis (Emamverdian et al. 2015; Shakya et al. 2008). Similarly, such decreases in the levels of photosynthetic pigments have been reported by other studies in many plants on exposure to heavy metals (Chandra and Kang 2016; Mirshekali et al. 2012; Shakya, Chettri, and Sawidis 2008; Vassilev et al. 2011).

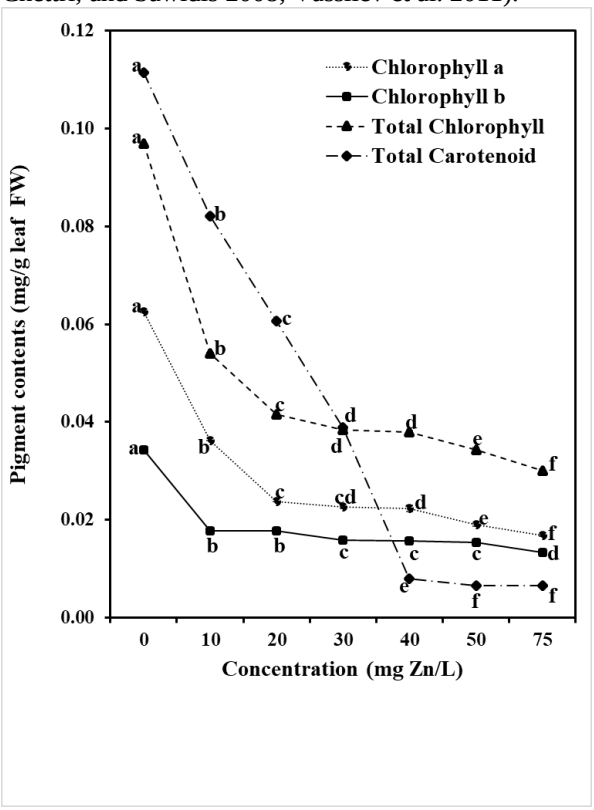

Fig. 3 Effects of increasing Zn concentration on pigment content of *E. hirsutum*

The results showed a significant difference at the probability level ($p < 0.001$) in leaf protein contents of *E. hirsutum*, which was exposed to different concentrations of Zn. The highest protein contents were found in solutions containing 10 and 20 mg Zn/L. Thus, this study calculated that plants grown at 10 mg/L Zn concentration had the highest protein content (4.39 mg/g leaf). A decrease in the amount of protein was detected at 40 mg Zn/L and above concentrations (Fig. 4). Zn is an essential component of particular proteins in all classes of enzymes, such as oxidoreductases, transferases, and hydrolases, and plays a

role as a protein cofactor (Chandra and Kang 2016; Emamverdian et al. 2015; Maret 2013; Vassilev et al. 2011). The decrease in the protein content of the plant exposed to high Zn concentrations may be due to changes in plant metabolism and plant response to heavy metal stress (Tsonev and Lidon 2012; Cakmak 2000). According to Jayasri and Suthindhiran (2017), high Zn concentration (20 mg Zn/L) negatively affected soluble proteins in *Lemna minor*. Plants respond to heavy metal stress by expressing genes that encode proteins (Chaudhary et al. 2019). Hence, from the above results, we can conclude that metal stress causes changes in plant protein content.

3.2. Tissue Culture Experiments

In our study, we determined the most suitable tissue culture conditions for using the *E. hirsutum* plant to detect metal toxicity and phytoremediation purposes in the future. The most crucial step of *in vitro* culture studies is selecting the appropriate tissue culture medium for the plant (Espinosa-Leal et al. 2018; Sarasan et al. 2006). This study obtained the highest (97 % ± 1.53) and fastest seed germination percentage in MS medium within 7.7 ± 0.26 days. Based on the results, the MS medium was selected as the optimum medium for the germination of *E. hirsutum.* In a study by Rogers (2003), *Typha angustifolia* seeds had a 60-95% germination rate when cultured in light in liquid MS or sterile water. According to Dreger et al. (2016), it was stated that MS medium is sufficient for the germination of *Epilobium angustifolium,* and they found that the highest germination was 99% within seven days. When the results are compared with the literature data, our study is similar to these studies.

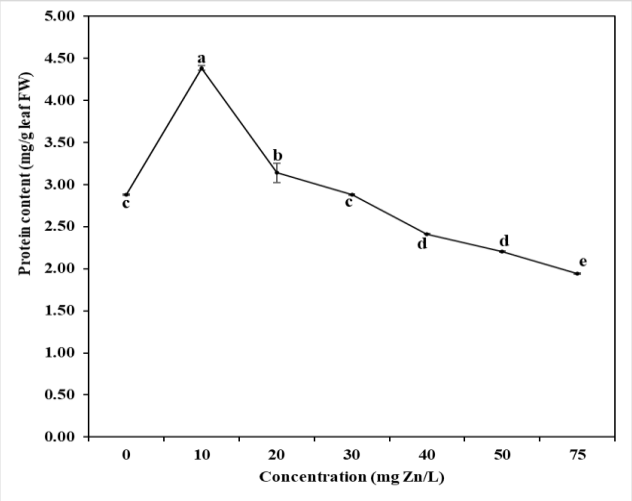

Fig. 4 Effects of different Zn concentrations on the leaf protein content of *E. hirsutum*

E. hirsutum seeds germinated in the MS medium did not occur adequate growth. Therefore, seedlings were first transferred to an MS medium containing different concentrations of GA. As shown in Table 1, when the growth parameters of the seedlings grown at different concentrations of GA (25, 50, and 75 mg GA/L) were evaluated, the number of shoots per explant (F=4.57; p=0.0168), shoot length (F=53.33; p<0.001), number of roots per shoot (F=24.87; p<0.0001), root length (F=74.76; p<0.0001), and the number of leaves per explant (F=29.58; p<0.0001, respectively) were increased by 50 mg GA/L. However, 75 mg GA/L treatment showed an inhibitory effect on root formation (Table 1). Endogenous hormones such as gibberellin are essential in seed germination (Hilhorst and Karssen 1992; Vishal and Kumar 2018). Furthermore, it has been found that exogenous GA treatment was an effective method to break seed dormancy and promote seed germination of *Pinus massoniana* (Guangwu and Xuwen 2014). Explant development depends on plant growth regulators and tissue culture medium content. Therefore, optimization of the concentration of growth regulators is crucial for promoting *in vitro* shoot cultures (Nasution and Nasution 2019; Khan et al. 2015).

The shoot tips grown in the MS medium containing 50 mg GA/L, where *E. hirsutum* shows the best growth, were transferred to the MS medium containing different plant growth regulator concentrations, as shown in Figure 5. The shoot tip explants taken into plant tissue culture exhibited different shoot growth behaviors depending on the concentration of plant growth regulator and type. Some researchers reported that BAP had superior effects over other cytokinins (Anish et al. 2008; Gümüşçü et al. 2008). Among the plant growth regulators containing different concentrations of BAP-NAA and BAP-IBA used in this study, 1.0 BAP/1.0 NAA has shown the best response to initiate multiple shoot formation. There were no statistical differences among all types of the medium; the highest number of shoots per explant was obtained in 1.0 BAP/1.0 NAA (3.96 ± 0.60, F=1.14; p=0.37). When shoot tip explants are cultured in an MS medium containing the combination of BAP and NAA or IBA, it has been found that shoot formation is interrelated with auxin concentration. An increase in the auxin concentration resulted in a significant increase in the number of shoots per explant.

However, the results showed that the highest shoot length was obtained on medium with 1.0 BAP/0.5 NAA (1.46 cm ± 0.08, F=2.30; p=0.005). The most extended length was also determined on medium with 1.0 BAP/1.0 IBA (0.28 cm ± 0.05, F=8.29; p=0.0001). According to the results in Figure 5, the combination of cytokinin with auxins in the MS medium significantly impacted the shoot multiplication of *E. hirsutum*. It has been determined that the most effective treatments for the shoot development of *E. hirsutum* are 1.0 BAP/1.0 NAA and 1.0 BAP/1.0 IBA treatments. Although no studies are detailed on the *in vitro* propagation of *E. hirsutum*, some researchers mentioned the regeneration procedure of *E. hirsutum*, *E. angustifolium*, and *E. parviflorum* (Akin et al. 2022; Tămaş et al. 2009; Turker et al. 2008; Akbudak and Babaoglu 2005; Rogers 2003). Thus, Tămaş et al. (2009) demonstrated that shoot cultivation of *E. hirsutum* on an MS medium supplemented with antioxidative agents and polyvinyl pyrrolidone (adsorbent agent) resulted in biosynthesis of higher concentrations of polyphenolic compounds than *E. hirsutum* plants collected from nature. Badkhane et al. (2016) tested the regeneration capacity of *in vitro* explants

of *Glycyrrhiza glabra* by adding different concentrations of BA, Kinetin (Ki), NAA, and IBA growth regulators to an MS nutrient medium. Besides, they obtained the best shoot regeneration percentage and shoot formation rate in a medium containing 2 BAP/0.5 NAA when they used the node and internode explants. As a result of our study, growth regulators used in plant culture medium, and their concentrations affected the organogenesis of the plant.

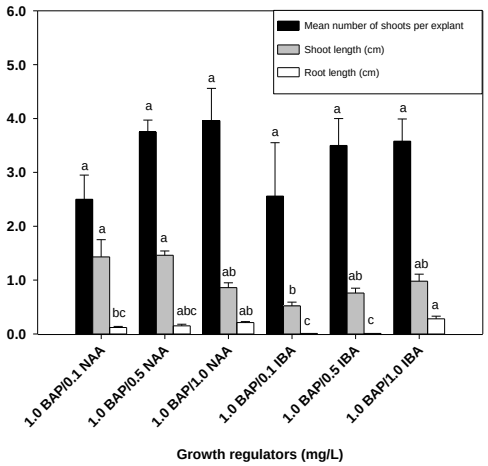

Fig. 5 Effects of different concentrations of BAP, IBA, and NAA on shoot formation of *E. hirsutum*

The more significant influence of BAP on proliferation and multiplication was also stated by several researchers (Akın et al. 2018; Akin et al. 2014; Arab et al. 2014; Waoo et al. 2013). In our study, mediums containing 1.0 BAP/1.0 NAA and 1.0 BAP/1.0 IBA induced the highest number of green and healthy adventitious shoots. Auxins are critical plant growth regulators that are involved in the process of adventitious root development. Thus, adventitious rooting is a crucial and essential step for the vegetative propagation of plants (Sauer et al. 2013). *In vitro*, regenerated shoots of *E. hirsutum* showed different behaviors during the rooting processes. This study transferred regenerated shoots in 1.0 BAP/1.0 NAA and 1.0 BAP/1.0 IBA to varying concentrations of root mediums (0.5 NAA, 1.0 NAA, 0.5 IBA, and 1.0 IBA) to test the rooting potential. The root parameters were changed significantly with different IBA concentrations. The best rooting was obtained in the MS medium containing 1.0 mg IBA/L. However, when we compared the effects of shooting mediums (1.0 BAP/1.0 NAA or 1.0 BAP/1.0 IBA) on the rooting of seedlings, 1.0 BAP/1.0 NAA medium (32.9 ± 6.71 number of root/shoots; F=6.65, p=0.002) proved to be superior to 1.0 BAP/1.0 IBA (25.0 ± 3.42 number of root/shoots; F=0.13, p=0.94, Table 2).

Table 1 Effect of different concentrations of GA on the shoot, root formation, and leaf number of *E. hirsutum*. According to the Tukey HSD test, mean values with the same letter are not statistically different ($p \leq 0.01$)

GA concentrations (mg/L)	Number of shoots/explant	Shoot length (cm)	Number of roots/shoot	Root length (cm)	Number of leaves/explant
25	1.0 ± 0.00^{b}*	2.1 ± 0.23^{b}	1.2 ± 0.32^{b}	0.3 ± 0.08^{b}	8.5 ± 0.36^{b}
50	1.6 ± 0.29^{a}	5.0 ± 0.29^{a}	4.0 ± 0.64^{a}	1.2 ± 0.10^{a}	10.9 ± 0.50^{a}
75	1.0 ± 0.00^{b}	2.3 ± 0.12^{b}	0.0 ± 0.00^{c}	0.0 ± 0.00^{b}	6.7 ± 0.27^{c}

Thus, 100% rooting was achieved in all root MS mediums (Table 2). *In vitro*, shoot formation may have resulted in different success levels depending upon the plant growth regulators used in the MS medium. Akın and Kocaçalışkan (2011) stated that the endemic plant *Arabis drabiformis* exhibited the best rooting at 0.5 mg IBA/L concentration. For decades, IBA was described as a 'synthetic auxin' most commonly used that induced root initiation and was preferred based on the results of prior studies (Akin et al. 2014; Akin and Kocaçalişkan 2011; Akın et al. 2018; Frick and Strader 2018; Kurt and Erdağ 2009; Prasad et al. 2004).

4. Conclusion

Even though *E. hirsutum*, a medicinal wetland plant, can accumulate heavy metals in its organs; there are few studies on the plant's metal accumulation ability and micropropagation. This study concluded that *E. hirsutum* tolerated Zn up to 75 mg/L and accumulated more Zn at a concentration of 30 mg/L without showing any Zn toxicity symptoms. Medicinal plants have been used as sources of pharmaceutical products for many years (Akın 2020). However, tissue culture methods reported herein are powerful for selecting, multiplying, and conserving crucial medicinal plants. The possibilities of plant regeneration through tissue culture technology were investigated in this study. As a result, the *in vitro* propagation protocol (for shoot formation 1.0 BAP/1.0 NAA and root formation 1.0 IBA) and *E. hirsutum* plantlets were developed in this study. The regeneration system described here can be used in future phytoremediation studies.

Authors' contributions: NB: obtaining data, editing, and writing; BA: obtaining data, experimental measurements, editing, and writing; NE: obtaining data, and experimental measurements.

Conflict of interest disclosure:

The authors declare that they have no conflicts of interest about the realization of this research.

Table 2 Effect of different culture media (1.0 BAP/1.0 NAA and 1.0 BAP/1.0 IBA) and growth regulators (IBA and NAA) on rooting *in vitro* regenerated shoots after four weeks of rooting treatments. According to the Tukey HSD test, mean values with the same letter are not statistically different ($p \leq 0.01$)

Medium (mg/L)	Growth regulators (mg/L)	Number of roots/shoots	Root length (cm)	Rooting (%)
1 BAP/ 1 NAA	0.5 NAA	$8.17 \pm 1.19^{b*}$	0.54 ± 0.11^{b}	100.00
	1.0 NAA	24.7 ± 2.31^{a}	1.57 ± 0.24^{a}	100.00
	0.5 IBA	27.5 ± 2.50^{a}	1.22 ± 0.21^{ab}	100.00
	1.0 IBA	32.9 ± 6.71^{a}	1.13 ± 0.10^{ab}	100.00
Medium (mg/L)	**Growth regulators (mg/L)**	**Number of roots/shoots**	**Root length (cm)**	**Rooting (%)**
1 BAP/1 IBA	0.5 NAA	21.0 ± 5.67^{a}	0.83 ± 0.17^{a}	100.00
	1.0 NAA	23.8 ± 8.97^{a}	1.10 ± 0.28^{a}	100.00
	0.5 IBA	21.7 ± 1.67^{a}	0.88 ± 0.21^{a}	100.00
	1.0 IBA	25.0 ± 3.42^{a}	1.50 ± 0.25^{a}	100.00

References

Ackova DG. 2018. Heavy metals and their general toxicity on plants. Plant Sci. Today 5(1):14-18. doi.org/10.14719/pst.2018.5.1.355

Adki VS, Jadhav JP, Bapat VA. (2014). At the cross roads of environmental pollutants and phytoremediation: A promising bio remedial approach. J Plant Biochem Biotechnol. 23(2):125-140. doi.org/10.1007/s13562-013-0250-6

Akbudak MA, Babaoglu M. 2005. Callus induction in small flowered willow herb (*Epilobium parviflorum* Schreb). J Plant Biochem Biotechnol. 14(2):189-191. doi.org/10.1007/BF03355957

Akın B. 2020. Tissue culture techniques of medicinal and aromatic plants: History, cultivation and micropropagation. JSR-A 45:253-266

Akin B, Kocaçalişkan I. 2011. In vitro propagation of *Arabis drabiformis* Boiss. (Brassiaceae) an endemic rare species of Uludağ mountain (Bursa-Turkey). AJB 10(80):18356-1836. doi.org/10.5897/AJB11.2831

Akin B, Bingol NA, Kocacaliskan I. 2022. Zinc stress alleviation by salicylic acid in hairy willowherb (*Epilobium hirsutum* L.) under i*n vitro* conditions. Fresenius Environ Bull. 31(03A):3735-3745

Akın B, Çetin B, Akanıl Bingöl N. 2018. In Vitro Propagation of Wetland Medicinal Plant *Lythrum salicaria* L. CBU Journal of Science 14(4):369-372. doi.org/10.18466/cbayarfbe.393626

Akin B, Kocaçalişkan I, Güleryüz G. 2014. Micropropagation of *Erodium sibthorpianum* subsp. *sibthorpianum*, an endemic threatened species of Uludağ mountain (Bursa-Turkey). Turk J Botany 38(1):148-155. doi.org/10.3906/bot-1304-24

Akpor OB, Ohiobor GO, Olaolu TD. 2014. Heavy metal pollutants in wastewater effluents: Sources, effects, and remediation. Adv Biosci Bioeng. 2(4):37-43. doi.org/10.11648/j.abb.20140204.11

Al Chami Z, Amer N, Al Bitar L, Cavoski I. 2015. Potential use of *Sorghum bicolor* and *Carthamus tinctorius* in phytoremediation of nickel, lead and zinc. Int J Environ Sci Technol. 2(12):3957-3970. doi.org/10.1007/s13762-015-0823-0

Ali H, Khan E. 2018. What are heavy metals? Long-standing controversy over the scientific use of the term 'heavy metals'–proposal of a comprehensive definition. Toxicol Environ Chem. 100(1):6-19. doi.org/10.1080/02772248.2017.1413652

Anish NP, Dan M, Bejoy M. 2008. Conservation using in vitro progenies of the threatened ginger-Boesenbergia pulcherrima (Wall.) Kuntze. Int J Botany 4(1):93-98. doi.org/10.3923/ijb.2008.93.98

Arab MM, Yadollahi A, Shojaeiyan A, Shokri S, Ghojah SM. 2014. Effects of nutrient media, different cytokinin types and their concentrations on in vitro multiplication of G × N15 (hybrid of almond × peach) vegetative rootstock. JGEB. 12(2):81-87. doi.org/10.1016/j.jgeb.2014.10.001.

Arán DS, Harguinteguy CA, Fernandez-Cirelli A, Pignata, ML. 2017. Phytoextraction of Pb, Cr, Ni, and Zn using the aquatic plant *Limnobium laevigatum* and its potential use in the treatment of wastewater. Environ Sci Pollut Res . 24(22):8295-18308. doi.org/10.1007/s11356-017-9464-9

Arnon DI. 1949. Copper enzymes in isolated chloroplasts, polyphenoloxidase in *Beta vulgaris*. Plant Physiol. 24(1):1-15. doi.org/10.1104/pp.24.1.1

Azubuike CC, Chikere CB, Okpokwasili GC. 2016. Bioremediation techniques–classification based on site of application: principles, advantages, limitations and prospects. World J Microbiol Biotechnol. 32(11):1-18. doi.org/10.1007/s11274-016-2137-x

Badkhane Govt Motilal Vigyan Mahavidyalayan Y, Bajaj Govt Motilal Vigyan Mahavidyalaya A. 2016. Effect of explant sources and different concentrations of plant growth regulators on in vitro micropropagation of *Glycyrrhiza glabra* L. Indo Am J Pharm Res. 6:5830-5840

Balafrej H, Bogusz D, Abidine Triqui Z el, Guedira A, Bendaou N, Smouni A, Fahr M. 2020. Zinc hyperaccumulation in plants: A review. Plants 9(5):562. doi.org/10.3390/plants9050562

Bingöl NA, Akin B, Kocaçalişkan İ, Nalbantoğlu B, Meşeli O. 2021. Effect of zinc on phytoremediation potential and carbonic anhydrase and polyphenol oxidase activities of *Lythrum salicaria* L. Turk J Botany 45(6):553-562. doi.org/10.3906/bot-2107-52

Bolat İ, Kara Ö. 2017. Bitki besin elementleri: Kaynakları, işlevleri, eksik ve fazlalıkları. BAROFD 19(1):218-228 (In Turkish).

Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem. 72:248-254. doi.org/10.1016/0003-2697(76)90527-3

Broadley MR, White PJ, Hammond JP, Zelko I, Lux A. 2007. Zinc in plants: Tansley review. New Phytol. 173(4):677–702. doi.org/10.1111/j.1469-8137.2007.01996.x

Broadley M, Brown P, Cakmak I, Rengel Z, Zhao F. 2012. Function of nutrients: micronutrients. In: Marschner P (ed) Marschner's mineral nutrition of higher plants, 3rd edn. Academic Press, London.

Cakmak I. 2000. Tansley review no. 111: Possible roles of zinc in protecting plant cells from damage by reactive oxygen species. New Phytologist 146(2):185-205. doi.org/10.1046/j.1469-8137.2000.00630.x

Chandra R, Kang H. 2016. Mixed heavy metal stress on photosynthesis, transpiration rate, and chlorophyll content in poplar hybrids. Forest Sci Technol. 12(2):55-61. doi.org/10.1080/21580103.2015.1044024

Chaudhary M, Varshney R, Shahnawaz M, Pandey RP. 2019. Various responses of plant proteins to heavy metal stress tolerance. Suresh Gyan Vihar University International Journal of Environment, Science and Technology 5(1):19-29

Davis PH. 1965. Flora of Turkey and the East Aegean Islands, Vol. 1-9. University Press, Edinburgh.

Edinburgh Univ. Press., Edinburgh.

Doğan M. 2011. Akuatik makrofitlerde ağır metal akümülasyonu. Türk Bilimsel Derlemeler Dergisi 4:33-36 (In Turkish)

Doran PM. 2009. Application of plant tissue cultures in phytoremediation research: Incentives and limitations. Biotechnol Bioeng. 103(1):60-76. doi.org/10.1002/bit.22280

Dreger M, Wegenke J, Makowiecka J, Michalik T, Wielgus K. 2016. Application of multi-shoots cultures in micropropagation of willow herb (*Chamaenerion angustifolium* (L.) Scop.). Herba Polonica. 62(3):28-39. doi.org/10.1515/hepo-2016-0015

Ehsan M, Lara Viveros FM, Hernández VE, Barakat MA, Ortega AR, Maza Av, Monter Jv. 2015. Zinc and cadmium accumulation by Lupinus uncinatus Schldl. grown in nutrient solution. Int J Environ Sci Technol. 12(1):307-316. doi.org/10.1007/s13762-013-0456-0

Eid EM, Shaltout KH, Almuqrin AH, Aloraini DA, Khedher KM, Taher MA, Alfarhan AH, Picó Y, Barcelo D. 2021. Uptake prediction of nine heavy metals by Eichhornia crassipes grown in irrigation canals: A biomonitoring approach. Sci. Total Environ. 782:146887. doi.org/10.1016/j.scitotenv.2021.146887

Emamverdian A, Ding Y, Mokhberdoran F, Xie Y. 2015. Heavy metal stress and some mechanisms of plant defense response. Sci. World J. Article ID 756120:1-18. doi.org/10.1155/2015/756120

Espinosa-Leal CA, Puente-Garza CA, García-Lara S. 2018. In vitro plant tissue culture: means for production of biological active compounds. Planta. 248(1):1-18. doi.org/10.1007/s00425-018-2910-1

Frick EM, Strader LC. 2018. Roles for IBA-derived auxin in plant development. J Exp Bot. 69(2):169-177. doi.org/10.1093/jxb/erx298

Fu F, Wang Q. 2011. Removal of heavy metal ions from wastewaters: A review. J Environ Manage. 92(3):407-418. doi.org/10.1016/j.jenvman.2010.11.011

García-Gonzáles R, Quiroz K, Carrasco B, Caligari P. 2010. Plant tissue culture: Current status, opportunities and challenges. Cienc Investig Agrar. 37(3):5-30. doi.org/10.4067/s0718-16202010000300001

Ghaderian SM, Ghotbi Ravandi AA. 2012. Accumulation of copper and other heavy metals by plants growing on Sarcheshmeh copper mining area, Iran. J Geochem Explor. 123:25-32. doi.org/10.1016/j.gexplo.2012.06.022

Granica S, Piwowarski JP, Czerwińska ME, Kiss AK. 2014. Phytochemistry, pharmacology and traditional uses of different Epilobium species (Onagraceae): A review. J Ethnopharmacol. 156:316-346. doi.org/10.1016/j.jep.2014.08.036

Guangwu Z, Xuwen J. 2014. Roles of gibberellin and Auxin in promoting seed germination and seedling Vigor in *Pinus massoniana*. For Sci. 60(2):367-373. doi.org/10.5849/forsci.12-143

Guittonny-Philippe A, Masotti V, Rabier J, Petit ME, Malleret L, Coulomb B, Laffont-Schwob I. 2015. Biomonitoring of epilobium hirsutum L. health status to assess water ecotoxicity in constructed wetlands treating mixtures of contaminants. Water (Switzerland) 7(2):697-715. doi.org/10.3390/w7020697

Gümüşçü A, Çöçü S, Uranbey S, Ipek A, Çalişkan M, Arslan N. 2008. In vitro micro-propagation of endangered ornamental plant-*Neotchihatchewia isatidea* (Boiss.) Rauschert. Afr J Biotechnol. 7(3):234-238

Hegazy AK, Abdel-Ghani NT, El-Chaghaby GA. 2011. Phytoremediation of industrial wastewater potentiality by Typha domingensis., Int J Environ Sci Technol. 8(3):1019-1027. doi.org/10.1007/BF03326249

Hesami R, Salimi A, Ghaderian SM. 2018. Lead, zinc, and cadmium uptake, accumulation, and phytoremediation by plants growing around Tang-e Douzan lead–zinc mine, Iran. Environ Sci Pollut Res. 25(9):8701-8714. doi.org/10.1007/s11356-017-1156-y

Hilhorst HWM, Karssen CM. 1992. Seed dormancy and germination: the role of abscisic acid and gibberellins and the importance of hormone mutants. Plant Growth Regul. 11(3):225-238. doi.org/10.1007/BF00024561

Jayasri MA, Suthindhiran K. 2017. Effect of zinc and lead on the physiological and biochemical properties of aquatic plant Lemna minor: its potential role in phytoremediation. Appl Water Sci. 7(3):247-1253. doi.org/10.1007/s13201-015-0376-x

JMP. 2005. JMP SAS statistical analysis system. Cary, North Carolina, USA.

Kaçar B, İnal A. 2008. Bitki analizleri. Nobel Yayın Dağıtım Ltd Şti, Ankara.

Khan N, Ahmed M, Hafiz I, Abbasi N, Ejaz S, Anjum M. 2015. Optimizing the concentrations of plant growth regulators for in vitro shoot cultures, callus induction and shoot regeneration from calluses of grapes. Int Sci Vigne Vin. 49(1):37-45. doi.org/10.20870/oeno-one.2015.49.1.95

Kocaçalışkan İ, Bingöl AN. 2017. Biyoistatistik. Nobel Akademik Yayıncılık Eğitim danışmanlık Tic Ltd Şti, Ankara.

Kurt S, Erdağ B. 2009. In vitro germination and axillary shoot propagation of *Centaurea zeybekii*. Biol. 64(1):97-101. doi.org/10.2478/s11756-009-0003-0

Mahdavian K, Ghaderian SM, Torkzadeh-Mahani M. 2017. Accumulation and phytoremediation of Pb, Zn, and Ag by plants growing on Koshk lead–zinc mining area, Iran. J Soils Sediments 17(5):1310-1320. doi.org/10.1007/s11368-015-1260-x

Malik N, Chamon A, Mondol M, Elahi S, Faiz S. 1970. Effects of different levels of zinc on growth and yield of red amaranth (*Amaranthus* sp.) and rice (*Oryza sativa*, Variety-BR49). J Bangladesh Assoc Young Res. 1(1):79-91. doi.org/10.3329/jbayr.v1i1.6836.

Maret W. 2013. Zinc biochemistry: From a single zinc enzyme to a key element of life. Adv Nutr. 4(1):2-91. doi.org/10.3945/an.112.003038.

Matthews DJ, Moran BM, Otte ML. 2004. Zinc tolerance, uptake, and accumulation in the wetland plants *Eriophorum angustifolium*, *Juncus effusus*, and *Juncus articulatus*. Wetlands 24(4):859-869

Mazumdar K, Das S. 2015. Phytoremediation of Pb, Zn, Fe, and Mg with 25 wetland plant species from a paper mill contaminated site in North East India. Environ Sci Pollut Res. 22(1): 701-710. doi.org/10.1007/s11356-014-3377-7

Mirshekali H, Hadi H, Amirnia R, Khodaverdiloo H, Ali H M, Hadi H, Amirnia R, Verdiloo HK. 2012. Effect of zinc toxicity on plant productivity, chlorophyll and Zn contents of sorghum (*Sorghum bicolor*) and common lambsquarter (*Chenopodium album*). IJRR. 2(3):247-254

Murashige T, Skoog F. 1962. A Revised Medium for Rapid Growth and Bio Assays with Tobacco Tissue Cultures. Physiol Plant. 15(3):473-497. doi.org/10.1111/j.1399-3054.1962.tb08052.x

Noulas C, Tziouvalekas M, Karyotis T. 2018. Zinc in Soils, Water and Food crops. J Trace Elem Med Biol. 49:252–260. doi.org/10.1016/j.jtemb.2018.02.009

Nardis BO, Silva EB, Grazziotti PH, Alleoni LRF, Melo L CA, Farnezi MMM 2018. Availability and zinc accumulation in forage grasses grown in contaminated soil. Int J Phytoremediation. 20(3):205-213. doi.org/10.1080/15226514.2017.1365347

Nasution NH, Nasution IW. 2019. The Effect of Plant Growth Regulators on Callus Induction of Mangosteen (Garcinia mangostana L.). IOP Conf Ser Earth Environ Sci. 305(1):012049. doi.org/10.1088/1755-1315/305/1/012049

Phillips DP, Human LRD, Adams JB. 2015. Wetland plants as indicators of heavy metal contamination. Mar Pollut Bull. 92, 227-232. doi.org/10.1016/j.marpolbul.2014.12.038

Prasad PJN, Chakradhar T, Pullaiah T. 2004. Micropropagation of *Cryptolepis buchanani* Roem. & Schult. Taiwania. 49:57-65. doi.org/10.6165/tai.2004.49(1).57

Reichman SM. 2002. The Responses of Plants to Metal Toxicity: A review focusing on Copper, Manganese and Zinc. Australian Minerals and Energy Environment Foundation, Melbourne, Australia.

Rodrigues ACD, Rocha MV de C, Lima ESA, Pinho CF de, Santos AM dos, Santos FS dos, Amaral Sobrinho NM B do. 2020. Potential of water lettuce (Pistia stratiotes L.) for phytoremediation: physiological responses and kinetics of zinc uptake. Int J Phytoremediation. 22(10):1019-1027. doi.org/10.1080/15226514.2020.1725868

Rogers SMD. 2003. Tissue culture and wetland establishment of the freshwater monocots Carex, Juncus, Scirpus, and Typha. In Vitro Cell Dev Biol Plant. 39(1):1-15. doi.org/10.1079/IVP2002358

Rout GR, Das P. 2003. Effect of metal toxicity on plant growth and metabolism: I. Zinc. Agronomie. 23(1):3-11. doi.org/10.1051/agro:2002073

Sabreena Hassan S, Bhat SA, Kumar V, Ganai BA, Ameen F. 2022. Phytoremediation of heavy metals: An indispensable contrivance in green remediation technology. Plants. 11(9):1255. doi.org/10.3390/plants11091255.

Sarasan V, Cripps R, Ramsay MM, Atherton C, McMichen M, Prendergast G, Rowntree JK. 2006. Conservation in vitro of threatened plants - Progress in the past decade. In Vitro Cell Dev Biol Plant. 42(3):206-214. doi.org/10.1079/IVP2006769

Sauer M, Robert S, Kleine-Vehn J. 2013. Auxin: Simply complicated. J Exp Bot. 64(9):2565-2577. doi.org/10.1093/jxb/ert139.

Schück M, Greger M. 2020. Screening the capacity of 34 wetland plant species to remove heavy metals from water. Int J Environ Res Public Health. 17(13):4623. doi.org/10.3390/ijerph17134623.

Shakya K, Chettri MK, Sawidis T. 2008. Impact of heavy metals (copper, zinc, and lead) on the chlorophyll content of some mosses. Arch Environ Contam Toxicol. 54(3):412-421. doi.org/10.1007/s00244-007-9060-y

Sharma A, Patni B, Shankhdhar D, Shankhdhar SC. 2013. Zinc - An Indispensable Micronutrient. Physiol Mol Biol Plants. 19(1):11-20. doi.org/10.1007/s12298-012-0139-1

Tămaş M, Toiu I, Oniga I, Deliu C, Oltean B, Coldea G. 2009. Quantitative determination of total polyphenols and flavonoids from indigenous species of Epilobium of wild origin and 'in vitro' regenerated plantlets. Contrib Bot. 44:119-123

Tiwari AK, de Maio M, Singh PK, Mahato MK. 2015. Evaluation of surface water quality by using GIS and a heavy metal pollution index (HPI) model in a coal mining area, India. Bull Environ Contam Toxicol. 95(3):304–310. doi.org/10.1007/s00128-015-1558-9

Tsonev T, Lidon FJC. 2012. Zinc in plants - An overview. Emir J Food Agric. 24(4):322-333

Turker AU, Mutlu EC, Yidirim AB. 2008. Efficient in vitro regeneration of fireweed, a medicinal plant. Acta Physiol Plant. 30(4):421-426. doi.org/10.1007/s11738-008-0136-8.

Vassilev A, Nikolova A, Koleva L, Lidon F. 2011. Effects of Excess Zn on Growth and Photosynthetic Performance of Young Bean Plants. J Phytol. 3(6):58-62

Vishal B, Kumar PP. 2018. Regulation of seed germination and abiotic stresses by gibberellins and abscisic acid. Front Plant Sci. 9:1-15. doi.org/10.3389/fpls.2018.00838

Waoo AA, Khare S, Ganguli S. 2013. In-vitro Propagation of *Datura innoxia* from nodal and shoot tip explants. World J Environ Eng. 1(1):1-4. doi.org/10.12691/wjee-1-1-1