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Original Research

In vitro Germination and Phytoremediation Potential of Endemic Plant Species *Verbascum phrygium* Bornm. Growing under Zinc Stress

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Abstract

Plant tissue culture techniques are a valuable tool to obtain suitable plant for phytoremediation and degraded areas because of heavy metals. In this study, the effects of different agar concentrations and different pH levels on *in vitro* germination parameters studied. In addition, the effects of various concentrations of zinc on growth parameters (root-shoot length, leaf number and fresh weight) and zinc accumulation for *Verbascum phrygium* endemic plant species investigated. During the *in vitro* germination period, the best development occurred at 0.7% agar and pH 6.5. In this research, zinc treatment (0, 5, 10, 30, 50, 100 and 250 Zn) was applied for seven days. The mean relative root length, shoot length and leaf number of *V. phrygium* increased up to 30 mg/L zinc concentration. However fresh weight did not show any significant differences in 0 to 50 mg/L Zn, but they decreased significantly in 100 mg/L Zn. Thus, *V. phrygium* exhibited high tolerance to increased Zn concentrations. Phytoremediation results indicated that, maximum zinc accumulations were obtained in 10 and 30 mg/L Zn, respectively (18,788.82 mg/kg DW, 18,325.33 mg/kg DW). Although Zn content in the roots and leaves increased with increasing Zn concentration, Zn predominantly accumulated in *V. phrygium* roots. In conclusion, *V. phrygium* can be used in contaminated areas due to its capacity to accumulate zinc in its organs.

Keywords: hydroponic culture, heavy metals, pollution, *Verbascum phrygium*, zinc accumulation

Introduction

Heavy metal pollution caused by human facilities is a global problem in both terrestrial and aquatic environments systems [1-2]. The release of large amounts of hazardous waste, heavy metals and pollutants into the environment by mining, agriculture, textile, industrial and human activities causes pollution on the ecosystem [1, 3-4]. Phytoremediation is a new technology that uses plants to clean-up polluted environments [1, 5-6]. Phytoremediation is one of the effective and feasible methods used today to remove

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pollutants from contaminated soil and groundwater. This environmentally friendly technology is potentially cost effective. The use of phytoremediation techniques in the restoration of contaminated areas has recently become an alternative to traditional techniques. Some plants can survive areas, which contaminated with heavy metal. The selection of suitable plants for remediation is an important factor for solving the heavy metal pollution. These contaminated areas can be remediated by plants that accumulate heavy metal in their aerial parts [1, 3, 7-10].

Zinc is an essential plant nutrient, is the second most abundant metal in organisms, play an important role in several plant metabolic functions [11-14]. However, it has a strong toxic effect at high concentrations and negatively affects the development of some plant species [15, 16]. Native plant species that can easily adapt to local soil conditions and develop strategies for survival in a polluted environment, that is important components of the biological diversity, can be used to reduce heavy metal concentration [10, 17].

The genus Verbascum L., which belongs to the Scrophulariaceae family, is represented by about 2500 species in the world [18, 19]. *Verbascum phrygium* BORNM. (Scrophulariaceae) is endemic to Anatolia and only grows in the Kütahya, Konya, İzmir and Afyon provinces [20]. Verbascum species are grow in a wide variety of habitats, including rocky mountains, meadows, open forests, roadsides and river banks [21-23]. The potential of Verbascum species to accumulate many elements in its organs were revealed in earlier studies [24-28]. Thus, many endemic plants are at higher risk of extinction due to changing environmental conditions and human-induced habitat degradation [29-31]. Therefore, *in vitro* culture methods have an important role in the conservation of endemic and critically endangered plant species with small populations [30, 32-34]. There are no reports *in vitro* germination and phytoremediation potential of *V. phrygium* growing under zinc stress. In this study, we aimed to put forward *in vitro* germination and phytoremediation potential of *V. phrygium* different organs (roots, leaves) under zinc stress.

Material and Methods

Plant Material and *in vitro* Germination

Mature seeds of the *V. phrygium* Bornm. were collected from subalpine belt between 1800 and 2000 m of Muratdagı Mountain (Kutahya-Turkey) Kesiksögüt location during July 2018. The seeds were air dried in the dark at room temperature and sealed in sample bags. *V. phrygium* seeds were sterilized in 3% sodium hypochlorite (NaOCl) for 15 minutes with a few drops of surfactant Tween-20, then rinsed three times with ultra-pure water. The seeds were germinated in Murashige and Skoog [35] medium supplemented with 3% sucrose, in a growth cabinet at 25°C temperature and 16/8 hours photoperiod.

To examine the effect of different agar concentrations on seed germination, germination rate and germination time, four different agar concentration (0.6, 0.7, 0.75, 0.80%) was added to MS medium. The pH of the medium was adjusted between 5.7-7.0 (5.7, 5.8, 6.0, 6.5, 7.0) and MS medium was autoclaved at 121°C and 1.1 atm for 20 min. After the seeds were aseptically transferred into hormone-free MS medium, seeds were incubated at 25ºC for 30 days. Each trial consisted of three glass bottles containing ten seeds. Three replicates used for each treatment. For adaptation, germinated plantlets were transferred to 2.5 lt pots, containing 20% Hoagland (pH 6.5), and placed in hydroponic culture and in this medium seedlings were kept for fourteen days. Seedlings reaching a height of about 9 cm transferred for zinc accumulation.

Experimental Setup

For the phytoremediation treatment, firstly, 20% Hoagland solutions with 0, 5, 10, 30, 50, 100 and 250 mg of Zn/L were prepared using $ZnSO_4$ for seven days to determine the highest Zn concentration that plant accumulate. Secondly, the accumulation of Zn in root and leaves of *V. phrygium* determined at the end of seven days. At the end of these exposed periods, Zn accumulations in all groups were measured.

Growth Parameters

After the experiment, seedlings were harvested and the plant roots were rinsed in Na-EDTA (1%) and washed with ultra-pure water to remove heavy metal adhering to the root surfaces. Seven days after accumulation, root length, shoot length and fresh weights were recorded before and after seedlings were treated with zinc. Then the roots and leaves were harvested separately and dried 70ºC for 48 hours.

Measurement of Zinc Content

Zn content of plants was analyzed by Atomic Absorption Spectrometer (Analytikjena ContrAA 300) at Advanced Technologies Centre of Kutahya Dumlupinar University by using Flame Atomic Absorption Spectrometry. In the last step of experiment, dried plant samples were digested by wet digestion method on nitric acid and hydrogen peroxide [36].

Statistical Analysis

Data analyses were performed by JMP 6 SAS program. Collected data were subjected to F-test to determine the differences between the treatments at $p<0.05$ level. Multiple comparison test was used on applications that were statistically different according to the F-test [37].

Table 1. Effect of agar concentrations on germination percentage (%), germination rate and germination time of *V. phyrigium* seeds cultured for 30 days on MS basal medium.

Agar concentration %	Germination %	Germination rate	Germination time (day)
0.60	80.00±5.77 ^{bc*}	26.42±0.80 ^{a}	4.67±0.33 ^{ab}
0.70	100.00±0.00 ^{a}	27.04±0.42 ^{a}	4.00±0.00 ^{b}
0.75	86.67±3.33 ^{ab}	23.74±0.36 ^{b}	4.67±0.33 ^{ab}
0.80	63.33±3.33 ^{c}	21.22±0.65 ^{b}	5.67±0.33 ^{a}

* Means having the same letter in each column do not different significantly at $p< 0.05$ (Tukey's test).

Results and Discussion

In this study, 0.60%, 0.70%, 0.75% and 0.80% agar concentration were used to identify the effect of agar concentration on germination percent, germination rate and germination time (days) of *V. phrygium* seeds. Differences were observed between the agar concentrations tested both in terms of germination%, germination rate and germination time. In MS medium with 0.70 agar, *V. phrygium* seeds had higher germination percent (100.00±0.00), germination rate (27.04±0.42) and needed shorter period to germinate (4.00±0.00) than seeds germinated on medium with lowest (0.60) and highest (0.75, 0.80) agar concentrations. Therefore, healthy germinated plants were obtained at low agar concentration (Table 1, Fig. 1). Gürel and Gülsen [38] showed that 0.7% agar is suitable concentration for shoot-tip culture of the almond cultivars. According to Gopal et al. [39] high agar concentrations in medium cause decrease in water potential and water stress in the plant. In the study of Gao et al. [40], high levels of agar (0.7%) decreased the hyperhydricity rate of *Dendrobium officinale*, whereas low agar level (0.3%) increased the hyperhydricity rate.

The present study show that the pH of the culture media is significantly affect *in vitro* germination of *V. phrygium* seeds. According to our results, it is concluded that germination percent, the rate of germination and germination time of *V. phrygium* was highly influenced by the pH of culture media. Although there were no statistically differences from pH 5.8 to 7.0, the highest germination percentages (100%) were obtained at pH 6.5 and 7.0 (Table 2). However, the best germination rate (25.44±0.44) and the shortest germination time was achieved at pH 6.5 (5.00±0.00) (Table 2, Fig. 1). The similar studies about medium pH show that, generally a better plant development was found at pH 5.5 and 6.0 [41-43]. However, Shi et al. [44] has reported that the medium pH between 5.5 and 7.5 is suitable for apple tissue culture. Yaacob et al. [45] stated that shoot formation occurred faster (30 days) and shooting percentage also higher at pH 5.8, compared to pH 4.8 and 6.8 in *Citrus assamensis*. Thus, seeds of *V. phyrigium* tolerate a range of pH in tissue culture conditions (5.5-7.0). There are similar studies showing that Verbascum genus grow in different pH ranges [46, 47].

We investigated the effects of different Zn concentrations on growth parameters of plant, containing 5, 10, 30, 50, 100 and 250 mg Zn/L in solution (Fig. 2). However, plant exposure to higher concentrations of zinc (250 mg/L) caused the death of seedlings. The mean relative root length, shoot length

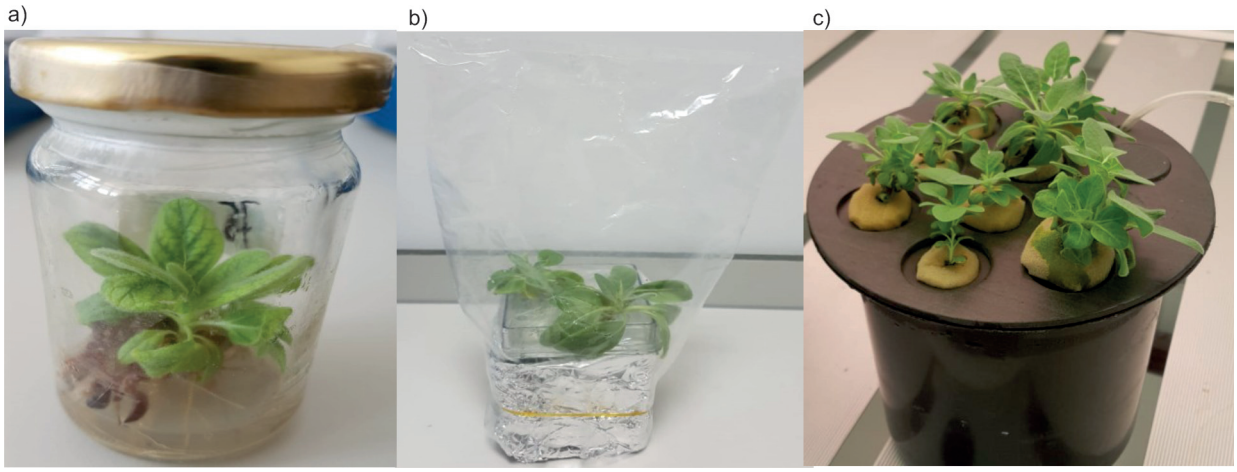

Fig. 1. Seed germination, adaptation and zinc accumulation of *V. phrygium* a) 30 days old seedling cultured on MS medium at pH 6.5 and 0.7% agar b) adaptation of germinated plantlets to hydroponic culture containing 20% Hoagland c) phytoremediation treatment.

Table 2. Effect of pH values on germination percentage (%), germination rate and germination day of *V. phyrigium* seeds cultured for 30 days on MS basal medium supplemented with 0.7% agar.

pH value	Germination %	Germination rate	Germination time (day)
5.5	80±0.00 ^{b*}	20.31±1.17 ^{c}	6.67±0.33 ^{a}
5.7	80±0.00 ^{b}	22.23±0.29 ^{bc}	6.33±0.33 ^{ab}
5.8	90±5.77 ^{ab}	23.24±0.51 ^{ab}	6.33±0.33 ^{ab}
6.0	90± 5.77 ^{ab}	23.89±0.23 ^{ab}	6.33±0.33 ^{ab}
6.5	100±0.00 ^{a}	25.44±0.44 ^{a}	5.00±0.00 ^{b}
7.0	100±0.00 ^{a}	24.82±0.54 ^{ab}	5.33±0.33 ^{ab}

* Means having the same letter in each column do not different significantly at $p< 0.05$ (Tukey's test).

and leaf number of *V. phrygium* increased, because of increasing zinc concentration up to 30 mg/L Zn (Fig. 2a, 2b, 2c). However, the root and shoots of *V. phrygium* continued to grow relatively even at the highest Zn (100 mg L^{-}) concentrations in the solution. The threshold of Zn toxicity varies depending on the increased zinc concentration. Despite the zinc concentration have no significant effect on fresh weight of plant excluding 100 mg/L Zn, the plants treated with 30 mg/L Zn showed a significant increase in root length, shoot height and leaf number, when compared to control (Fig. 2a, 2b, 2c, 2d). Zinc toxicity thresholds are determined depending on the stage of plant development and the plant part [48]. Zn is an essential element for plant and involved in several plant metabolic processes such as enzyme activation, protein synthesis, photosynthesis and carbohydrate metabolisms [15, 49-52]. Decrease in *V. phrygium* leaf number and fresh weight with increasing zinc concentration; it can be caused by decreased photosynthesis rate and low protein synthesis. Similarly to our results, the decrease in leaf area, the fresh and dry weight of the shoot has been shown previously, in Zn exposed *Brassica napus* and *B. juncea* [53]. As Tsonev and Lidon [52] stated in their reviews, *Artemisia annua* and sugarcane plants root and shoot growth were negatively affected by the

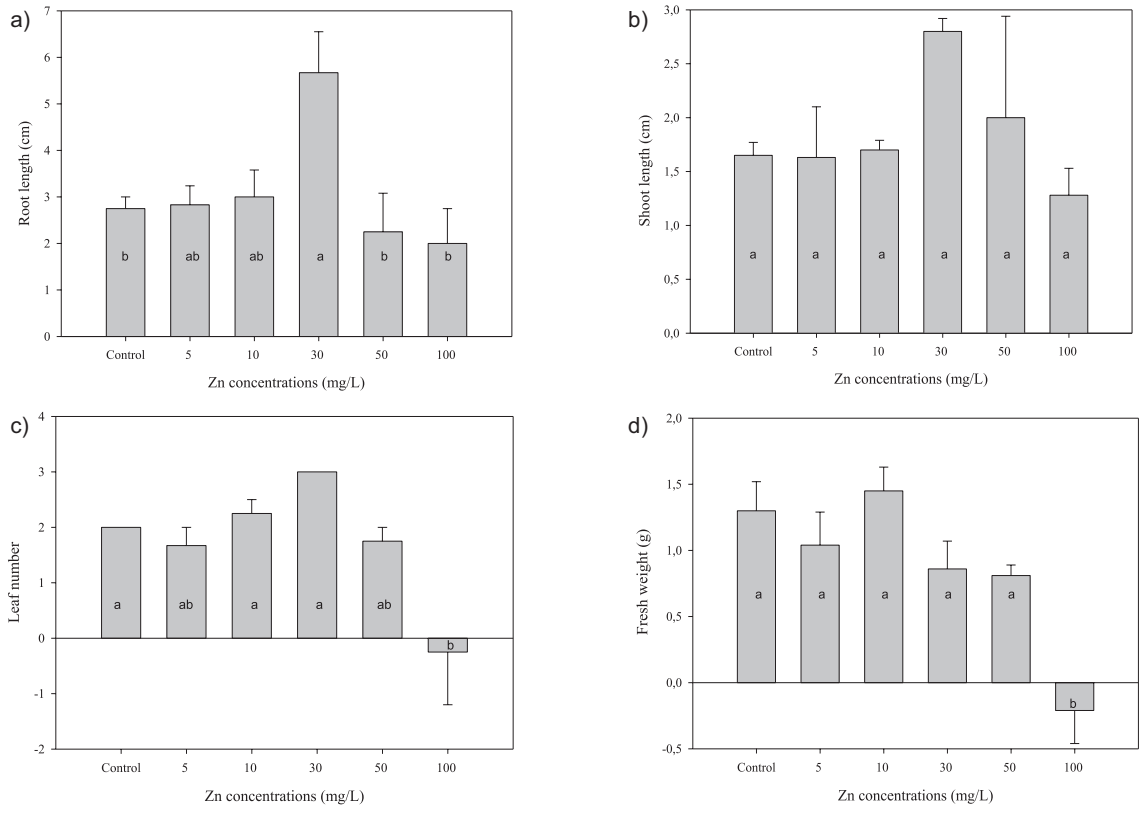

Fig. 2. The effects of zinc concentrations on growth parameters of *V. phrygium* a) Root length (cm) b) Shoot length (cm) c) Leaf number d) Fresh weight (g).

increase in zinc concentrations. Long et al. [54] reported that shoot growth was significantly inhibited and shoot fresh weight decreased at Zn concentrations above 200 mg L^- for Chinese cabbage, pakchoi and celery. However, exposure of plant with zinc heavy metal in low concentration (10 mg/l) promotes *Lemna minor* growth, but it cause toxic effect at higher concentration (20 mg/l) [55].

When we evaluate the effects of different Zn concentrations on Zn accumulation in plant, maximum zinc accumulations were obtained in 10 and 30 mg/L Zn, respectively (18,788.82±217.14 mg/kg DW, 18,325.33±368.50 mg/kg DW) (Fig. 3a.). Thus, Zn accumulation in the whole plant increased with increasing zinc concentrations up to 30 mg Zn/L. In the present study, it was also determined that *V. phrygium* accumulate zinc 70 times greater than the control. Zinc is not only an essential element, it is also a heavy metal. Besides at higher concentrations, it is become toxic [56-59]. However, in our study, it has been determined that our plant continues its development in high zinc concentrations up to 50 mg Zn/L. Morina et al. [60] reported that, the leaves of *Verbascum thapsus* which treated with different concentrations of zinc, accumulated zinc 60 times higher at 10 mM compared to control. Güleryüz et al. [22] stated that *V. olympicum* plant could be a bio-indicator for Cu, Fe, Mn, Ni, Pb, Zn and these metals could be a useful tool for monitoring changes in the environment. According to Sagiroglu et al. [61], when the metal content of *Verbascum cheiranthifolium* root and leaf samples were evaluated, it reported that *V. cheiranthifolium* accumulates a high rate of Cu, Pb, Zn and Cd and therefore this species is a hyperaccumulator. In a study conducted by Nouri [62] et al. to determine the ability of different wild plant species that grow in heavy metal contaminated areas to accumulate heavy metals, it has been shown that *Verbascum speciosum* species can accumulate an average of 15,343 µg/g Fe in the above-ground tissues and 9,226.3 µg/g Fe in the underground tissues. Some plant species have metal concentrations higher than toxic, indicating that these plant species may have metal detoxification tolerance mechanisms [63].

Zinc accumulations in the roots and leaves of *V. phrygium* were measured on 7th day of experiment (Fig. 3b). When the zinc accumulation in organs compared with each other, the zinc accumulation of roots was higher than leaves. The accumulation of these elements in plant organs varies depending on species, heavy metal levels and element types [64]. The zinc content in roots of *V. phrygium* is up to 17,044.54 mg/kg DW and it was approximately 7.8 times more than leaves (Fig. 3b). According to Neilson and Rajakaruna [65] a plant is considered hyperaccumulator that accumulate heavy metals 10 to 500 times higher in their aerial parts. We determined that in present study *V. phrygium*, with concentrations of 2,180.67 mg Zn/kg DW of leaves (30 mg Zn/L) and it was approximately 35 times more than control. Our study demonstrates that it can be considered to be highly tolerant to zinc and has a strong phytoremediation property (Fig. 3b). Morina et al. [47] reported that *Verbascum thapsus* (MET1-zinc contaminated area) and *Verbascum lychnitis* (MET2-open cast copper mine) exposed to excessive Zn or Cu in hydroponic culture showed high tolerance to both Zn and Cu. Moreover, the roots of these species accumulated higher Zn in the roots than the shoots. Furthermore, *V. thapsus*, inoculated with mycorrhizal fungus, is among the few species that can be successfully spread through seeds in the industrial waste area contaminated with high concentrations of Zn, Fe and Pb [66]. A study conducted by Kırat [24] in *Verbascum euphraticum* at Görgü Pb-Zn mine site Malatya-TURKEY, this plant can be used as an indicator plant for environmental contamination and can also be said as an accumulator or hyperaccumulator for Cd, Pb and Zn. In a related study about common medicinal plants toxic metal contents of Dir Lower (Pakistan) showed that, *Verbascum thapsus* accumulated the highest concentration of Cr (5.10 ppm), Fe (129.04 ppm) and Ni (4.57 ppm) [25]. According to

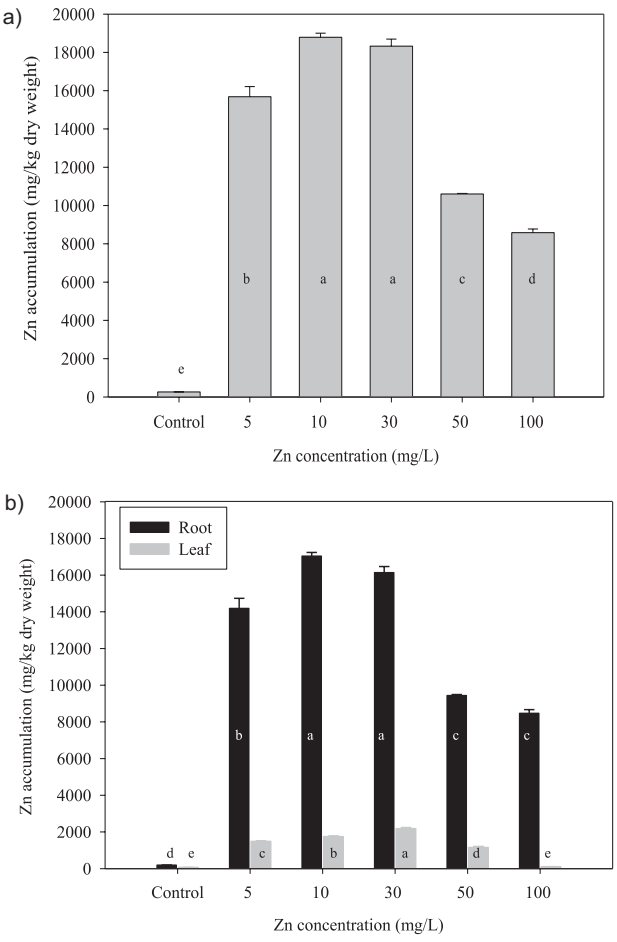

Fig. 3. The accumulation of Zn (mg/kg dry weight) in *V. phrygium* at pH 6.5 and 7th days of experiment; a) different concentrations of Zn in whole plant (Control, 5, 10, 30, 50 and 100 mg Zn/L), b) different concentrations of Zn in different organs (root and leaf) of *V. phrygium*

Arslan et al. [27], *Verbascum bombyciferum* plant can be used as a bioindicator species in monitoring Cd, Cr, Pb, and Zn pollution in the environment.

Conclusions

In conclusion, many endemic plant species are germinated *in vitro*, as they do not respond well to conventional germination methods. The *in vitro* techniques offer a powerful tool for propagation of threatened endemic plant species. The possibilities of germination through tissue culture technology and phytoremediation potential of *V. phrygium* under zinc stress were explored in this study. It has been shown that *V. phrygium* plant has high germination rate *in vitro* culture. An ideal hyperaccumulator, should be able to thrive in heavy metal contaminated environments, not requiring much maintenance and producing high biomass [67]. Verbascum species, which have a long and developed root system, have a high growth rate and can significantly increase their biomass in the second year [47]. In addition, the zinc contents in whole plant of *V. phrygium* were significantly higher than the normal for trace element value in a plant (50 mg/kg dry weight) [68]. *V. phrygium* plant accumulating metals mainly in the roots and this species can be used in contaminated areas due to its capacity to accumulate Zn. Thus, it is very important for phytostabilization of polluted areas. Conclusively, this study describes a proper *in vitro* germination protocol and phytoremediation capacity for *V. phrygium* for the first time.

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Conflict of Interest

The authors declare no conflict of interest.

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