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Original Article

Bioaccumulation potential of *In vitro* regenerated plants of *Ceratophyllum demersum* against Chromium – A lab study

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Abstract

Phytoremediation of contaminated environment using hyperaccumulator plants is considered as ecofriendly and cost-effective technique. Aquatic plants like *Ceratophyllum demersum* (Coontail) is used for the phytoremediation of aquatic environment contaminated by heavy metals. In this study, in vitro regenerated plants of *C. demersum* were exposed to different concentrations of Chromium (Cr) (0, 3, 6, 9, 12 and 15 mg L^{-1}) for different exposure time (1, 3 and 5 days) in order to investigate the phytoremediation potential. The plants contained in Cr containing liquid medium were cultured in growth room having 16 hr light photoperiod aided by white Light Emitting Diodes (LEDs) and temperature of 24±1 ^{0}C. The plant samples were taken out from liquid medium after 1, 3 and 5 days for taking data regarding fresh weight, dry weight, Cr uptake by plants and bioconcentration factor (BCF). The clear relationship between Cr concentration and exposure time on phytoremediation was revealed. Both fresh and dry weight of plants was recorded higher at variable Cr concentration compared to control plants. The exposure time of 3 days to Cr caused the maximum Cr uptake, followed by further uptake of Cr at relatively slow rate after 5 days. The highest Cr uptake and BCF was achieved from medium provided with 12 mg L^{-1} and 3 mg L^{-1} of Cr respectively. Comparison of exposure time exposure time × Cr concentration revealed the highest Cr uptake (9145 mg kg^{-1}) and BCF value (2076.5) from the combination of 12 mg L^{-1} × 5 d and 3 mg L^{-1} × 5 d respectively. The results revealed that in vitro regenerated plants of *C. demersum* can be used for phytoremediation of Cr and possibly use against other heavy metals.

Keywords: Bioconcentration factor, Chromium, *C. demersum*, Phytoremediation, Water

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Introduction

Heavy industrialization in the 20th century in both developed and underdeveloped countries lead to accumulation of heavy metal to the environment (Fu and Wang, 2011) and water (Sadik et al., 2015). The issue of heavy metal pollutions is now considered as one of the major threat to humans (Zheng et al., 2016) and other living organisms due to its cycle of transfer from non-living to living organisms (Akpor and Muchie, 2011). The water bodies all over the world are more prone to heavy metal contaminants due to release of heavy metals to aquatic environment especially in under developed countries where, violation of rules and lack of resources are main hinders to overcome this issue. To overcome this issue of heavy metal contamination in the water bodies, different methods like physical, chemical, and biological processes are used either singly or in combinations (Ali et al., 2017). Phytoremediation is one of the popular, ecofriendly and cost-effective system used for cleaning water and soil (Ali et al., 2013; Cao et al., 2017). This system has been reported for bioremediation of water bodies or aquatic environment (Harguinteguy et al., 2016; Xu et al., 2019) using submerged aquatic plants (Thiébaut, 2012) like *C. demersum* L. (Chen et al., 2015). The coontail is rootless, submerged and perennial aquatic macrophyte which prefers to live in shallow water with low light intensity (Polechonska et al., 2018). Its rapid propagation and high biomass in water bodies make it an important macrophyte which absorbs elements easily from its surrounding (Wang et al., 2014; Polechonska et al., 2018). However, the main issue related with this system is the availability of plants which must be free of heavy metals (Dogan et al., 2018). Plant tissue culture technique offers a solution of plant availability propagated under controlled conditions and without adhering to external conditions (George et al., 2008). Keeping in view, this study was designed to check the phytoremediation potential of in vitro propagated *C. demersum* plants subjected to variable concentrations of Chromium (Cr) under lab conditions. This study will open the window for using such type of plants under natural ecological environments.

Material and Methods

Experimental labs

The experiments about in vitro propagation and phytoremediation studies were carried out at Plant Biotechnology Laboratory, Necmettin Erbakan University, Department of Biotechnology. Whereas, work related to Cr analysis was performed at Environmental Engineering Laboratory of Necmettin Erbakan University, Konya, Turkey.

In vitro propagation of plant material

For the continuous availability of plant material, nodal segment explants were excised from *in vitro* stock material available at Plant Biotechnology Laboratory. Explants were isolated from top part of the *in vitro* stock material and transferred to liquid (Karataş et al., 2014) Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) containing 1.0 mg L^{-1} 6-Benzylaminopurine (BAP). The MS medium was also enriched with 3.0% sucrose, 0.44% MS and adjusted to 5.6-5.8 pH prior to autoclave. The explants were cultured under white LEDs (1500 LUX) for 16 h light photoperiod in growth room at 23±1 °C.

Phytoremediation studies

All reagents used in phytoremediation study were of analytical grade (Merck®, Germany). $CrCl_2$ (Merck) with mol. wt. of 122.9 was used as salt for phytoremediation studies. The stock solution was prepared at the rate of 50 mg L^{-1} and stored at 4 °C. Cr were used at the rate of 3, 6, 9, 12 and 15 mg L^{-1} along with control containing 0 mg L^{-1} $CrCl_2$. Plastic magenta GA^7 boxes (100 × 100 × 80 mm) available as sterilized were used for phytoremediation studies. Each Cr concentration was mixed in 400 mL solution containing distilled water and later used for culture of plants for phytoremediation. The pH of the water samples were 7.0 at the time of use of Cr. *In vitro* regenerated plants of 5-7 cm were used for phytoremediation studies (7.5 g/L) and exposed for three different time intervals of 1, 3 and 5 days under growth conditions of 24 °C and 16 h light photoperiod using white LEDs.

Plant sampling and analysis

After phytoremediation for three different days (1, 3 and 5 days), samples were taken out from phytoremediation medium and immediately weighed for fresh wt. The samples were dried on Whatman paper 42, oven dried at 70 oC using oven (Memmert) for 4 days, followed by weighing to measure dry wt. Thereafter, plant samples were mixed with 10 mL 65% HNO_3 followed by digestion in microwave (CEM, MarsXpress, USA). The material was sieved using Whatman filter papers no 42 and brought the

volume up to 25 mL by adding ultra-distilled water, followed by analysis of Cr by using Atomic Absorption Spectrophotometer (AAS) - Perkin Elmer-800 equipped with flame and graphite furnace. The calibration standard was prepared using 1000 mg L^{-1} stock solution for Cr. Reagent blanks' concentrations were below the detection limit for the Cr. 10% HNO_3 were applied for glassware and plastic ware followed by using deionized water for washing. The analysis of each sample was repeated thrice in each replicate for quality assurance. The analysis of mid-concentration of metal was performed after every ten samples. Detection limit of AAS was determined as 0.188 µg/L for Cr.

Heavy metal (Cr) contents in the plants were expressed as mg kg^{-1} dry weight (dw) using appropriate conversion formula. Bioconcentration Factor (BCF) of *C. demersum* plants subjected to different concentrations was formulated by using formula (Zayed et al., 1998; Dogan et al., 2018).

BCF = Trace element concentration in plant tissue (mg kg^{-1}) / First concentration of the element added to water (mg L^{-1}).

Statistical analysis

Data about shoot fresh and dry wt, Cr concentration in plants and BCF were tabulated and subjected to statistical analysis for One Way ANOVA (analysis of variance) with the help of SPSS 21 for Windows (SPSS Inc. Chicago, IL, USA) program. Duncan's Multiple Range Test (DMRT) at $p<0.01$ level of significance was used for comparing means. The data given in percentages (%) were transformed arcsine (√X) form (Snedecor and Cochran, 1997) before subjecting them to ANOVA and DMRT.

Results and Discussion

The application of hyperaccumulator aquatic plants are highly significant for the cleaning of water bodies contaminated with wide arrays of pollutants like heavy metals. There are number of aquatic plants which are recommended for phytoremediation studies like *C. demersum* (Chen et al., 2015; Dogan et al. 2018), *Lemna minor* and *Lemna gibba* (Sasmaz et al., 2016). Among these, *C. demersum* is highly effective aquatic plants used for phytoremediation against different pollutants found in water bodies (Abdallah 2012; Vahdatiraad and Khara 2012; Hassan and Al-Khalidi 2018).

The phytoremediation potential of *C. demersum* plants subjected to different concentrations of Cr exhibited significant impact on plant growth during phytoremediation phase (Terzi and Yildiz 2011). The exposure time significantly ($p<0.01$) affected the fresh wt, dry wt, Cr uptake by plant and BCF in similar fashion. All parameters increased with increase in exposure time (Table 1) and recorded as 2.99-5.14 g (fresh wt), 57-92 mg (dry wt), 2586.92-6509.67 mg kg^{-1} (Cr uptake by plants) and BCF value of 406.58-927.50 (Table 1). Previously, elevated Cd and Pb uptake by *C. demersum* with increase of exposure time has been reported respectively by Al- Ubaidy and Rasheed (2015) and Chen et al., (2015). Whereas, Dogan et al., (2018), linked the decreased plant growth and chlorophyll contents of *C. demersum* plants with exposure time. Similarly, the impact of exposure time on metal accumulation and plant growth have been reported by other researchers using different aquatic macrophytes (Singh et al. 2010; Sasmaz et al. 2016).

Table-1: Impact of exposure time on plant growth and phytoremediation potential of *C. demersum* plants

Day	Fresh Wt (g)	Dry Wt (mg)	Cr Uptake (mg kg^{-1})	BCF
1	2.99a	57b	2586.92c	406.58c
3	4.91±0.51b	90a	4916.08b	724.58b
5	5.14±0.59a	92a	6509.67a	927.50a

Means followed by different small letters within columns are significantly different using DMRT test at $p<0.01$

Application of Cr concentration exerted statistical effect ($p<0.01$) on all parameters tested. Fresh wt and dry wt in response to Cr concentration ranged 4.06-4.64 g and 76-84 mg respectively (Table 2). Both highest fresh wt and dry wt was recorded for medium supplemented with 9 mg L^{-1} Cr. Earlier, Doğan et al., (2018) reported toxic effects of Cr on plant growth and pigmentation of *C. demersum* exposed to different concentrations. The Cr uptake by plants was statistically similar up to 9 mg L^{-1} of Cr. However, the maximum Cr uptake by plants was attributed to medium having 12 mg L^{-1} Cr but further increase of Cr led to decreased uptake by plants. The BCF values decreased significantly with increased Cr concentration in the medium and ranged 373.67-1679.83 (Table 2). Decreased BCF value with elevated concentration of Cr (Doğan et al., 2018) or Cd

(Bunluesin et al., 2004; Das et al., 2016; Dogan et al., 2018) have also been reported.

Table-2: Impact of Cr concentration on plant growth and phytoremediation potential of *C. demersum* plants

Conc (mg L^{-1})	Fresh Wt (g)	Dry wt (mg)	Cr Uptake (mg kg^{-1})	BCF
0	4.18bc	76c	0.00c	0.00
3	4.06	77c	5039.67b	1679.83a
6	4.35abc	81ab	5667.67b	944.83b
9	4.64a	84a	5145.33b	571.50c
12	4.45ab	80bc	6569.83a	547.50c
15	4.39abc	81ab	5602.83b	373.67d

Means followed by different small letters within columns are significantly different using DMRT test at $p<0.01$

The exposure of plants to different Cr concentrations and time interval (exposure time × Cr conc.) significantly affected the plant growth which in turn resulted in variable response on fresh and dry wt of phytoremediated plants. Both fresh and dry wt parameters reflect the toxicity level of phytoremediated plants. Different researchers highlighted the variable response of plants exposed to different heavy metals at variable concentrations (Cedergreen, 2008; Duman and Koca, 2014). There was slight change in fresh wt after 1 day but after 3 days, plants showed significant growth and gained significant fresh wt in response to different concentrations of Cr. Maximum fresh wt was achieved from medium supplemented with 9 mg L^{-1} Cr followed by 12 mg L^{-1} Cr after 3 days (Table 3). The further increase of Cr concentration was highly detrimental and resulted in reduced fresh wt.

Comparing exposure time, clear change in fresh wt. was recorded between one and three days of exposure. Whereas, slight change of fresh wt. between three and five days exposure time was recorded (Table 3). These results suggested that plants took time for adaptation in the Cr medium within 24 hr (1 day) followed by enhanced growth in the medium up to 3 days. Thereafter, plants growth remained slow due to exposing further to Cr concentration for 2 more days. The results further suggest that plants can survive and continue to grow at higher concentration of Cr up to 3 days but further exposure time may lead to slow plant growth. Negative impact of Cr on plant biomass of *C. demersum* has been reported by Doğan et al., (2018). Similarly, negative impact of Cd or Pb concentration and exposure time on fresh wt of *C. demersum* has also reported (Dogan et al., 2018). Similar types of results were also tested by Mishra et al., (2006) and Chen et al., (2015), when exposed *C. demersum* plants to Pb for 1-7 days.

Table-3: Impact of exposure time × Cr concentration on fresh wt (g) and dry wt (mg) of phytoremediated *C. demersum* plants

Cr (mg L^{-1})	Fresh Wt (g)			Dry Wt (mg)		
	1 d	3 d	5 d	1 d	3 d	5 d
0	2.848f	4.574de	5.134bcd	52g	94bc	82e
3	2.827f	4.765cde	4.593de	55fg	88cde	87cde
6	3.376f	4.813cde	4.862cde	60f	94bc	89cde
9	3.021f	5.557ab	5.343abc	59f	93bc	99ab
12	2.899f	5.328abc	5.120bcd	60f	89cde	90cd
15	3.001f	4.398e	5.762a	57fg	82de	104a

Means followed by different small letters within columns are significantly different using DMRT test at $p<0.01$

Results exhibited the clear impact of exposure time and Cr concentration on dry wt. of phytoremediated plants. Dry wt ranged 51-60 mg with highest dry wt. from 12 mg L^{-1} Cr after 1 day. After 3 days, fresh wt. increased significantly and ranged 79-94 mg (Table 3) with minimum dry wt. was achieved from samples containing 15 mg L^{-1} Cr. After 5 days, variable dry wt. was observed and ranged 82-104 mg with highest from 15 mg L^{-1} Cr (Table 3). The dry wt. of phytoremediated plants was in general higher than control plants. These results do not support the findings of Doğan et al (2018), who reported decreased dry wt. compared to control plants. Dogan et al., (2018) also reported decreased dry wt. with increased Cd or Pb concentration compared to control group of in vitro *C. demersum* plants. These results suggested that dry wt of phytoremediated plants is not related to their respective fresh wt. and can increase or decreased with with exposure time, heavy metal type or concentration.

Ceratophyllum demersum is well known plant for heavy metals phytoremediation in the water bodies. The analysis of phytoremediated plants revealed the clear relationship between exposure time and Cr concentration on the uptake by plants. The uptake of Cr by plants was recorded between 2766 to 3393 mg kg^{-1} after 1st day, 5103 to 7536 mg kg^{-1} after 3 days and 6229-9145 mg kg^{-1} after 5 days of phytoremediation. The highest uptake was recorded from medium containing 12 mg L^{-1} Cr after 3 and 5 days (Table 2). There was no detection of Cr in the control plants. These results confirmed the previous findings of

Doğan et al., (2018), who also observed increased Cr uptake with increase in Cr concentration. Earlier, Duman et al. (2010) also reported accumulation of Cr by *C. demersum* plants up to 6 days, when used at the rate of 1, 5, and 10 mM.

Table-4: Impact of exposure time × Cr concentration on uptake of Cr conc (mg kg^{-1}**) by phytoremediated *C. demersum* plants**

Cr (mg L^{-1}**)**	**1 d**	**3 d**	**5 d**
0	0f	0f	0f
3	3302e	5588d	6229cd
6	3393e	5382d	8227ab
9	3033e	5103d	7301bc
12	3029e	7536b	9145a
15	2766e	5887d	8156ab

Means followed by different small letters within columns are significantly different using DMRT test at $p<0.01$

Table-5: Impact of exposure time × Cr concentration on bioaccumulation factor (BCF) of phytoremediated *C. demersum* plants

Cr (mg L^{-1}**)**	**1 d**	**3 d**	**5 d**
0	.00i	.00i	.00i
3	1100.50c	1862.50a	2076.50a
6	565.50efg	897.50cd	1371.50b
9	336.50gh	567.00efg	811.00de
12	252.50hi	628.00ef	762.00de
15	184.50hi	392.50fgh	544.00efg

Means followed by different small letters within columns are significantly different using DMRT test at $p<0.01$

BCF exhibits the metal uptake or accumulation capacity of the phytoremediated plant (Kara and Zeytunluoglu, 2007) from ecological site either soil or aquatic environment (Zayed et al., 1998). The results revealed the significant impact of Cr concentration and exposure time on BCF. The highest BCF after 1 day was 1100.50 from medium supplemented with 3.0 mg L^{-1} Cr. The same pattern was also followed after 3 days (1862.50) and 5 days (2076.50) (Table 3) and confirmed the previous findings of Doğan et al., (2018). It was also noted that BCF concentration showed decreased pattern with increase of Cr concentration in the medium irrespective of exposure time. Decreased BCF value with increase of Cd (Bunluesin et al., 2004) or Pb (Abdallah, 2012) concentration has been reported for *C. demersum* used for phytoremediation.

Conclusion

The study reflects the possible phytoremediation potential of *in vitro* regenerated *C. demersum* plants against Cr under lab conditions. The results suggest that Cr in the medium significantly affected the fresh wt. and dry wt. of phytoremediated plants compared to control plants. The uptake of Cr from the medium by *in vitro* regenerated plants was fast and highest after 3 days. Although, plants continuously uptake Cr from the medium, the speed was relatively slow. The BCF was highest to lowest with elevated Cr concentration. These results clearly emphasize the potential of *in vitro* regenerated plants for phytoremediation studies for Cr under lab conditions and there is need to carry experiments for phytoremediation studies under natural ecological environments or subjecting the plants to polluted water under lab conditions.

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Contribution of Authors

Aasim M: Conceived idea, designed research methodology, statistical analysis and manuscript writing
Aydın S: Conceived idea, designed research methodology, data analysis, manuscript final reading and approval
Karataş M: Conceived idea, literature review and manuscript final reading and approval
Aydın ME: Literature review, data analysis and data interpretation
Soğukpinar C: Designed research methodology, data collection and data analysis
Sevinc C: Literature review, data analysis and manuscript final reading