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Application of *in vitro* test system for evaluating the tolerance of *Ageratum houstonianum* and *Petunia x hybrida* to cadmium toxicity

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Abstract

Milusheva, D. I., Atanassova, B. Y., & Iakimova, E. T. (2019). Application of *in vitro* test system for evaluating the tolerance of *Ageratum houstonianum* and *Petunia x hybrida* to cadmium toxicity. *Bulgarian Journal of Agricultural Science*, *25*(2), 300–309

Cadmium (Cd) is a highly toxic heavy metal pollutant of the environment and biosphere. Phytoremediation of soils and waters contaminated with Cd and other heavy metals by the use of agricultural crops aimed for food is a hazardous approach. In this sense the ornamental plants can be an appropriate alternative. The current study was undertaken to develop *in vitro* test system for evaluating the physiological response to Cd stress of the annual ornamentals *Ageratum houstonianum*, cv. Blue Hawaii and *Petunia x hybrida*, cv. Lavender. *In vitro* cultured explants were exposed to a range of Cd concentrations exceeding the maximum permissible concentration (MPC), which for Cd in soil is 2 mg kg^{-1} dry matter. The stress impact was judged by the indices height and width of microplants, root length and diameter, fresh and dry biomass and the electrolyte leakage. The symptoms of toxicity were determined by visual observations. *In vitro*, the plants expressed different degree of tolerance at Cd levels exceeding MPC. Ageratum appeared medium tolerant to Cd concentrations elevated up to 5 x MPC whereas petunia showed lower tolerance and developed successfully only up to 2 x MPC. Pot experiments with ageratum were carried out to find out whether the *in vitro* established degree of tolerance may correspond to the response of whole plants grown on Cd contaminated substrate. In comparison to *in vitro* conditions, *in vivo* the plants tolerated even higher doses of Cd (up to 25 x MPC). The applied *in vitro* model system represents an innovative experimental approach suggested as promising tool for early diagnosis of the potential of annual ornamental plants to grow at conditions of Cd and other heavy metals stress. For establishing the potency of the examined plants for phytoremediation, further analyses of their phytoextraction capacity are envisaged.

Keywords: tolerance; heavy metals; cadmium; *Ageratum houstonianum*; *Petunia x hybrida*; phytoremediation

Introduction

The pollution of the environment with heavy metals creates unfavorable conditions for the growth and development of plants in the ecosystems and in agricultural and urban areas, threatens the existence of beneficial soil and water microorganisms and poses in risk the animal and human health (Alkorta et al. 2004; Nagajyoti et al., 2010; Tóth et al., 2016). Lead (Pb), Zn, Cd, Cr, Hg, Cu, As and Al are among the most widespread contaminants of soil, water and biosphere, especially in areas with anthropogenic influence, such as mining, ore processing, production of alloys, batteries, fuel, electricity and other industrial activities (Nagajyoti et al., 2010; Liao et al., 2016). An environmentally friendly strategy for cleaning the contaminated sites from heavy metals is phytoremediation. It is based on the use of plants able to extract the metals (phytoextractors), accumulate them in their tissues (hyperaccumulators) and grow successfully on highly contaminated background (Salt et al., 1996; Susarla *et* al., 2002; Sarma, 2011; Rascio and Navari-Izzo, 2011; Pandey et al., 2015; Cristaldi et al. 2017). More than 500 species from about 100 families are established as hyperaccumula-

tors (Sarma, 2011; Rascio and Navari-Izzo, 2011; Tran and Popova, 2013). It should be however taken into consideration that, in the context of food safety, phytoremediation by agricultural crops meant for food is hazardous (Tóth et al. 2016). The ornamental plants can be an appropriate alternative for growing on polluted areas (Wang, 2005; Liu et al., 2006; Sun et al., 2011). Some ornamentals can hyperaccumulate and tolerate high excess of heavy metals; others are tolerant to low and medium high concentrations but are not established as phytoextractors and some are sensitive and do not develop properly at elevated doses of the metals (Table 1).

In comparison to pot experiments and field trials with whole plants, reliable and quicker results for the tolerance to heavy metal stress can be obtained through experimental systems *in vitro* (Golan-Goldhirsh et al., 2004; Doran 2009). Among the recommended models are cell suspension and tissue cultures of *Arabidopsis*, the unicellular alga *Chlamydomonas reinhardtii* and the alpine pennygrass *Thlaspi caerulescens* (Assunção et al. 2003; Cobbett, 2003; Suzuki, 2005; Doran, 2009; Dimitrova et al., 2014). *In vitro* cultured ornamental plants can be another promising object with a perspective for practical application for landscaping and remedy of polluted areas. However this research direction is still poorly explored. Among the few works is the recently elaborated tissue culture system to investigate the tolerance of *Dianthus carthusianorum* to Cd (Muszyńska and Hanus-Fajerska, 2017).

Cadmium is one of the most dangerous pollutants. Its annual release into environment is about 25 000 tons, almost half of which comes from natural sources such as volcanic eruptions, forest fires and release from the rocks. The rest is due to anthropogenic activity (Tran and Popova, 2013). According to Yotova et al. (2018) the geochemical threshold values in the surface level of the agricultural soils in Bulgaria, as estimated by median+2Median Absolute Deviations (median+2MAD) and Tukey inner fence (TIF) methodology, are 0.38 and 0.78 mg/kg respectively. In the 'Ordinance for regulation and the way for utilization of sludge from waste

Table 1. Examples of the capacity of some ornamental plants to tolerate excess of heavy metals

Species and cultivars	Heavy metal	Tolerance	References
Althaea rosea *Calendula officinalis* *Chrysanthemum indicum* *Gladiolus grandiflorus* *Helianthus annuus*, cv. 'Pacino' *Salvia splendens* *Tagetes erecta*	Cd, Pb	hyperaccumulator	Chakravarty and Srivastava, 1992; Bosiacki, 2008, 2009; Liu et al., 2008a,b; Lal et al., 2008; Ramana et al., 2009; Mani et al., 2016
Iris lactea var. *chinensis*	Cd	hyperaccumulator	Han et al., 2007
Iris tectorum	Cd	tolerant	Han et al., 2007
Impatiens walleriana	Cd	hyperaccumulator	Bosiacki, 2009; Lin et al., 2010; Wei et al., 2012; Lai, 2015
Tagetes patula	Fe, Cr, Cu, Pb, Ni, Cd	hyperaccumulator	Chaturvedi et al., 2014; Wei et al., 2012; Lai, 2015
Celosia cristata pyramidalis	Pb	hyperaccumulator	Cui et al., 2013
Pellargonium spp.	Cd, Ni, Pb	hyperaccumulator	Dan et al., 2002; Sarma, 2011
Tagetes erecta x *Tagetes patula* *Pteris vittata*	As	hyperaccumulator	Zhao et al., 2003; Chintakovid et al., 2008; Milusheva et al., 2016
Viola baoshanensis	Cd	hyperaccumulator	Liu et al., 2004
Osmanthus fragrans var. *thunbergii*	Cd, Pb, Zn, Cu	tolerant	Wu et al., 2011
Armeria maritime ssp. *halleri*	Zn	tolerant	Heumann, 2002
Zinnia merylandica, cv. 'Zahara Yellow'	Cd	tolerant	Milusheva et al., 2015
Zinnia merylandica, cv. 'Zahara Yellow'	Pb	sensitive	Milusheva et al., 2015
Zinnia elegans	Cd	sensitive	Thamayanthi et al., 2011
Antirrhinum majus *Quamolit pennata*	Pb	sensitive	Cui et al., 2013
Tagetes patula, cv. 'Janie Primrose' and cv. 'Roodkapje'	Zn	sensitive	Ivanova et al., 2006, 2007; Milusheva et al., 2016
Salvia splendens	Pb, Cu, Zn	sensitive	Atanassova and Zapryanova, 2009
Ageratum houstonianum, cv. 'Delphy' *Callistephus sinensis*, cv. 'Royal Red Ball'	Pb, Cu, Zn	sensitive	Ivanova et al., 2006, 2007

waters through the use in agriculture' (Decree of Ministry Council No. 201 of 4.08.2016) the maximum permissible concentration (MPC) for Cd in soil with pH 6.0-7.4 is 2 mg/kg dry soil matter (DS). This chemical element may cause adverse changes in the physiological, biochemical and molecular processes in plants, leading to suppression of seed germination, disturbance of growth and development, premature senescence, inhibition of photosynthesis and respiration, water imbalance, oxidative stress, DNA damage, anomalies in cellular structures etc. Cadmium can also alter the uptake, transport and utilization of water and important ions such as Ca, Mg, Fe, P and K (Das et al., 1997; di Toppi and Gabrielli, 1999; Cuypers et al., 2010; Thamayanthi et al., 2011; Irfan et al., 2013; Tran and Popova, 2013; Choppala et al., 2014; Yousefi et al., 2018). Cadmium is also a potent inducer of programmed cell death (Yakimova et al., 2006; de Pinto et al., 2012; Kutik et al., 2014). Among the visual symptoms of Cd toxicity are delayed growth, leaf chlorosis and necrosis, root tip browning, withering and dead at severe stress impact (di Toppi and Gabbrielli, 1999;).

The aim of present study was to develop *in vitro* test system for evaluating the effect of Cd stress on growth and development of explants from the annual ornamental plants *Ageratum houstonianum* and *Petunia x hybrida*. The introduced tissue culture model is suggested as a tool for early diagnosis of the tolerance/sensitivity to Cd and potentially other heavy metals.

Materials and Methods

The experiments were carried out with *in vitro* propagated plants of *Ageratum houstonianum* F1 cv. Blue Hawaii (PanAmerican Seed, USA) and *Petunia x hybrida* Fanfare, cv. Lavender (cat. No. 13044, Vitroflora, Co., Poland) (Fig. 1A). *Ageratum houstoianum* Mill. is an annual plant from genus *Ageratum*, fam. *Asteraceae*, grown in gardens or as pot and balcony flower. The leaves are ovate to triangular, 2-7 cm long; the flowers are of light blue color; the stems grow between 0.3 and 1 m high. *Petunia x hybrida* is summer flowering hybrid from genus *Petunia*, fam. *Solanaceae*. The plants of cv. Lavender form compact, 10-60 cm high small bushes with many attractive trumpet-shaped light pinkish-purple colored flower petals and green simple leaves (Fig. 1B). Usually they are used for balcony and interior decoration.

Microplants were propagated in sterile conditions following a procedure routinely established in the Institute of Ornamental and Medicinal Plants, Sofia on nutrient medium (Murashige and Skoog, 1962) with vitamins, supplemented with 30 g l^{-1} sucrose and solidified with 6 g l^{-1} agar; pH 6.0.

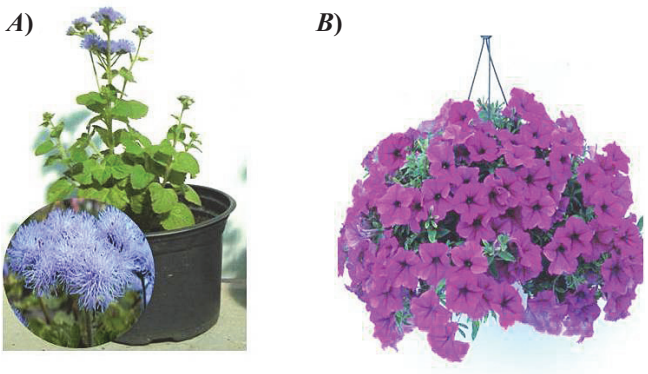

Fig. 1. Representative images of (*A*) *Ageratum houstonianum* F_1, cv. Blue Hawaii (this study); (*B*) *Petunia x hybrida,* cv. Lavender (catalogue Vitroflora, Co., Poland)

For testing the effect of Cd, 3 mm long explants isolated from stem apex of 20 days old ageratum and 30 days old petunia microplants were cultured in the same medium and maintained in controlled environment – temperature 22°C, photoperiod 16/8 (day/night) and light intensity 30 µmol.$m^{-2}s^{-1}$ provided by 40 W cool white fluorescent tubes (Philips, Bulgaria). Cadmium was applied into the medium in the form of water solution of $CdSO_4$ at final concentrations per liter medium: 2 mg l^{-1} (mimicking the MPC in soil) and 4, 10, 20, 50, 100 and 200 mg l^{-1}.

The growth of explants was assessed by the height and width of the microplants and by root length and diameter, scored 10 and 20 days after Cd treatment. In the time point of 20 days the fresh weight (FW) and dry weight (DW) (presented as percentage of FW) were measured. The procedure included drying out the samples at 80°C for 24 h, cooling down for 2 h in a dessicator and weighting, next the samples were transferred back to the oven for additional 24 h and the weight was measured again followed by subsequent measurements at intervals of 2 h in the oven until reaching constant weight.

The physiological state of explants was estimated also by electrolyte leakage (an indicator of cellular membrane integrity, marker of stress response and senescence). The exoosmosis of electrolytes was analyzed 10 and 20 days post-treatments by recording electroconductivity, according to the procedure described in Song et al. (2012). The analysis was undertaken with an average sample of 100 mg FW gently excised leaves (size approximately 3/2 mm) collected from at least 60 explants. The samples were floated with their abaxial part up in 15 ml deionized water and incubated at room temperature (21°C) and constant stirring. After 2 hours the electrical conductance (in 10^{-3} siemens (mS)) of the solution was measured by using a con-

ductivity meter (pH/Cond, Meetinstrumenten Aalsmeer Holland Nieuwkoop B.V. SN 10196). This value was determined as EC1. In the next step the samples were boiled for 20 min in order to destroy the tissue and allow the release of all electrolytes. After cooling to room temperature, the conductivity was measured again and recorded as total conductivity (EC2). Electrolyte leakage (EL) is expressed in percentage, calculated using the formula: EL (%) = EC1/EC2 × 100.

Similar trials *in vitro* were carried out with petunia explants.

Pot experiments with whole ageratum plants were undertaken to compare the effect of Cd toxicity *in vitro* and *in vivo*. Twenty days old *in vivo* produced seedlings were grown in pots under controlled greenhouse conditions (air temperature 23-25°C, soil temperature 19-23°C, relative air humidity 70%; natural photoperiod). The substrate (soil/peat, ratio 1:1, pH 6.2) was contaminated with Cd supplied as $CdSO_4$ solution at final concentrations in the substrate ranging from MPC (2 mg kg^{-1} DS) to 100 x MPC (200 mg kg^{-1}). Height and width of the plants were measured 20 days and 40 days after treatment; root system dimensions were measured after 40 days when the experiment was terminated.

The symptoms of toxicity in *in vitro* and *in vivo* experiments were estimated by visual observations for occurrence of leaf chlorosis, necrotic spots and wilting.

Data were statistically processed by one-way analysis of variance (ANOVA) at probability level $p \leq 0.05$ (Microsoft Office Excel). The presented values of the biometrical indices and FW and DW in the experiments *in vitro* are average of 3 independent experiments, each with at least 60 explants per treatment (n=180). Data from pot experiments are means of n=36 (12 plants per variant of treatment in each of 3 separate sets of experiments). For electrolyte leakage the data are average of n = 9 (3 repeated measurements of average samples collected from 60 explants per treatment in 3 independent sets of experiments).

Results

The biometrical indices of *in vitro* cultured ageratum showed that in general the effect of Cd toxicity on explants height and width and root development was better pronounced in 20 days old plants (Fig. 2).

In comparison to control (non-treated), 10 days after the exposure to Cd the height of explants was not affected in presence of Cd up to 10 mg l^{-1} (5 x MPC). Significant decrease of the height of 20 days old explants was detected

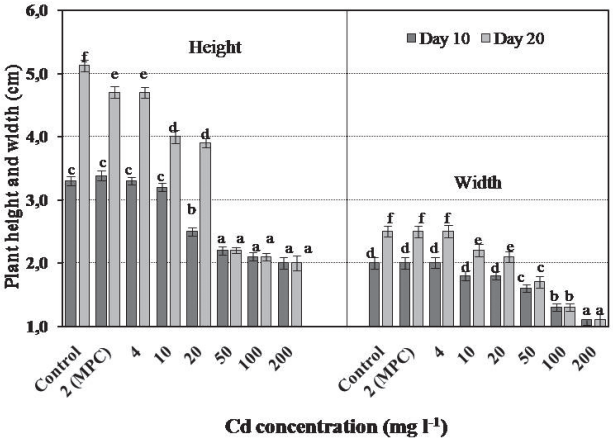

Fig. 3. Effect of Cd on biometric indices of *in vitro* cultured explants of *Ageratum houstonianum*, cv. Blue Hawaii

Data sets for height and width are statistically analyzed separately. For each treatment the values are average of 3 independent sets of experiments (n = 180). Error bars show standard error of the means – $SEM_{(n-1)}$. Values indicated with different letters are significantly different from each other ($p \leq 0.05$) within the measurements separately for height and width; MPC – maximum permissible concentration

Fig. 2. Effect of Cd on development of 20 days old *in vitro* cultured *Ageratum houstonianum*, cv. 'Blue Hawaii'; MPC - maximum permissible concentration

both at 5 x and 10 x MPC. At 20 mg l^{-1} (10 x MPC) the height diminished with about 20% and further decreased at elevation of the concentrations from 50 to 200 mg l^{-1} (Fig. 3). Twenty days after treatment shortening of internodes and reduction of the height were observed also at Cd corresponding to MPC but the plants were fresh and with well preserved leaf turgor. At concentrations above 50 mg l^{-1} the growth of explants was strongly retarded and the plants wilted after 20 days (Fig. 3). The changes in plant width followed similar tendency (Fig. 3).

Root formation started after 10 days of culturing. At day 20 the root length and diameter at MPC and Cd doses of 4 mg l^{-1} did not differ significantly from non-treated plants. At 10 mg l^{-1} Cd the root system was not fully developed but overall the explants were well preserved with turgescent leaves. Smaller roots were formed at 20 mg l^{-1} whereby the leaves showed symptoms of initial yellowing. At concentrations exceeding this level, the root formation was entirely inhibited (Fig. 2 and 4).

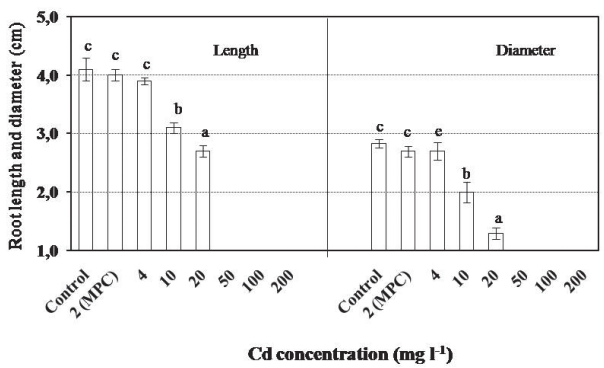

Fig. 4. Effect of Cd on root development of *in vitro* cultured 20 days old *Ageratum houstonianum*, cv. Blue Hawaii

Data sets for length and diameter are statistically analyzed separately. For each treatment the values are average of 3 independent sets of experiments (n = 180). Error bars show standard error of the means – $SEM_{(n-1)}$. Values indicated with different letters are significantly different from each other ($p \leq 0.05$) within the measurements separately for length and diameter; MPC – maximum permissible concentration

The FW of 20 days old Cd-treated explants decreased at Cd concentration of 20 mg l^{-1}. No difference from control was found at Cd up to 10 mg l^{-1}. At Cd level of 50 - 200 mg l^{-1} the FW sharply diminished (Fig. 5A). In presence of Cd up to 10 mg l^{-1} the accumulation of DW was similar to non-treated plants but declined in explants subjected to 20 mg l^{-1}. At further increase of Cd dose, in accordance with the advanced wilting, DW remarkably increased and presented a substantial part of the FW (Fig. 5B). In the apparently wilted explants (Fig. 3) the increase of the percent of DW rather reflected the desiccation of tissues and not an accumulation of more biomass. These results indicate that low to medium Cd concentrations do not suppress the accumulation of FW and dry mass whereas extremely high Cd levels inhibit the growth and accelerate the process of senescence.

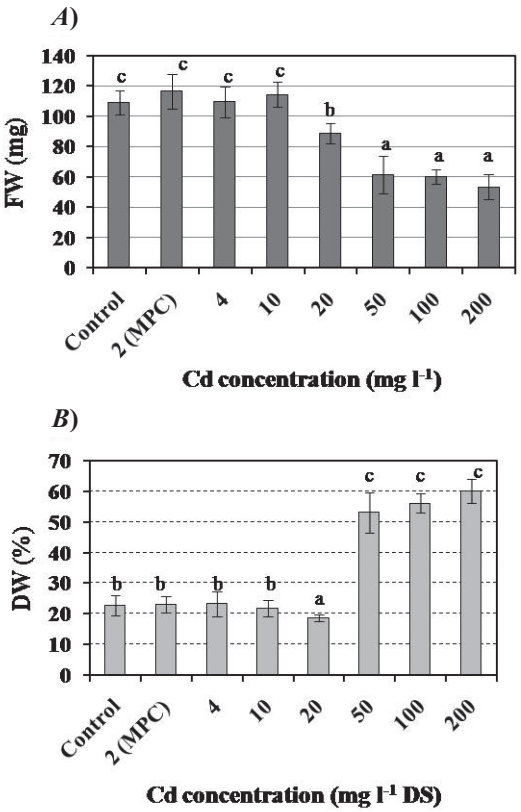

Fig. 5. Fresh (FW) and percentage of dry weight (DW) from FW of 20 days old *in vitro* cultured *Ageratum houstonianum*, cv. Lavender

Presented values are average of 3 independent sets of experiments (n = 180). Error bars show standard error of the means – $SEM_{(n-1)}$. Values indicated with different letters are significantly different from each other ($p \leq 0.05$); MPC – maximum permissible concentration

Electrolyte leakage increased at Cd concentrations over 20 mg l^{-1} and reached the maximum in the dying 20 days old explants (Fig. 6). This indicated that high doses of Cd cause destructive processes related to increased permeability of cellular membrane. Visibly the symptoms of damaged explants appeared as senescence – leaf yellowing and wilting (Fig. 3).

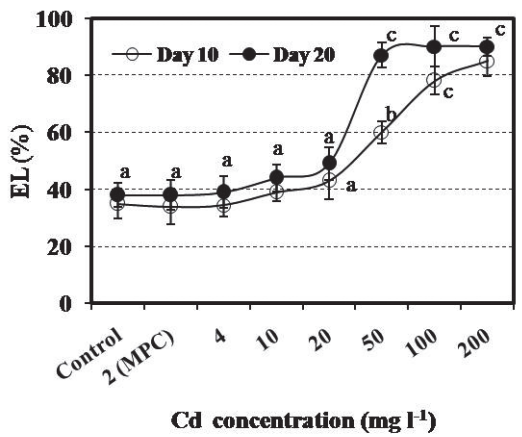

Fig. 6. Electrolyte leakage from Cd-treated explants of *Ageratum houstorianum*, cv. Blue Hawaii

The data for each treatment are average of n = 9 (3 repeated measurements of average samples collected from 60 explants per treatment in 3 independent sets of experiments). Error bars show standard error of the means – $SEM_{(n-1)}$. Values indicated with different letters are significantly different from each other at $p \leq 0.05$; MPC – maximum permissible concentration

Taken together, the biometrical parameters, the changes in FW and DW and electrolyte leakage as well the visual observations soundly pointed that in *in vitro* conditions the studied ageratum cultivar can overcome the toxicity and develop successfully at Cd levels up to 5 x MPC.

The changes in growth and development of petunia cultured *in vitro* in presence of Cd followed similar pattern as that of ageratum (data not shown). The effect of Cd on length and width of plants, FW and DW and electrolyte leakage was pronounced also at Cd concentration of 5 times over MPC. However, in difference to ageratum where the leaf yellowing occurred at 20 mg l^{-1} MPC, the senescence of 20 days old petunia explants was initiated at 10 mg l^{-1} (5 x MPC). In addition, petunia did not develop roots at 5 x MPC (Fig. 7).

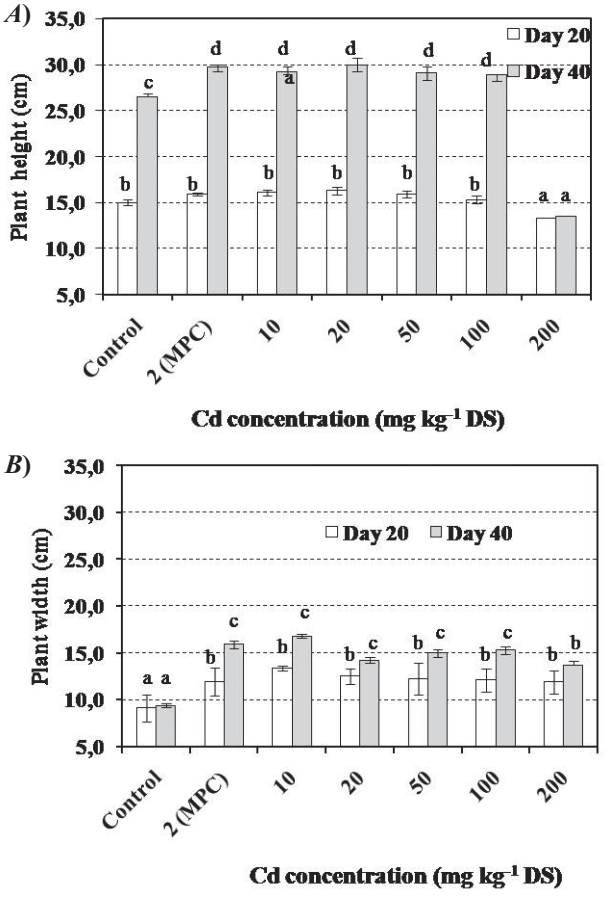

Fig. 8. Effect of Cd on growth of whole plants of *Ageratum houstonianum*, cv. Blue Hawaii (pot experiments): *A*) plant height; *B*) plant width

Data are average of 3 independent sets of experiments each with 12 plants per treatment (n = 36). Error bars show standard error of the means – $SEM_{(n-1)}$. Values indicated with different letters are significantly different from each other ($p \leq 0.05$); MPC – maximum permissible concentration

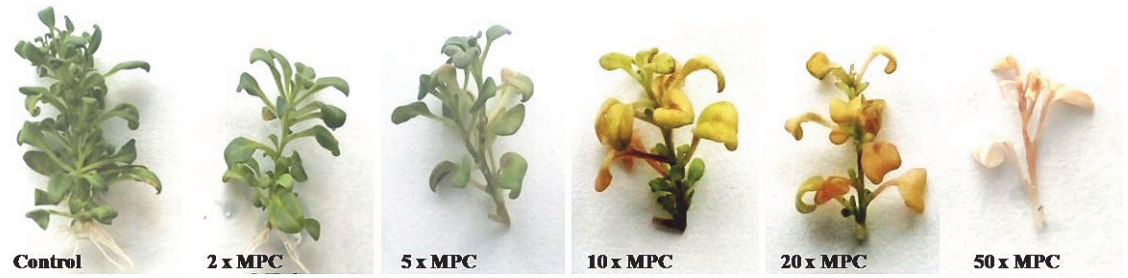

Fig. 7. Effect of Cd on development of *in vitro* cultured *Petunia x hybrida*, cv. Lavender; MPC – maximum permissible concentration

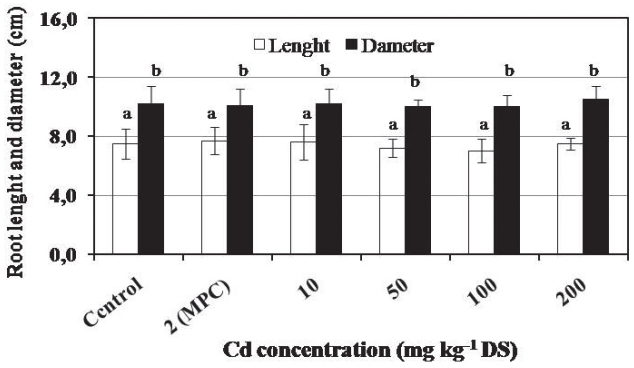

Fig. 9. Effect of Cd on root development of whole plant of *Ageratum houstonianum*, cv. 'Blue Hawaii' (pot experiments)

Data are average of 3 independent sets of experiments each with 12 plants per treatment (n = 36). Error bars show standard error of the means – $SEM_{(n-1)}$. Values indicated with different letters are significantly different from each other ($p \leq 0.05$); MPC – maximum permissible concentration

Judging by the observed physiological performance of *in vitro* cultured plants, ageratum can be determined as tolerant to Cd toxicity at levels exceeding MPC up to 5 times and petunia behaves as low tolerant (shows sensitivity to Cd concentrations higher than 2 x MPC).

The pot experiments were undertaken with whole plants from ageratum genotype used in the experiments *in vitro*. Height and width of plants were measured 20 and 40 days from the beginning of Cd treatment (Fig. 8). Interestingly, after day 20 in presence of Cd at concentration range up to 50 x MPC (100 mg kg^{-1} DS) the plant height and width increased as compared to non-treated control plants. The growth was retarded at concentration of 200 mg kg^{-1} DS (100 x MPC (Fig. 8A, B). The elevated Cd doses did not affect timing of initiation of the phenophases of but development and flowering. Up to Cd concentrations not exceeding 50 mg kg^{-1} DS (25 x MPC), the vegetative organs (stems and leaves) and the flowers appeared fresh and with maintained ornamental value. However, at Cd level higher than 50 mg kg^{-1} DS the plants showed symptoms of toxicity – leaf yellowing and occurrence of necrotic spots.

Cadmium contamination did not influence the length and diameter of root system of the whole plants (Fig. 9).

The observations on the plants grown *in vivo* indicated that out of tissue culture conditions the studied ageratum may tolerate much higher Cd levels (up to 25 x MPC).

Discussion

Here we introduce *in vitro* test system for evaluating the effect of Cd on growth and development of genotypes from the annual ornamental plants ageratum and petunia. The response *in vitro* was compared with that of whole plants grown on Cd contaminated substrate. Ageratum explants successfully survived at Cd concentrations up to 5 x MPC. In the pot experiments the plants showed tolerance to Cd doses up to 25 x MPC. This degree of tolerance was substantially higher than that of the explants but still in the range of medium level. At conditions *in vivo* enhancement of the growth of tolerant whole plants was observed. Earlier, stimulation of vegetative growth in the presence of low to medium high Pb, Cu and Zn doses was described for *in vivo* grown *Callistephus sinensis*, cv. Royal Red Ball and *Ageratum houstonianum*, cv. Delphy (Ivanova et al., 2006, 2007). To the extent of our experiments the reason for this phenomenon is hard to explain, but it is very probable that at moderate stress magnitude this can be natural reaction in the attempt of the species to accommodate to adverse condition. Some of defense mechanisms against heavy metal stress are sequestration of the metal in the cellular vacuole, metabolic and gene dependent control over the occurrence of oxidative stress, protein modifications, synthesis of heavy metal binding proteins (phytochelatins and metallothioneins) and of hormones with protective functions such as salicylic and jasmonic acids (Cobbett and Goldsbrough, 2002; Alkorta et al., 2004; Maksymiec and Krupa, 2006; Sarma, 2011; Rascio and Navari-Izzo, 2011; Tran and Popova, 2013; Choppala et al., 2014).

In vitro the effect of the metal on the root system occurred as shortening of the length and diameter of the roots and at extremely high Cd levels root formation was entirely suppressed whereas *in vivo* the root development was not affected even at extremely high Cd doses. *In vitro* cultured petunia explants did not develop roots and senescence commenced at Cd excess more than 2 x MPC. Experiments with whole petunia plants (in progress) are expected to clarify whether the established low tolerance of the explants *in vitro* corresponds to the physiological response to Cd at conditions *in vivo*.

At least hypothetically, the difference in Cd tolerance *in vitro* and *in vivo* might be dependent on difference in the potency of the naturally developed and young artificially grown plants to detoxify the metal or to prevent its uptake. It might be related to the maturity of the roots and root anatomy. It is found that at excessive Cd levels, the xylem and endodermis tissues of the roots that are in closest contact with Cd, mature faster and change the structure in order to restrict the uptake of the metal and prevent Cd penetration into the cells thus limiting Cd availability to the aboveground organs (Lux et al., 2011). In our trials the *in vitro* cultured plants from ageratum and petunia with underdeveloped or lacking roots showed lower degree of tolerance. In presence even

of highly elevated Cd concentrations the whole ageratum plants developed well formed roots and the plants expressed higher tolerance. This suggests that indeed the difference of plant behavior in the two experimental systems might be attributed, at least partially, to the development and the structural and functional properties of the root system. For better understanding the processes underlying the difference in Cd tolerance of ageratum in artificial conditions and in conditions closer to the natural, supplementary cytological, biochemical and molecular analysis are necessary.

Out of the sterile conditions, the plants are exposed to multiple abiotic and biotic stresses that may augment the harmful effect of heavy metals or restrict the phytoextraction capacity. This may be limiting circumstance for direct use of *in vitro* propagated plants for phytoremediation (Doran, 2009). With this in mind, a relevant question is whether the tolerance of *in vitro* cultured plants is commensurate with the response of whole plants (Doran, 2009). Our study shows that the results obtained from the experiments with ageratum tissue cultures offer reliable preliminary information that the genotype is tolerant to moderate Cd excess. There are also other reports that the heavy metal tolerance of *in vitro* cultured plants occurs at higher degree in the whole plants grown on contaminated substrate. For example, Muszyńska et al. (2018) demonstrated that mature plants of *Dianthus carthusianorum* grown in soil containing excess of Cd showed improved vitality and physiological and biochemical activity than those cultured in presence of Cd *in vitro*. Additionally, it was found that micropropagated *Gypsophila fastigiata* is less sensitive to soil pollution with Cd and Pb (Muszyńska and Hanus-Fajerska, 2017). Together with the mentioned literature data, our finding substantiate the view that *in vitro* test systems can be useful for forecasting the behavior of plants exposed to heavy metals at conditions *in vivo*.

Conclusions

The presented research led to establishment of an *in vitro* test system for preliminary evaluation of the tolerance of *Ageratum houstonianum*, cv. Blue Hawaii and *Petunia x hybrida*, cv, Lavender to Cd stress. Ageratum propagated in tissue culture system appeared as medium tolerant and petunia was determined as low tolerant. The physiological performance estimated by biometrical indices, fresh and dry mass accumulation, electrolyte leakage and overall vitality showed tolerance of ageratum microplants to Cd excess up to 5 x MPC and of petunia to 2 x MPC. The results obtained *in vitro* provided guiding information for the growth response of whole plants in presence of elevated Cd doses. In the pot experiments it was found that the whole plants are less sensitive to Cd toxicity than the *in vitro* cultured ones. The obtained results give a reason to propose *Ageratum houstonianum,* cv. Blue Hawaii as a candidate for cultivation on sites polluted with Cd exceeding MPC up to 25 times.

To the best of our knowledge, the elaborated *in vitro* test system for evaluating the Cd tolerance of studied genotypes has not been reported before. This experimental design is suggested as efficient tool for early diagnosis of the potential of the plants to overcome Cd stress at conditions *in vivo*. The use of *in vitro* models of ornamental plants is an innovative approach that proves to be reliable and promising for investigating and predicting the tolerance of annual ornamental plants to Cd and other heavy metals. However, considering the specificity of the stress response of the diverse genotypes, for forecasting the tolerance of *in vivo* grown plants, case by case *in vitro* tests are needed. The determination of phytoextraction capacity of *in vitr*o and *in vivo* cultured plants may explain the augmented tolerance at conditions *in vivo*. Moreover, such analysis will provide important information for the perspective the studied annual ornamentals to be grown on contaminated soils not only as a safe alternative of trophic horticultural crops but also for eventual application for the purpose of phytoremediation.

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