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Cadmium-induced turion formation of *Spirodela polyrhiza* (L.) Schleiden

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ABSTRACT *Spirodela polyrhiza* (L.) Schleiden (giant duckweed) is an extensively studied representative of duckweeds (Lemnoideae subfamily, Araceae). Despite its widespread application in numerous research areas and well-known capability for producing turions, the effects of various toxicants on turion production are sporadically discussed in the scientific literature. Our aim was to study the growth of *S. polyrhiza* under cadmium stress and to test its potential interference with turion production. Effects of cadmium were assessed in axenic cultures of a local *S. polyrhiza* clone in 7-days-long duckweed growth inhibition tests. Our results indicated that higher cadmium concentrations decreased the relative growth rates of cultures within 3 days and growth inhibition reached its maximum between the 3rd and 5th days with 0.080 mg l⁻¹ IC₅₀. Parallel with slowed frond production - by mirror image pattern - induction of turion formation was observed. The first turions protruded by the 5th day of treatments in the 0.075-0.75 mg l⁻¹ concentration range. Such rapid onset of turion formation is indicative of an almost immediate switch between normal and turion mode of meristem function upon cadmium stress. The consideration of this mechanism in practical applications of *S. polyrhiza* is recommended.

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duckweed
cadmium
growth inhibition
turion
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Spirodela polyrhiza (L.) Schleiden (giant duckweed) is a cosmopolite representative of the Lemnoideae subfamily (Araceae). Although *Lemna gibba* L. and *L. minor* L. are the commonly used species in standardized ecotoxicological test procedures (Environment Canada 2007) *S. polyrhiza* is also widely applied as a model organism in plant physiology, ecotoxicology and bioremediation studies (Gaur et al. 1994; Oláh et al. 2008). This special attention could partly be attributed to its special way of propagation (Chaloupková and Smart 1994). Duckweeds usually produce descendants vegetatively by their meristematic 'pockets' at the proximal end of their fronds. A considerable portion of the subfamily (15 out of 37 species according to Appenroth 2002), however, can produce not only daughter fronds but turions or turion-like fronds as well, which serve as dormant buds for surviving unfavorable periods (Jacobs 1947). *S. polyrhiza* is the most extreme example of this strategy since in temperate regions its winter survival relies exclusively on its turions because normal fronds cannot tolerate low temperatures (Appenroth 2002). Turions are produced by the same meristematic regions as normal vegetative fronds. Jacobs (1947) observed that the growing primordium loses its ability to reversibly switch between normal and turion developmental paths when it reaches approximately 0.2 mm length. According

to Smart and Trewavas (1983) the developing primordium crosses this limit when cell expansion tends to dominate over cell division and the developmental switch is regulated by the internal level of abscisic acid (ABA). The mesophyll of turions is less differentiated, contains high amounts of starch, and lacks aerenchyma, and, as a result, turions sink to the water bottom during unfavorable periods (Jacobs 1947). Structural simplicity and small size of fronds, rapid growth, and easy observation of turion formation qualify *S. polyrhiza* as an ideal model system for investigating regulation of morphogenesis and dormancy in plants (Chaloupková and Smart 1994). Besides their popularity in plant physiological and ecotoxicological research, common advantages of duckweed species make these plants suitable for various fields of practical applications (Wang et al. 2014). Recently duckweeds are considered as potential candidates for bioremediation, waste water treatment, and raw material (e.g. protein and starch) production (Cheng and Stomp 2009).

Despite its extensive application in ecotoxicology and phytoremediation research, the effects of heavy metals on turion formation of *S. polyrhiza* are sparingly discussed and the results are rather contradictory. Xyländer et al. (1993) found that the presence of either cadmium (Cd) or nickel in the nutrient medium inhibited formation of turions. Contrary to that, Srivastava and Jaiswal (1989) found Cd to induce turion formation. Susplugas et al. (2000) reported similar effect of hexavalent chromium. Since the induction or suppression

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Table 1. Relative growth rates (RGR, mean±SE, n=12) of control samples, lowest observed effect Cd-concentrations (LOEC), median growth inhibiting Cd-concentrations (IC₅₀, ±95% confidence intervals), mortality- ('Mortality') and turion formation-inducing concentrations ('Turion formation') of Cd as calculated for the periods between counting days 0th, 3rd, 5th and 7th, respectively. 'Mortality' and 'Turion formation' denote the Cd concentration ranges where negative mean RGRs or protruding turions, respectively, were observed.

	0 th -3 rd days	3 rd -5 th days	5 th -7 th days
RGR _{control} (frond frond ⁻¹ day ⁻¹)	0.401 (±0.011)	0.398 (±0.013)	0.275 (±0.012)
LOEC (mg Cd l ⁻¹)	0.075	0.01	0.05
IC ₅₀ (mg Cd l ⁻¹)	0.297 (±0.088)	0.080 (±0.016)	0.100 (±0.030)
Mortality (mg Cd l ⁻¹)	-	-	0.5-1
Turion formation (mg Cd l ⁻¹)	-	0.075-0.75	0.075-0.75

of turion formation could affect both realized growth rates and quality of the produced biomass, the effects of various chemicals on turion formation are important factors considering the practical applicability of duckweeds. The aim of the present study was to assess the effects of Cd on growth of *S. polyrhiza* over a wide concentration range and to test its potential interference with turion production.

Materials and Methods

Test conditions

Effects of Cd were investigated in duckweed growth inhibition tests. Axenic stock culture of *Spirodela polyrhiza* (L.) Schleiden. was initiated from plants collected in 2004 at lake Kis-Balaton (W. Hungary). Stock cultures were maintained on Steinberg medium (pH 5.5±0.2, Environment Canada 2007) in tissue culturing room of Department of Botany, University of Debrecen (Hungary), under constant temperature (22±2 °C) and irradiation (warm white fluorescent lamps, PPFD: 60±10 µmol m⁻² s⁻¹). 7-10 days prior to growth inhibition tests several plants were subcultured aseptically in 300 cm³ Erlenmeyer's flasks containing 100 ml sterile Steinberg medium in order to maintain exponential growth of cultures. The applied conditions assured that no turion formation occurred during pre-culturing and tests in control cultures.

7-days-long, static growth inhibition tests were performed in 80 mm (diameter) crystallizing dishes containing 100-100 ml test solutions. Cd was applied in form of 3CdSO₄ x 8H₂O salt (Reanal, Hungary) in final concentrations of 0, 0.001, 0.0075, 0.01, 0.025, 0.05, 0.075, 0.1, 0.175, 0.25, 0.375, 0.5, 0.75 and 1 mg Cd l⁻¹, respectively. 2-3 colonies (10±2 fronds) per vessel were used as starting inoculum at the beginning of tests. Growth was characterized by the change in total frond number (FN) of cultures. Each culture was photographed digitally at 0th, 3rd, 5th, and 7th days of tests (Environment Canada 2007). Then digital images were used for counting FN by means of ImageJ (Abramoff et al. 2004). All living, distinguishable fronds were considered.

The same digital images were used for assessing turions produced during the tests. All distinguishable turion pri-

mordia were counted irrespective of their detachment from mother frond.

Data analysis

Relative growth rate (RGR) of cultures was calculated for periods between counting days according to the Environment Canada (2007) guidelines:

$$\text{RGR (frond frond}^{-1} \text{ day}^{-1}) = (\ln(\text{FN}_j) - \ln(\text{FN}_i)) / t_j - t_i$$

where FN_i and FN_j are the observed frond numbers at days t_i and t_j

Based on newly formed turions and fronds the turion yield ratio (T%) of cultures that is production of turions in percentage of total new offshoots during a given period was calculated as follows:

$$\text{T\%} = \text{turions} / (\text{turions} + \text{new fronds})$$

Each applied Cd concentration was tested in 4 parallel cultures per test, and in 2 or 3 independent experiments. For assessing effects of Cd respective results of all parallel treatments were pooled (n = 8-12). Medians were compared by Kruskal-Wallis test and *post-hoc* Mann-Whitney pairwise comparisons using Past 3.01 (Hammer et al. 2001). Effects of different Cd concentrations were considered as significantly different at p<0.05 levels. The lowest observed effect concentration (LOEC) was considered as the lowest applied Cd concentration which resulted in significant (p<0.05) growth inhibition as compared to control. IC₅₀ that is Cd concentration resulting in 50% growth inhibition for a given period of treatments was calculated performing non-linear regressions by means of Sigmaplot 10 (Systat Software). Four parameter logistic curves were fit to mean RGRs plotted as percentage of respective control means.

Results

Inhibiting effects of Cd on FN of *S. polyrhiza* cultures evolved very fast. By the 3rd day of treatments 0.075 mg l⁻¹ and higher concentrations significantly (p<0.05) reduced RGR and 0.75 and 1 mg l⁻¹ Cd almost completely stopped growth of cultures (Fig. 1, Table 1). Prolonged exposure to low Cd concentrations slowed the growth of plants as well. Growth of cultures

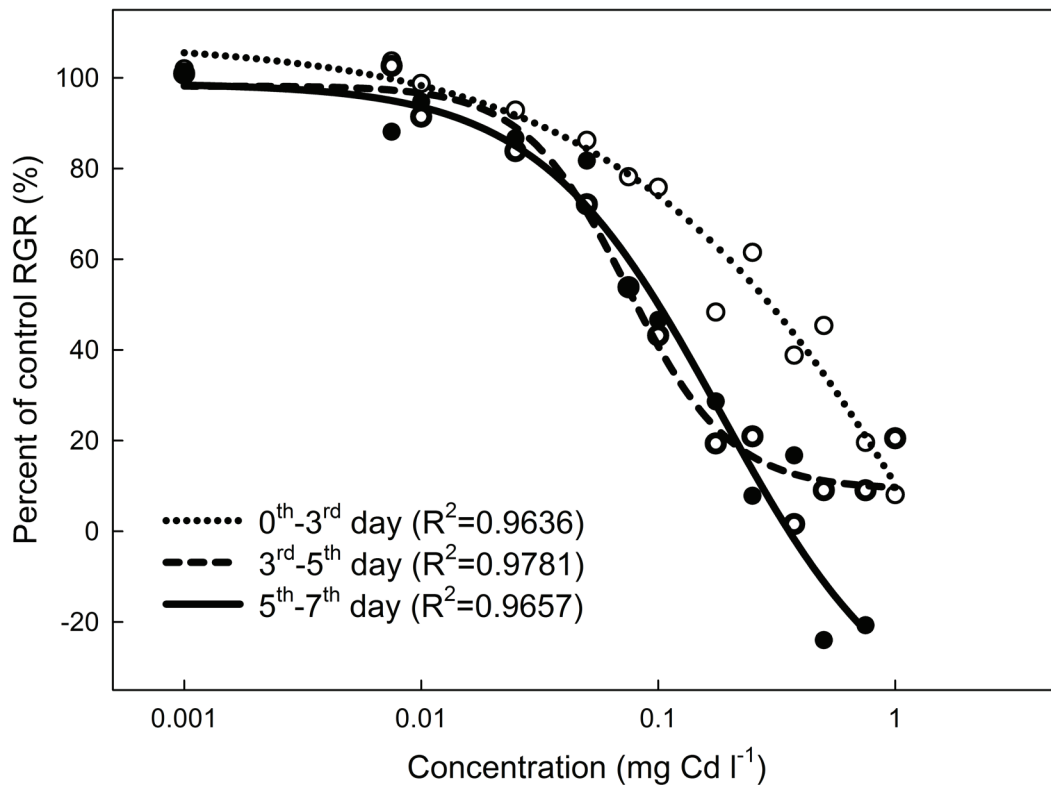


Figure 1. Relative growth rates (RGR) of *S. polyrhiza* cultures calculated on the basis of frond number changes between 0th-3rd, 3rd-5th and 5th-7th days of cadmium treatments, respectively. Regression lines are 4 parameter logistic curves with coefficients of determination in brackets. Symbols denote mean of 8-12 samples as percentiles of mean control RGR.

was significantly ($p < 0.05$) inhibited between the 3rd and 5th days of treatments at 0.01 mg dm⁻³ and higher concentrations. In this period 0.175-1 mg l⁻¹ and higher concentrations lowered growth rates of cultures below 0.1 frond frond⁻¹ day⁻¹ that is increased the doubling times of fronds over 8 days. There was no considerable difference in pattern of growth inhibition over the applied Cd concentration range between periods of 3rd-5th days and 5th-7th days (Fig. 1). 0.25 mg l⁻¹ and higher concentrations stopped entirely production of new fronds or even induced 'negative growth' that is resulted in mortality of existing fronds (Table 1). At 1 mg l⁻¹ Cd proved to be lethal for test cultures within 7 days.

The LOEC and IC₅₀ values showed strong decrease as comparing the periods of 0th-3rd and 3rd-5th days, respectively. However, later (5th-7th days) both LOEC and IC₅₀ showed slight increase (Table 1).

Formation of turions was first observed at the 5th day of exposures. At this point, protruding turions were visible in the 0.075-0.75 mg l⁻¹ concentration range (Table 1). Comparing the turion-inducing Cd concentrations, there was no difference at the 5th and 7th days of exposures, respectively (Table 1). Differentiation of primordia between the 3rd and 7th days of exposures was concentration-dependent and showed clearly

mirror image pattern (Fig. 2). Increase in Cd concentration elevated T% suggesting that a growing proportion of newly formed offshoots became turion. Beyond 0.1 mg l⁻¹ production rate of new fronds dropped strongly and in line with that T% exceeded 45% (Fig. 2). At 0.5 mg dm⁻³ Cd formation of turions became almost exclusive (85±8%) among offshoots in the test cultures. 0.75 mg l⁻¹ Cd -the highest applied non-lethal concentration- accelerated the inhibition on the meristematic activity further and thus both formation of fronds and turions declined. This resulted in still high but somewhat lower T% (63±15%) than 0.5 mg l⁻¹ Cd.

Discussion

Due to rapid doubling of duckweed cultures Cd-induced growth retention took place within a short time interval, even 3 days of exposure proved to be effective. The calculated IC₅₀ values for the assessed three periods (0.297; 0.080 and 0.100 mg l⁻¹ for 0th-3rd, 3rd-5th, and 5th-7th days, respectively) indicate that growth inhibition reached its maximum by the 5th day of exposure stopping completely the production of new fronds at higher concentrations after which the mortality of fronds took place resulting in negative growth rates of cultures. The observed effective concentrations were in the similar range as

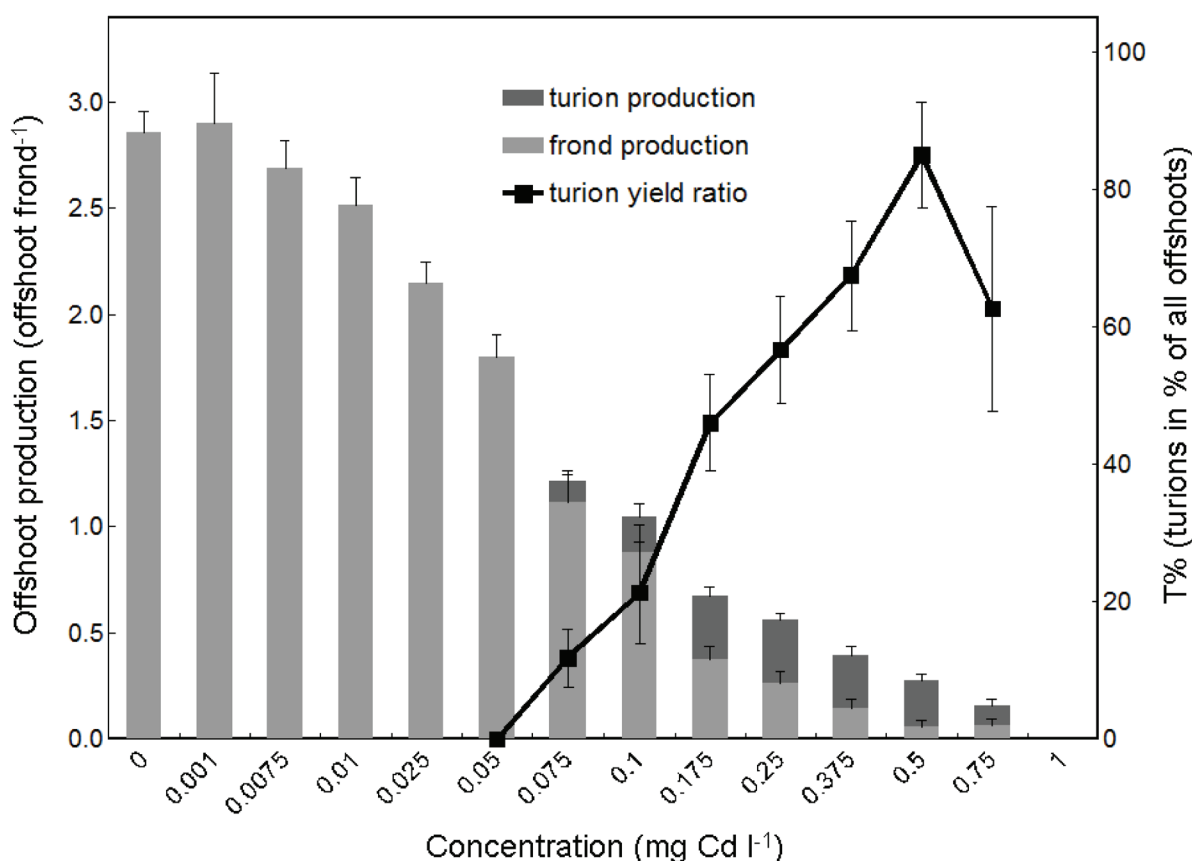


Figure 2. Average number of fronds and turions produced by one mother frond between the 3rd and 7th days of cadmium treatments (offshoot production) and relative proportion of turions in total offshoot production (T%), respectively. Error bars denote standard errors of means (n=8-12).

the literature data. On the basis of fresh weight change Gaur et al. (1994) calculated 0.09 mg l⁻¹ IC₅₀ for *S. polyrhiza* after 4 days of Cd treatments. In 7 days long growth inhibition tests with similar test conditions to ours Naumann et al. (2007) reported 0.323 mg l⁻¹ IC₅₀ based on FN of *L. minor* cultures.

Although *S. polyrhiza* is widely studied in ecotoxicology and bioremediation research effects of heavy metals on its turion formation are scarcely mentioned. The only available report on turion-inducing effect of Cd was provided by Srivastava and Jaiswal (1989). In their paper growth and turion formation of duckweed cultures was monitored at 10th, 20th and 30th days of treatments. Turion formation was first observed after 10 days due to 10 mg l⁻¹ Cd. Although this concentration is one order of magnitude higher than the lethal concentration in our experiments, it induced similar extent of inhibition in growth rate (35%) also leading to turion production. Since they applied similar light and temperature conditions to those in our experiments we assume that the different growth media might be the potential reason for the higher inhibiting dose of Cd. Srivastava and Jaiswal (1989) used different concentrations of major nutrients and also ap-

plied 1% sucrose in the growth medium which might improve the tolerance of plants to Cd.

Rapid onset of turion production in our study suggests that switch of mother frond meristems from normal mode to turion formation takes place almost immediately after exposure to Cd. Since developmental path of offshoot primordium is irreversibly determined after a distinct period of morphogenesis (Jacobs 1947; Smart and Trevas 1983) production of normal fronds continued during the first few days irrespective of Cd treatments. After protrusion of those fronds, the next generation of offshoots clearly reflected turion-inducing effects of Cd. The observed lag in turion formation fits the time range reported for plants under phosphate deficiency (3 days, Appenroth 2002) and ABA treatments (within 4 days, Smart et al. 1995).

Appenroth (2002) grouped turion inducing factors into 2 classes: those in the first one influence turion yield by total productivity of the system (e.g. via conditions of carbon assimilation), while those in the second one are signals for switching between normal and turion mode of meristematic pockets. Rapid appearance of turions in our experiments

suggests that Cd stress serves as a factor for triggering turion mode similarly to the latter group of factors.

Differentiation of primordia into normal fronds versus turions between the 3rd and 7th days of exposures showed distinct bell-shaped response of T% as a function of the applied Cd concentration (Fig. 2). This pattern is very similar to that reported by Smart and Trewavas (1983): they found that turion formation induced by ABA treatments was always accompanied by decreasing rate of frond production and after reaching maximum T% it decreased in parallel with the declining meristematic activity. Srivastava and Jaiswal (1989) and Susplugas et al. (2000) also observed a decrease in vegetative growth when turion formation had started as a result of Cd and chromate stress, respectively.

Although the regulating factors of Cd-induced turion formation were not assessed directly in our study we suggest at least two mechanisms for explanation for formation of turions under Cd stress as based on former reports:

i) Induction of turion formation by Cd might be attributed to its interference with mineral nutrition especially to imbalanced uptake of phosphate (Srivastava and Jaiswal 1989), a well described factor triggering turion formation (Appenroth 2002). Cd is known to inhibit phosphate nutrition of plants (Das et al. 1997) and in the rapidly growing duckweed plants P-deficiency might develop within short time. Appenroth (2002) observed rapid onset of turion formation (3 days) as a result of phosphate limitation which supports the former assumption.

ii) Similarly to other abiotic stress factors heavy metals influence the hormonal regulation of plants (Manara 2012). Enhanced synthesis of stress-related hormones such as ABA, ethylene and salicylic acid and decreased concentration of cytokinins might promote senescence of plants. Internal level of ABA and its relative concentration to cytokinins were proved to be directly linked to turion formation of *S. polyrhiza* by e.g. Smart and Trewavas (1983), Chaloupková and Smart (1994) and Smart et al. (1995).

Further efforts are needed to reveal which of the above mechanisms or perhaps a third one is responsible for alteration of primordium developmental pathway in response to Cd stress. Latest transcriptome analyses on both active *S. polyrhiza* fronds and turions published by Wang et al. (2014) revealed that ABA-responsive element binding factors, ethylene-responsive element binding factors, heat-shock transcription factors, and WRKY transcription factors –which also regulate plants' stress defense – play role in switch from normal to turion mode of meristems.

Based on our results it could be concluded that turion formation of *S. polyrhiza* should have been taken into consideration when this species is used in basic and applied research. Despite the widespread application of giant duckweed in ecotoxicology and plant physiology, turion formation as a response to various toxicants is reported scarcely. Our results

indicate that the switch to turion mode of meristems can occur within the time period of frequently applied growth inhibition tests. Thus, the onset of turion formation may provide important additional information on special physiological effects of a given toxicant.

Another interesting aspect of turion formation might emerge when potential use of duckweeds is discussed as tools for phytoremediation. Such techniques apply plants rapidly increasing their biomass and exhibiting a relatively high affinity to absorb toxicants. As some of these toxicants seem to induce or at least enhance turion production, this mechanism might significantly reduce the expected growth rates of the applied species.

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