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A Kinetic Modelling of the Growth Rate of *Lolium perenne* for Phytotoxicity Bioassays

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Plant growth rate represents one of the main factors in the vegetal-model bioassays design and the development of phytoremediation technologies. *Lolium perenne* is a promising plant to heavy metals monitoring and phytoremediation, but bioassays protocols and toxicity limits are needed. In this research, the effects of cadmium and mercury on germination time and emergence as toxicity markers of *Lolium perenne* plants were determined. Seeds were exposed to increasing concentrations in the range of 0 to 25 mg/L of cadmium (Cd^{2+}) and mercury (Hg^{2+}) in Petri dishes, by independent experiments carried out for 14 d. Emergence, root and shot length were assessed and kinetic parameters of growth were calculated. After 14 d, the maximum germination index of control seeds was 78.7 ± 4.8 %. The treatment of 25 mg/L Cd^{2+} posed an inhibitory effect on the seeds emergence of 24.7 %, and reduced the velocity germination Index (VGI). The maximum germination index of Hg^{2+} treated seeds had not significant differences to control. Toxic effects of Cd^{2+} and Hg^{2+} were found on the development of stem and roots of *Lolium perenne*, however, the range of concentrations which the plant grows well, is considered a suitable condition to potentially act as a phytoremediator. The kinetic parameters of growth are useful to perform toxicity tests and phytoremediation protocols of *Lolium perenne*.

1. Introduction

Environmental changes determine plant development, including seed germination and growing rate. Abiotic stresses, such as salinity, cold, heat, drought and pollutants can potentially affect plants when their different tolerance mechanisms are insufficient (Mithöfer et al., 2004). Several responses are involved from molecular to morphological level to face stress conditions, thus, sensitive and tolerant species are used as sentinels for environmental monitoring and remediation.

Heavy metals are high concern pollutants, which at present need actions aimed to minimize their environmental impact (Qi and Zhang 2017). Metals occurs naturally in ecosystems, some of them are essential for multiple biological processes such as the growth of plants, while others are toxic to living organism, even in low doses, becoming a public health and environmental concern (Prieto Méndez et al., 2009). Due to industrial development, there has been a substantial increase in the concentrations of metals in water ecosystems and soils (Londoño Franco et al., 2016). Likewise, significant quantities of cadmium and mercury are found in soils areas because the use of metal containing products and its poor waste disposal (Reyes et al., 2016). Agriculture, smelter, mining, and tannery are common sources of metal pollution when waste management are poorly addressed. The role of plants in heavy metals issues have been highlighted as promising because their responses to low metal concentrations and accumulation ability (Ali et al., 2013). Thus, *in vitro* to field biomonitoring approaches and plant toxicity tests have been proposed to assess the environmental impacts of heavy metals. Phytoremediation technologies to soil and water treatment has been studied as well. These plants potentialities have encouraged extensive research to the identification of efficient species, and to the characterization of their performance under metal exposure (Rahman et al., 2016).

Soil, sediments and water are the main sinks of heavy metal wastes, becoming pathways to exposure and bioaccumulation to living organisms. Cadmium (Cd) and mercury (Hg) are of special interest due to their biodiversity and human health impacts. Cd has been related to kidney disease and endocrine disorders,

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meanwhile Hg is known because the impact in the nervous system, liver, among others (Rana et al., 2018). Thus, there is a need to monitor and remediate the pollution of heavy metals.

At present, many soil treatment techniques have been developed (Rufino et al., 2012), including physical-chemical, biological, thermal or mixed treatments (Ortiz et al., 2007). Phytoremediation are among the biological treatments, which are characterized by a much lower cost than other alternatives. Phytoremediation emerged as an alternative for the removal of heavy metals, due to the problems and limitations presented by many of the traditional methods. The high costs, secondary waste, the great demands of chemicals, among others, are some of the implications of the traditional methods of removal of soil contaminants (Beltrán et al., 2016).

In *Poaceae* family, *Lolium perenne* is a promising plant to heavy metals monitoring and remediation (Huang et al., 2018) because of its rapid growth, root development, sensitivity to metals (Inostroza-Blancheteau et al., 2017), and adaptability to soil degradation. *Lolium perenne* L. is largely cultivated in tropical soils, however, few studies have been developed on the heavy metal toxicity and phytoremediation capacity. Hence, the aim of present study is to realize a kinetic model of the growth of seeds of *Lolium perenne* in order to evaluate its response under different concentrations of mercury and cadmium, and to develop further metal bioassays and phytoremediation protocols.

2. Materials and methods

2.1 Seed germination and plant growing tests

The emergence and growth *Lolium perenne* were evaluated under different concentrations of cadmium (Cd^{2+}) and mercury (Hg^{2+}) in independent experiments in a completely randomized design. Petri dishes and filter paper were used as growing medium, and 100 commercial seeds of the *Lolium perenne* species (Rye Grass Bestfort plus X CEBA, Colombia) were carefully placed per experimental unit. $CdCl_2$ $2.5H_2O$ (Alfa Aesar ®, United States) and $HgCl_2$ (Sigma-Aldrich ®) were used to prepare stock solutions. The test concentrations of Cd^{2+} and Hg^{2+} were 0.05, 0.10, 0.25, 1.00, 5.00 and 25.00 mg/L, and 5.0 mL were added to each Petri dish respectively. Five replicates were used for each treatment. The growing conditions were room temperature at 18 °C and a 12/12 h light-dark regime. The experiment was checked every day to observe desiccation in the filter paper, and it was moistened with the respective solution when needed. The number of germinated seeds were registered every day, where 1 mm of radical seed emergence was considered as seed germination. After 14 d, the plants were carefully removed and the length of the root and stem were measured on a millimetric basis. To assess the kinetics of plant germination and growing, seed emergence indexes of final germination percentage (FGP), first day of germination (FDG), last day of germination (LDG), and velocity germination index (VGI) and mean germination time (MGT) were calculated (Al-Ansari, F. & Ksiksi, 2016).

2.2 Statistical analysis

Data are presented by the mean and the standard deviation (SD). Germination and length data were checked for normality with Kolmogorov-Smirnov test, and homogeneity of variances with Levene test. In order to determine if there is any relationship between the tested concentrations of the metals and the development of the plants, an ANOVA analysis was performed on the morphology data. Dunnett's multiple comparison test was conducted to analyze significant differences among means of each treatment and control. Differences were considered significant when $p < 0.05$. Simple regression analysis was used to assess possible models to describe the concentration effect on morphological parameters of *Lolium perenne.* The statistical analyses were performed using Statgraphics Centurion XVI and GraphPad Prism 5.0 software.

3. Results

3.1 Effect of cadmium and mercury on *Lolium perenne* germination

The germination rate was reported daily during the 14 d of the experiment. Figure 1 shows the germination rate (%) of *Lolium perenne* seeds exposed to different Cd^{2+} and Hg^{2+} concentrations. In order to assess the effect of the metals on the germination rate in comparison to the control seeds, ANOVA and Dunnett's multiple comparison test were performed when the first germinated seed was observed in control group, when the 50 % of emergence was observed in control seeds, and after 14 d.

The germination of the control seeds started after 4 d (12.7 ± 4.7 %). A later beginning of germination was observed in treated seeds, suggesting a significant effect of the tested concentrations of Cd^{2+} ($p<0.0001$; $F = 25.7$) and Hg^{2+} ($p<0.0001$; $F= 34.3$) on the time of emergence of *Lolium perenne* seeds. An emergence rate greater than 50 % was observed in the control seeds after 7 d. The seeds exposed to Cd^{2+} emerged more than 50 % except for the 25 mg/L Cd^{2+} treatment (38,7 ± 7.8 %), which means a significant inhibitory potential of 42.5% on seeds emergence in comparison to the control seeds ($p = 0.004$; $F = 3.9$).

At the same experiment time, Hg^{2+} treated seeds emerged from 50.0 to 58.7 % without significant differences to the control seeds (p = 0.235). Control seeds showed a maximum germination index of 78.7 ± 4.8 % after 14 d of the experiment. Cd^{2+} had a significant effect (p = 0.015; F = 3.16) for the 25 mg/L treatment (59.3 ± 4.68 %), which posed an inhibitory effect on the seeds emergence of 24.7 %. The maximum germination index of Hg^{2+} treated seeds was lower than average germination of control seeds, ranging from 60.7 to 73.3 %, however, significant differences were not considered (p = 0.070; F = 2.17).

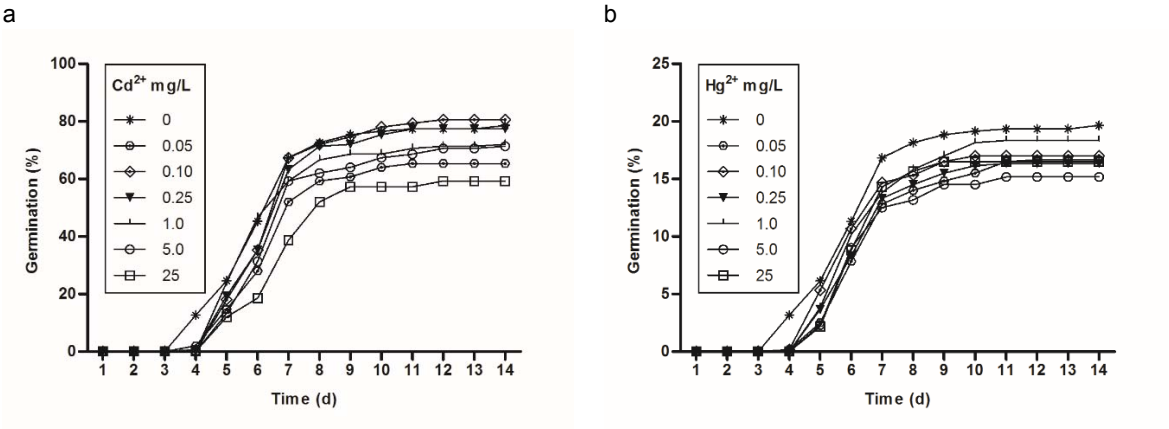

Figure 1: Germination rate of Lolium perenne. a) seeds under Cd^{2+} exposure, b) seeds under Hg^{2+} exposure

The first process of exchange of the seeds with the surrounding medium occurs in the germination stage (Guerrero et al., 2014), and it is in this stage where the changes of the environmental conditions turn out to have a greater impact due to the sensitivity of the seeds (Shah et al., 2010). The toxicity of Hg on seed germination was asessed by Morales and Gallego (2013) on the grass *Brachiaria dictyonerura*, and significant differences were found for 50 and 75 mg/L Hg $^{2+}$. Cd2+ toxicity was reported on seed germination of rice and alfalfa plants by Al-Helal, A. A. (1995). In this study was found that the final germination percentage (FGP) and velocity germination Index (VGI) were different for 25 mg L^{-1} Cd^{2+} treatment.
Effects on the average amount of germinated seeds and VGI were not observed for Hg^{2+} treatments at the conditions of this experiment. Cd^{2+} and Hg^{2+} affected the first day of germination (FDG) at concentrations equal or greater to 0.05 mg/L. The germination parameters are shown in the Table 1.

Table 1: Germination parameters of Lolium perenne under Cd^{2+} and Hg^{2+} exposure

	Parameter				
	FGP (%)	FDG (d)	LDG (d)	VGI	MGT (d)
Cd^{2+} (mg L^{-1})					
0.05	65.3 (13.8)	4.7 (0.5)*	10.2 (0.8)	1.2 (0.2)	13.9 (3.1)
0.10	80.7 (11.4)	5.0 (0.0)*	10.8 (1.0)	1.4 (0.2)	16.7 (2.9)
0.25	77.3 (13.1)	5.2 (0.4)*	10.2 (0.8)	1.4 (0.2)	16.8 (3.3)
1.0	72.0 (9.1)	5.2 (0.4)*	10.2 (2.3)	1.3 (0.2)	14.1 (1.8)
5.0	71.3 (12.8)	5.3 (0.5)*	10.8 (2.0)	1.3 (0.2)	16.3 (3.5)
25	59.3 (4.7)*	5.3 (0.5)*	9.7 (1.9)	1.1 (0.1)*	12.4 (1.1)
Hg^{2+} (mg L^{-1})					
0.05	66.7 (12.0)	4.8 (0.4)*	10.7 (1.4)	1.2 (0.2)	13.8 (2.6)
0.10	68.0 (6.7)	4.8 (0.4)*	9.8 (3.3)	1.2 (0.1)	14.6 (1.5)
0.25	65.3 (15.3)	5.0 (0.0)*	9.8 (0.8)	1.2 (0.3)	14.3 (3.7)
1.0	73.3 (8.3)	5.3 (0.5)*	10.0 (0.9)	1.3 (0.1)	14.2 (1.3)
5.0	60.7 (3.0)	5.2 (0.4)*	10.2 (1.3)	1.1 (0.1)	14.2 (1.1)
25	66.0 (12.1)	5.3 (0.5)*	8.3 (0.8)	1.2 (0.2)	14.4 (2.8)
Control	78.7 (4.8)	4.0 (0.0)	10.3 (2.9)	1.4 (0.1)	14.5 (0.5)

3.2 Effect of cadmium and mercury on *Lolium perenne* growth

Root and shoot length were assessed after 14 d of the experiment. In the case of Hg an inhibition effect was observed in the root and not in the stem. For the root, it is necessary that at higher concentrations its elongation decreases, however, for the response curve of concentration-growth of the stem we have the U-shape typical of response to hormetic metal ions (Poschenrieder et al., 2013). This duality in the response of the organs of the plant can be explained by the tolerance mechanism used by each one (Guerrero et al., 2014).With respect to the response caused by the Cd, it was found that there is no clear growth response with respect to the increase of contaminant for any of the organs of the plant. However, for a concentration of 0.25 ppm a positive response was observed for the root and stem. Therefore, this could be considered the dose where the stimulation of growth is presented as a response to a compensatory adaptation process (Poschenrieder et al., 2013).

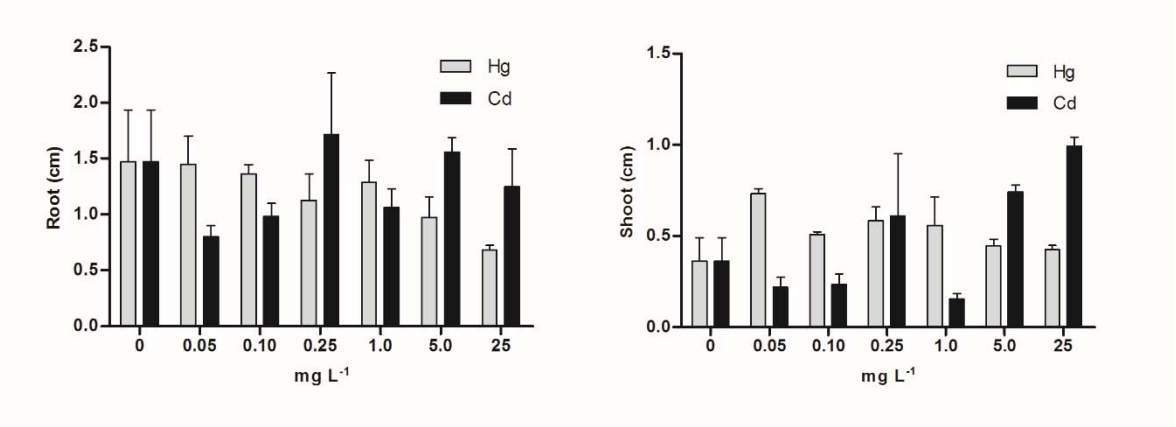

Figure 2: Cd^{2+} and Hg^{2+} effect on Root (a) and Shoot (b) length development.

Cd and Hg toxicity on roots was assessed by the *L. sativa* bioassay, and the toxicity was reported at 0.26 mg/L Hg and 0.15 mg/L Cd (Lyu et al., 2018). Morales and Gallego (2013) reported root toxicity for 25 mg/L Hg^{2+} and suggested regression models to assess the dose-dependent effect of metals. In this study, regression tests were performed to analyze the model of inhibition of root and shoot of *Lolium perene* under mercury and cadmium exposure. The best predictive function (R^2 = 77,80 %) was found for root length under mercury effect (figure 3). The resulting equation Eq(1) corresponds to the model Inverse-Y Square Root-X, with a correlation coefficient of 0.882 (p=0.000).

$$Root\ (cm) = \frac{1}{0.71 + 0.15\sqrt{Hg^{2+}}} \quad (1)$$

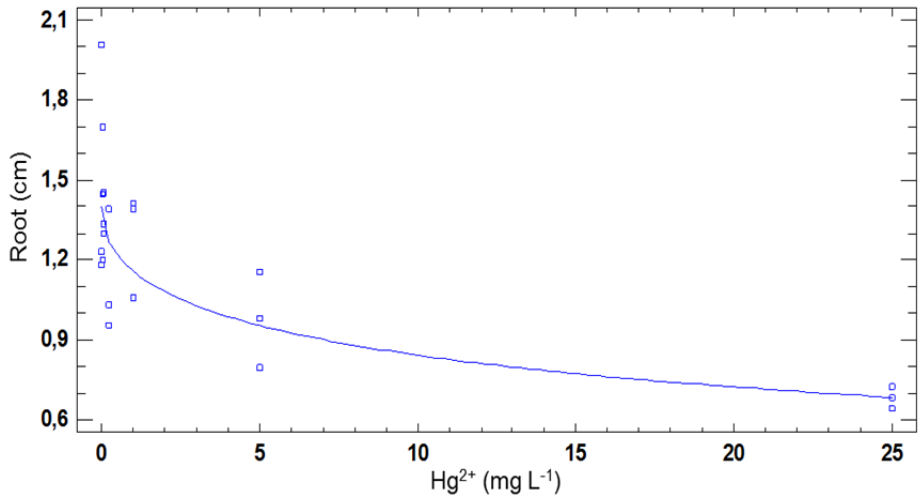

Figure 3: Regression model of root inhibition under Hg^{2+} exposure.

With the statistical analysis, it was determined that the effect of growth inhibition caused by the contaminant was greater when the plant was exposed to Cd than to Hg, this for both the stem and the root (Figure 2). However, when comparing the different levels with the control, it was observed that not necessarily the metal in all the concentrations has a negative effect on the seed (Kumar et al., 2016). This may be the result of hormesis and homeostasis processes, in which the plant uses the contaminant load as a stimulant for growth, or has the ability to maintain a stable system despite environmental changes (Poschenrieder et al., 2013). Plants grow at widely differing rates, and had developed different adaptations to environmental stressors. Thus, the kinetic model and the assessment of *Lolium perenne* growing is important for the understanding of future bioassays and phytoremediation technologies.

4. Conclusion

Seeds emergency and early root and shoot development are crucial for an effective plant bioassay or phytoremediation technology. Germination and growing parameters of *Lollium perene* were assessed under heavy metal stress. *Lolium perenne* seeds had the ability to germinate under a wide range of Hg and Cd concentrations, showing none significant changes on their kinetic parameters. Shot and root development was sensitive to Hg and Cd concentrations, which suggest the plant as a monitoring and bioassay model. *Lolium perenne* had the ability to grow despite toxicity conditions, in addition, growth parameters are suitable for a wide range of Hg and Cd concentrations. Therefore, *Lolium perenne* is a candidate for phytoremediation protocols and heavy metal bioassays.

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