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*Original Research*

# Cadmium Phytotoxicity on Seed Germination, Early Growth and the Differential Antioxidant Response of Guaiacol Peroxidase in *Phragmites australis* Seedling Organs

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**Abstract**

The Cd-inhibition effects on *Phragmites australis* germination characteristics, early seedling growth, and the antioxidant activity response of Guaiacol peroxidase enzyme (GPOx) in seedling organs were investigated under various cadmium (Cd) levels. The final germination percentage (GP%), germination index (GI), mean germination time (MGT), and time to 50% of germination (T50%) indices indicated that Cd inhibited the germination percentage and delayed the start of germination in a dose-dependent manner. A slight increase in the order of parts per billion of Cd negatively affected the rate and speed of germination. This high susceptibility partly explains why the common reed depends mainly on vegetative propagation. The root and shoot length indices (RLI and SLI) measured at 10-day intervals for 30 days showed that shoot growth was susceptible during the first ten days of germination, whereas root growth was more affected after 30 days. It is most likely related to the damaging effects of Cd sequestered in roots, as *P. australis* is an excluder species. The GPOx antioxidant response was found to be dose and organ-dependent. The leaf/root GPOx ratio was >1 in control, and seedlings grew under 10, 30, and 100 ppb. In contrast, the leaf/root GPOx ratio was <1 in seedlings grown under 100, 500, and 1000 ppm, where some phytotoxicity symptoms such as necrotic roots, yellowing, and abnormal leaves were observed. The pattern of changes in GPOx activity observed in the current study indicates that *P. australis* may have a mechanism to regulate its GPOx

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activity response, not only according to the intensity of the Cd dose and plant organ sensitivity but also according to the stage of seedling development, probably for optimal nutrient uptake and growth.

**Keywords**: cadmium, germination indices, seedling early growth, guaiacol peroxidase, *Phragmites australis*

## Introduction

During the past decade, cadmium (Cd) has received increasing attention due to its involvement in food chain contamination [1, 2]. Plants are considered the main pathway of Cd entry into the trophic chain. Cd enters plants via the roots through a similar pathway for zinc or calcium transport and then is translocated to shoots in an ionic form and/or via the ascent of sap [3, 4]. Cadmium is probably the most damaging metal to plant species; with a long biological half-life of about 25-30 years, it can be taken up by plants, disrupting cell homeostasis and triggering several metabolic pathways [5, 6]. Cd is a phytotoxic metal and is non-essential for plant metabolism. However, many species take up this element, which inhibits several physiological processes, including nutrient and water uptake, photosynthesis, respiration, enzymatic activity, seed germination, and seedling growth [7-9]. To cope with Cd stress, plants have evolved multifarious adaptive mechanisms [10]. Resistance mechanisms may be accomplished by one of two strategies: avoiding or tolerating the effects of internal stress [11]. Plants implement avoidance strategies to prevent metal access to root cells and restrict entry and movement into plant tissue through the production of organic acids, chelation, sequestration, and complexation by root exudates [12]. Tolerance strategies refer to plant responses to cope with heavy metal toxicity after cell intrusion. The tolerance mechanism includes uptake, transport, sequestration, and chelation of heavy metals by binding them to peptides and proteins [13], changes in gene expression, metal ion homeostasis, and compartmentalization of ligand-metal complexes [10]. Maintaining high enzymatic and non-enzymatic antioxidant capacities is also crucial to eliminating toxic levels of reactive oxygen species (ROS). ROS are oxygen-containing radicals very harmful to plant cells, over-generated under Cd stress, such as hydrogen peroxide ($H_2O_2$), superoxide anion radical (O2• −), and hydroxyl radical (OH•) [14, 15]. The main ROS-eliminating enzymes involved in detoxification include guaiacol peroxidase (GPOx), catalase (CAT), glutathione (GSH), superoxide dismutase (SOD), ascorbate peroxidase (APX), and monodehydroascorbate reductase (MDHAR). The non-enzymatic antioxidants involve proline, ascorbic acid, carotenoids, flavonoids and polyphenols [16, 17]. Heavy metal tolerant plants or metallophytes can be classified into three categories: *(i)* excluders when the concentration of heavy metals in the shoot remains constant and low over a wide range of soil concentrations [18]. The excluder plants have a high level of heavy metal in their roots and exhibit a transfer factor shoot/root <than 1 [19]; *(ii)* indicators, where uptake and transport of metals to shoots are regulated so that internal concentration reflects external levels [18], whereas *(iii)* accumulator plants can transport the majority of absorbed heavy metals to the shoots, with a transfer factor shoot/root >than 1 [18, 20]. *Phragmites australis* (Cav.) Trin. ex Steud, or common reed, is an excluder plant for Cd [21, 22]. One of the most widely distributed of all angiosperms, it is found worldwide, mainly in estuaries or wetlands, where it forms reed beds, which are a key habitat for several wetland bird species [23]. This species has a high ability to accumulate various nutrients, heavy metals, and micropollutants. It is also widely used in the treatment of heavy metal-contaminated wastewater [24, 25]. Common reed is propagated laterally by vegetative multiplication in the local area, but it spreads to new zone and habitats through seed [26]. Although extensive research has been carried out on mechanisms involved in tolerance strategies of *P. australis* reeds to Cd, few studies have been interested in the effects of Cd on juvenile seedlings of this excluder species. Furthermore, rare studies were conducted to investigate the effects of Cd on seed germination, early growth, and the antioxidant enzymatic responses of *P. australis* seedlings emerging from the seed. In addition, the rare phytotoxicological studies conducted on *P. australis* seed germination have not used sufficient indices to examine the effect of Cd on germination characteristics and seedling growth. Using a single index as the final germination rate may not provide a realistic assessment of cadmium's effects on the germination process. In the current study, employing six different indices (GP%, GI, MGT, T50%, RLI, and SLI) could satisfactorily differentiate the germination patterns and the seedling growth aspects under the various used Cd levels. To the best of our knowledge, the GPOx activity response in *P. australis* seedlings that emerged from seeds exposed to cadmium has not been investigated. Therefore, assessing this enzyme in the seedling organs is essential to understanding its involvement in the antioxidant defense system. Investigating Cd effects on germination, early seedling growth, and the antioxidant enzyme responses constitutes an interesting tool, not only for estimating the potential of juvenile seedling to cope with Cd stress but also for assessing the potential of this species to spread and establish through seed in contaminated habitats.

In this context, the main aims of the present study are: 1) to evaluate the Cd-inhibition effect on *P. australis* germination characteristics; 2) to estimate

the sensitivity degree of seed germination, initial growth (early seedling stages) to low and high concentrations of Cd using germination indices; and 3) to discuss the differential antioxidant activity response of GPOx in seedlings' leaves, stems, and roots under various Cd levels.

## Materials and Methods

### Germination Experiment

The germination test was conducted following the standard method of the United States environmental protection agency (USEPA) using the ecological effects test guidelines protocol [27], and the seedling emergence and growth test of the Organization for Economic Cooperation and Development [28]. Seeds of *P. australis* were obtained from two-year-old reeds cultured in an outdoor hydroponic system using a 1/4 dilution of NPK (20-20-20) as a source of nutrients. The hydroponic system was installed in Tébéssa department, in the North-East of Algeria. The two-year-old reeds were grown from rhizomes obtained from a natural population growing at Babar dam (35°10'31.1"N and 7°01'15.4"E) in the North-East of Algeria. No heavy metals or organic pollutants were signaled in the dam water. We collected seeds from the middle part of the inflorescence to obtain homogeneous seeds in terms of weight and size by using sieves (seed weight: mg/100 seeds = 16.77±0.11 mg/n = 21). After collecting, all seeds were stored in the dark at 4ºC until germination trial commencement.

### Phytotoxicity Test

Seeds were first checked for viability by suspending them in deionized water, and those which settled to the bottom with unbroken seed coats were selected for further study. The selected seeds were soaked in a 6% sodium hypochlorite solution for 5 minutes and rinsed with deionized water for 10 minutes. Germination plates (14 cm in diameter) were autoclaved for 15 min at 121ºC prior to experiments to minimize fungal and bacterial contamination. The bioassay experiment was carried out with seven groups: an untreated control group and six groups treated with increasing Cd concentrations (10, 30, and 100 ppb) and (100, 500, and 1000 ppm). The three high concentrations (100, 500, and 1000 ppm) were tested to assess whether seeds and seedlings could also tolerate high levels of Cd as in mature *P. australis* reed. The three low concentrations (10, 30, and 100 ppb) were used to evaluate the sensitivity level of *P. australis* seed and seedlings to Cd. Doses of Cd were analyzed immediately after preparation by NexION® 300D inductively coupled plasma mass spectrometry (ICP-MS: Perkin Elmer Inc., Waltham, MA, USA). The results were as follows: Cd (10 ppb) = 10±2.4; Cd (30 ppb) = 30±3.1; Cd (100 ppb) = 100±1.8; Cd (100 ppm) = 100±6.73; Cd (500 ppm) = 500±4.41 and Cd (1000 ppm) = 1000±6.02 (n = 4). The bioassay experiment was conducted using 2100 seeds and carried out with 3 independent replicates, for a total of 700 seeds per replicate and 100 seeds per treatment. One hundred (100) seeds were placed in each germination plates, the germination medium contains 15ml of Cd chloride ($CdCl_2$, $H_2O$) (98% of purity, Merck chemicals) and 1/4 of Hoagland nutrient solution (Basal Salt Mixture: Sigma-Aldrich, Cat. no: H2395): $(NH_4)_3PO_4$ (115.03 $mgl^{-1}$), $H_3BO_3$ (2.86 $mgl^{-1}$), Ca $(NO_3)_2$ (656.4 $mgl^{-1}$), $CuSO_4$ (0.08 $mgl^{-1}$), $Fe_2(C_4H_4O_6)_3$ (5.32 $mgl^{-1}$), $MgSO_4$ (240.76 $mgl^{-1}$), $MnCl_2$ (1.81 $mgl^{-1}$), $MoO_3$ (0.016 $mgl^{-1}$), $KNO_3$ (606.6 $mgl^{-1}$), $ZnSO_4$ (0.22 $mgl^{-1}$). The germination plates were then maintained under greenhouse conditions at temperatures 23±3ºC (day) and 11±4ºC (night) with a light intensity of ≥5400 lux and 12 h photoperiods. The alternating temperatures and photoperiods were similar to the field conditions during spring in northeast Algeria, providing favorable conditions for *P. australis* germination and seedlings [29, 22].

### Germination Indices

During 20 days, newly germinated seeds were counted. The seed was considered germinated when the radicle was about 1 mm in length [29]. Alternatively, when 2 mm of the shoot had emerged, if no radicle had emerged [30]. In our study, germination was considered complete when no further germination occurred for five successive days. Six germination indices were determined: Final germination percentage (GP%), Mean germination time (MGT), Time to 50% of germination (T50%), Germination index (GI), Radicle and root length index (RLI), Shoot length index (SLI) (Fig. 1).

*Final germination percentage (GP%)*

$$GP\% = \frac{Ng}{Nt} \times 100 \quad (1)$$

*Mean Germination Time (MGT)*

$$MGT = \frac{\sum(t \times n)}{\sum n} \ (day) \quad (2)$$

where: t = the time in days from day (0) to the end of the germination test, and n is the number of germinated seeds on the day t [31].

*Time to 50% of Germination (T50%)*

Time to 50% of germination is the time to reach 50% of final germination, computed according to the formula by Farooq et al. ( 2005) [32] as follows:

$$T50\% = t_i - \frac{(n/2 - n_i)\ x\ (t_j - t_i)}{(n_j - n_i)} \ (day) \quad (3)$$

Where: *n* is the final number of seeds germinated, *ni* and $n_j$ are total number of seeds germinated by adjacent counts at time $t_i$ and $t_j$, where: $n_i < \frac{(n+1)}{2} < n_j$

*Germination Index (GI)*

The germination index is the germination rate in terms of the total number of seeds that germinate in a time interval. The index was calculated as described by the Association of Official Seed Analysts (AOSA) by the following equation [33]:

$$GI = \frac{No.of\ germinated\ seeds}{days\ of\ first\ count} + \cdots + \frac{No.of\ germinated\ seeds}{Days\ of\ final\ count}\ (seed \cdot day^{-1}) \quad (4)$$

*Seedling Length Indices*

The root and shoot length indices of germinated seeds were measured at 10-day intervals (10, 20, and 30 days) for 15 randomly chosen seedlings for each treatment and per period (n = 15). The 3 measurement periods were chosen to evaluate the effect of Cd on seedlings at different stages of growth. Seedlings were scanned (Epson perfection v700) and measured by the image software Mesurim Pro version 3.4 (Madre, Academy of Amiens, France). The radicle and root lengths were measured from the root-shoot junction to the tip of the root using the following formula:

$$RLI\ (\%) = \frac{Length\ of\ the\ longest\ radicle\ in\ treatment}{Length\ of\ the\ longest\ radicle\ in\ control} \times 100 \quad (5)$$

Shoot length was measured from the root-shoot junction to the tip of the shoot using the following formula:

$$SLI\ (\%) = \frac{Length\ of\ the\ shoot\ in\ treatment}{Length\ of\ the\ shoot\ in\ control} \times 100 \quad (6)$$

*Assay of Guaïcol-Peroxidase Activity (GPOx)*

Guaiacol peroxidase (EC 1.11.1.7; GPOx) activity was measured in the leaves, stems and roots of *P. australis* seedlings exposed to different doses of cadmium. 0.5 g of each sample was frozen in liquid nitrogen and stored at -80ºC until assay commencement. Samples were homogenized (separately) in 5 ml of phosphate buffer (50 mM, pH 7.5). The homogenate was then centrifuged at 12000 g for 20 min at 4ºC, and the resultant supernatant was used to measure the activity of GPOx [34]. The assay is based on using Guaïcol (2-Methoxyphenol) as a hydrogen donor. In the presence of hydrogen peroxide, the tetra Guaïcol is formed, which has an absorption maximum at 470 nm. The kinetic evolution of absorbance at 470 nm was measured during 1 min [35]. GPOx activity was calculated using the molar extinction coefficient of tetra Guaïcol (ε = 26.6 $\mu M^{-1}.cm^{-1}$) and expressed as µmol of guaiacol oxidized per min per mg of protein at 25ºC. For a final volume of 3 ml, the reaction mixture contains 100 µl of enzyme extract, 50 µl of 0.3% $H_2O_2$, and 2850 µl of Guaïcol-phosphate buffer (50 mM NaK, 8 mM of Guaïcol, pH 7.2). The reaction is initiated by adding hydrogen peroxide [36].

Statistical Analysis

A split-plot design was used for the data analysis. Germination indices and guaïcol-peroxidase activity (GPOx) data were presented as the mean values±standard deviation (SD) of three independent experiments. Data were tested for significant differences between treatments using a one-way analysis of variance (ANOVA), $p \leq 0.05$. Data of seedling length indices RLI and SLI of each period (10, 20, and 30 days) was tested separately for significant differences between the groups (including the control group). Tukey t-test at p = 0.05 was used as a mean separation test for significant indices. Finally, a principal component analysis (PCA) was performed to identify correlations between data and the major patterns of variation of germination characteristics, early growth indices, and guaiacol peroxidase activity (GPOx). Data analyses were conducted using IBM SPSS Version 25 (SPSS Inc. 2011).

## Results

Final Germination Percentage (GP%)

The germination response of *P. australis* under six Cd treatments and control group is shown in Fig. 1. The results showed that *P. australis* seeds germinated in all tested doses. However, treated seeds started germination one day after the control seeds. In addition, all tested doses reduced the final germination percentage, and the downward trend in germination was dose-dependent.

The highest final germination percentage was recorded in the control group 95.33% (±4.61 SE). On day 20 of the test, all treated groups showed a significant decrease when compared to the control group except in the 10 ppb and 30 ppb groups, where we recorded 91% (±3 SE) and 87.33% (±4.04 SE), respectively ($F_{6,14}$ = 50.6; p = 0.000<0.05). While the lowest germination rate of 52.66% (±3.05 SE) has been recorded at 1000 ppm Cd treatment (Table 1).

Mean Germination Time (MGT)

The mean germination time (MGT) index results are shown in Table 1. The results indicate that all Cd doses

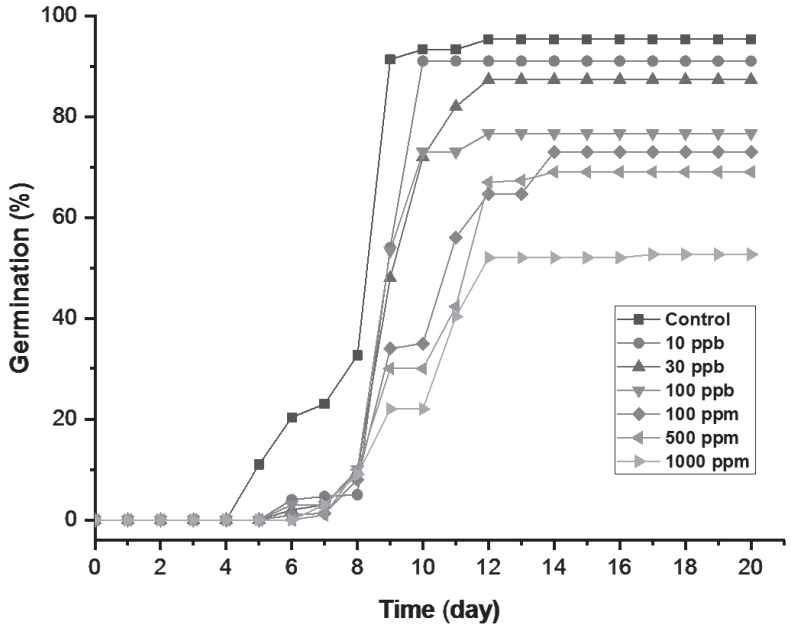


Fig. 1. Final germination percentage of *P. australis* seeds during 20 days exposed to different concentrations of Cd *(n = 3)*

delayed and inhibited *P. australis* seed germination. Compared to the control group, the differences were statistically significant ($F_{6,14}$ = 90.79; p = 0.000<0.05). The post-hoc Tukey test revealed three homogeneous sets: the control group, the second (groups treated with 10, 30, and 100 ppb), and the third (groups treated with 100, 500, and 1000 ppm). Compared to what was recorded in the control group MGT = 8.13 days, the largest mean germination time was recorded in the 100, 500, and 1000 ppm groups, with 10.54, 10.40, and 10.20 days, respectively. The MGT index revealed that *P. australis* seed was susceptible to Cd; even the lowest dose (10 ppb) delayed seed germination, where MGT was 9.37 days.

## Time to 50% of Germination (T50%)

The time required to reach 50% of the final germination corroborates the MGT index results; all Cd doses caused a delay in germination (Table 1). The T50% index values were higher in the treated groups compared to the control group ($F_{6,14}$ = 36.93; p = 0.000<0.05). However, differences between the control group and those treated with 10, 30, and 100 ppb were not significant. In contrast to groups treated with 100, 500, and 1000 ppm, the differences were significant. Compared to the control group, 500 and 1000 ppm doses delayed the T50% of germination by more than two days.

## Germination Index (GI)

The germination index (GI) is defined as a weighted sum of the daily numbers of germinated seeds [37]. The index value is the correlation between the daily germination rate and the maximum germination rate. In the present study, the GI values in all treated groups were significantly lower than in the control group (Table 1). The GI index decreased gradually with increasing Cd concentrations. The control group had the highest index value GI = 12.24 seed.day -1 (±0.18 SE), while the group treated with 1000 ppm had the lowest index value of GI = 5.31 seed.day -1 (±0.36 SE). The phytotoxicity test revealed a significant negative effect of Cd on ($F_{6,14}$ = 147.32; p = 0.000<0.05). However, no significant differences were observed between groups of seeds treated with 10 and 30 ppb, also between 100 and 500 ppm groups.

## Seedling Length Indices

Root and shoot length indices of germinated seeds measured at 10, 20 and 30 days are shown in Fig. 2.

### *Root Length Index (RLI)*

The results of the bioassay after 10 days revealed a dose-dependent effect of Cd on root growth ($F_{6,98}$ = 143.70; p = 0.000<0.05) (Fig. 2a). The inhibition in length growth was evident on all seedlings. Compared to the control group, all treated seeds showed a significant reduction, except for the group in which the seeds were treated with 10 ppb; we recorded a very slight reduction of root length index RLI = 95,5% (±8.12 SE). Unlike the other groups, especially those treated with 100, 500, and 1000 ppm, the values of the root length index were RLI≤51%. Regarding RLI values after 20 days (Fig. 2b), and 30 days (Fig. 2c) the results were comparable to those found after 10 days ($F_{6,98}$ = 80.55; P = 0.000<0.05) and ($F_{6,98}$ = 180.21;

Table 1. Final germination percentage (GP%), Mean germination time (MGT), Time to 50% of germination (T50%), Germination index (GI), of *P. australis* seed at different concentrations of cadmium measured at the 20th day. The values with different superscript letters in a column are significantly different (p≤0.05, Tukey's test). The values represent Means (±Sd) of 3 replicates of 100 seeds each. The highest values receive the letter (a).

| Treatment | Germination Indices | | | |
|---|---|---|---|---|
| | GP (%) | MGT (Day) | T 50% (Day) | GI (Seed day$^{-1}$) |
| Control | 95.33±4.61 $^{a}$ | 8.13±0.27 $^{c}$ | 8.25±0.067 $^{b}$ | 12.24±0.18 $^{a}$ |
| 10 ppb | 91.00±3.00 $^{a}$ | 9.37±0.61 $^{b}$ | 8.81±0.046 $^{b}$ | 9.87±0.29 $^{b}$ |
| 30 ppb | 87.33±4.04 $^{a}$ | 9.33±0.14 $^{b}$ | 8.79±0.070 $^{b}$ | 9.47±0.48 $^{b}$ |
| 100 ppb | 76.66±1.52 $^{b}$ | 9.16±0.11 $^{b}$ | 8.65±0.072 $^{b}$ | 8.50±0.13 $^{c}$ |
| 100 ppm | 73.00±2.64 $^{b}$ | 10.54±0.12 $^{a}$ | 9.90±0.59 $^{a}$ | 7.17±0.32 $^{d}$ |
| 500 ppm | 69.00±5.00 $^{b}$ | 10.40±0.16 $^{a}$ | 10.36±0.18 $^{a}$ | 6.81±0.40 $^{d}$ |
| 1000 ppm | 52.66±3.05 $^{c}$ | 10.20±0.15 $^{a}$ | 10.25±0.12 $^{a}$ | 5.31±0.36 $^{e}$ |

P = 0.000<0.05), respectively. Compared to the control group, we found a significant reduction in root growth in all treated groups except in seeds treated with 10 ppb after 20 days. Overall, the reduction in root length growth was more significant in the groups treated with 100, 500, and 1000 ppm of Cd. The negative effect of Cd was more pronounced after 30 days than after 10 and 20 days of treatment. In addition, we observed necrosis tissue in the roots of seeds treated with 100, 500, and 1000 ppm after 20 days of the experiment.

### *Shoot Length Index (SLI)*

The results of the shoot length index (SLI) of *P. australis* seedlings under six Cd doses are shown in Fig. 2 (d, e, and f). After 10 days of germination, the obtained results showed a strong negative effect of Cd on shoot length growth (Fig. 2d) ($F_{6,98}$ = 231.86; p = 0.000<0.05). Compared to the control group, a significant decrease in SLI index value was found in all treated groups. Except for the 10 ppm Cd dose, the inhibitory effect on shoot length growth was similar in all other doses. The highest SLI was 70.30% (±8.23 SE) in the group treated with 10 ppb while the lowest was SLI = 29.75% (±9.40 SE) in the group treated with 1000 ppm. Compared to the control group of each period, the results after 20 and 30 days of the experiment were similar ($F_{6,98}$ = 220.97; P = 0.000<0.05), and ($F_{6,98}$ = 181.92; P = 0.000<0.05), respectively. The values of the SLI index in most treated groups showed a significant decrease compared to the control group, except for the group treated by 10 ppm, where a slight increase in SLI index was recorded. Compared to day-10 and day-20 of the experiment, the inhibition effect of Cd on shoot growth appears to be less significant at day-30. In particular, in the groups of seeds treated by 10, 30, and 100 pbb. This indicates that the growth in length of the shoot was more sensitive to Cd in the first 10 days. The seedling's morphological structure revealed an increase in the prevalence of abnormal seedlings, including leaf atrophy, asymmetric development, and twisted leaves, particularly in the 500 and 1000 ppm groups.

## Guaïcol-Peroxidase Activity (GPOx)

After 30 days of germination, a positive dose-response effect of cadmium on GPOx activity was observed in leaves, stems, and roots (Table 2). In all seedling's organs, GPOx activity increased gradually as cadmium concentrations increased. The maximum activity was recorded under 500 or 1000 ppm except in leaves, where the peak induction was recorded under 100 ppm (GPOx = 37.80 µM. min$^{-1}$ mg$^{-1}$ Fw), then the activity declined significantly in groups treated with 500 and 1000 ppm to 33.85 and 29.03 µM. min$^{-1}$ mg$^{-1}$ Fw, respectively. In contrast to what was observed at high Cd levels (100, 500, and 1000 ppm), the stimulation of GPOx activity in all seedling organs at low levels (especially at 10 and 30 ppb) was not significantly different from that of the control group. In roots, there were no statistically significant differences between GPOx activity in the control group and the groups treated with 10, 30, and 100 ppb. Also, no significant differences were found between groups exposed to 500 and 1000 ppm of cadmium. The leaf/root GPOx activity ratio was greater than 1 in the control group and groups treated with low levels of Cd. However, the ratio was less than <1 under the high Cd levels groups. This demonstrates a doubling of GPOx activity in the roots as compared to the leaves (Table 2).

## The Principal Component Analysis

The principal component analysis (PCA) performed on the 15 selected parameters (Fig. 3) indicates that the first two principal components ($\lambda_1$ = 12.57, $\lambda_2$ = 1.41) explain cumulatively 93.24% of the total variance. Variations in the measured indices are mainly described

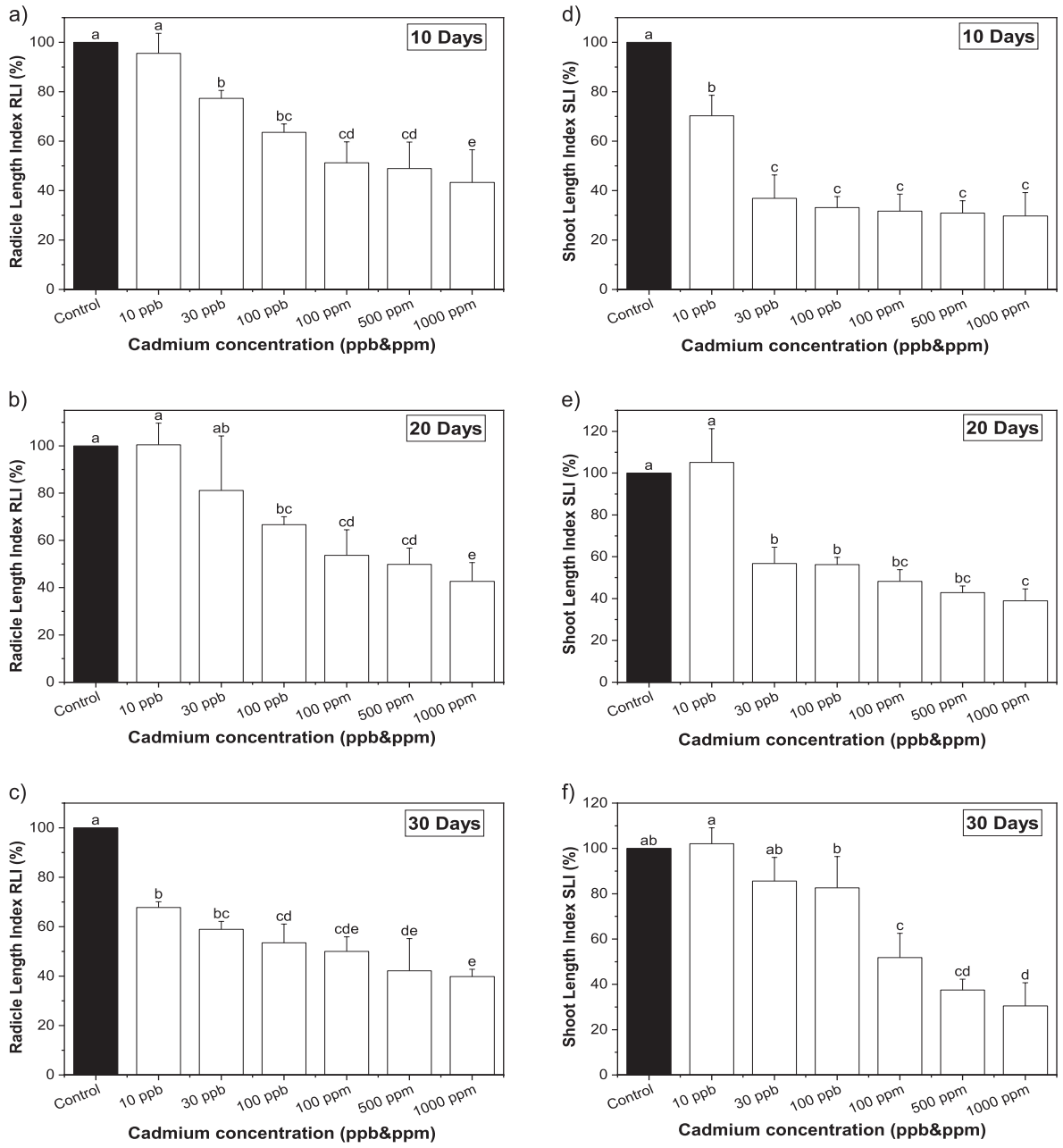


Fig. 2. Radical and shoot length indices of germinated seeds at 10, 20, and 30 days exposed to different cadmium concentrations. The data is an average observation from three independent experiments, each with five replications *(n =15)*.

by the first principal component (PC-1) axis; PC-1 accounted for 86.51 % of the observed variation. It was positively correlated with GP%, GI, RLI (10, 20, and 30 day), SLI (10, 20, and 30 day) and GPOx leaf/root %. In contrast, it was negatively correlated to MGT, T50%, and GPOx activity in leaves, stems, and roots. The second principal component axis (7.52 %) was positively correlated with most variables except GP%, MGT, SLI 30 day, and GPOx leaf/root %. The first principal component axis separates high Cd level treatments (100, 500, and 1000 ppm) from control and low Cd level treatments (10, 30, and 100 ppb); the second principal component axis separates treatments with 30 and 100 ppb from control and 10 ppb of Cd treatment. Overall, the principal component analysis (PCA) identified two distinct clusters: the first represented GP%, GI, GPOx leaf/root % and growth seedling indices (RLI and SLI); the second cluster gathered the enzymatic activity of GPOx in leaf stem and root, T50%, MGT.

## Discussion

### Germination Indices

The decreasing trend of the final germination percentage (GP%) and germination index (GI) values was dose-dependent; the result in the control group

Table 2. Guaiacol peroxidase activity in *P. australis* seedlings organs exposed to various cadmium concentrations after 30 days of germination. The data is an average observation from three independent experiments, each with three replications (n = 9). The highest values receive the letter (a). The values with different superscript letters in a column refer to significant differences ($p \leq 0.05$, Tukey's test).

| Treatment | GPOx activity (µM. $min^{-1}$ $mg^{-1}$ Fw) | | | |
|---|---|---|---|---|
| | Leaf | Stem | Root | Leaf/ Root % |
| Control | 5.15±0.54 $^{e}$ | 1.26±0.09 $^{e}$ | 2.19±0.17 $^{c}$ | 2,35±0,04 $^{b}$ |
| 10 ppb | 4.74±0.69 $^{e}$ | 1.79±0.13 $^{de}$ | 2.55±0.34 $^{c}$ | 1,86±0,20 $^{b}$ |
| 30 ppb | 5.68±0.33 $^{e}$ | 1.87±0.07 $^{de}$ | 2.76±0.26 $^{c}$ | 2,06±0,26 $^{b}$ |
| 100 ppb | 11.87±1.19 $^{d}$ | 3.11±0.23 $^{d}$ | 2.91±0.45 $^{c}$ | 4,07±0,99 $^{a}$ |
| 100 ppm | 37.80±3.62 $^{a}$ | 22.90±1.29 $^{c}$ | 62.43±2.89 $^{b}$ | 0,61±0,05 $^{c}$ |
| 500 ppm | 33.85±3.22 $^{b}$ | 25.91±1.63 $^{b}$ | 68.88±0.55 $^{a}$ | 0,49±0,04 $^{c}$ |
| 1000 ppm | 29.03±2.73 $^{c}$ | 31.35±1.28 $^{a}$ | 68.39±0,69 $^{a}$ | 0,42±0,03 $^{c}$ |

was found to be maximum and gradually decreased with increasing Cd concentration. This finding is similar to that reported by Peng et al. (2010) [38], who recorded a reduction in *P. australis* seed germination percentage and a decrease in germination index value as $Cd^{2+}$ concentration in solution increased. Similar results were observed when *P. australis* seeds were treated with 0.5 and 1 $\mu$g $ml^{-1}$ of $CdSO_4$ [39]. In our study, by testing lower Cd levels (10, 30, and 100 ppb) than those tested by Peng et al. (2010) [38] and Ye et al. (1997) [39], which were 500 ppb and 1 ppm of Cd, respectively. We found that the seed was more sensitive than what was reported, and 100 ppb of Cd significantly inhibited seed germination percentage. Concerning the mean germination time index (MGT) used to measure duration and germination rate, a lower value of MGT corresponds to quicker germination. It was found in our study that all Cd doses had delayed and inhibited seed germination. The increase in Cd concentrations also increased the time needed to reach 50% of germination,

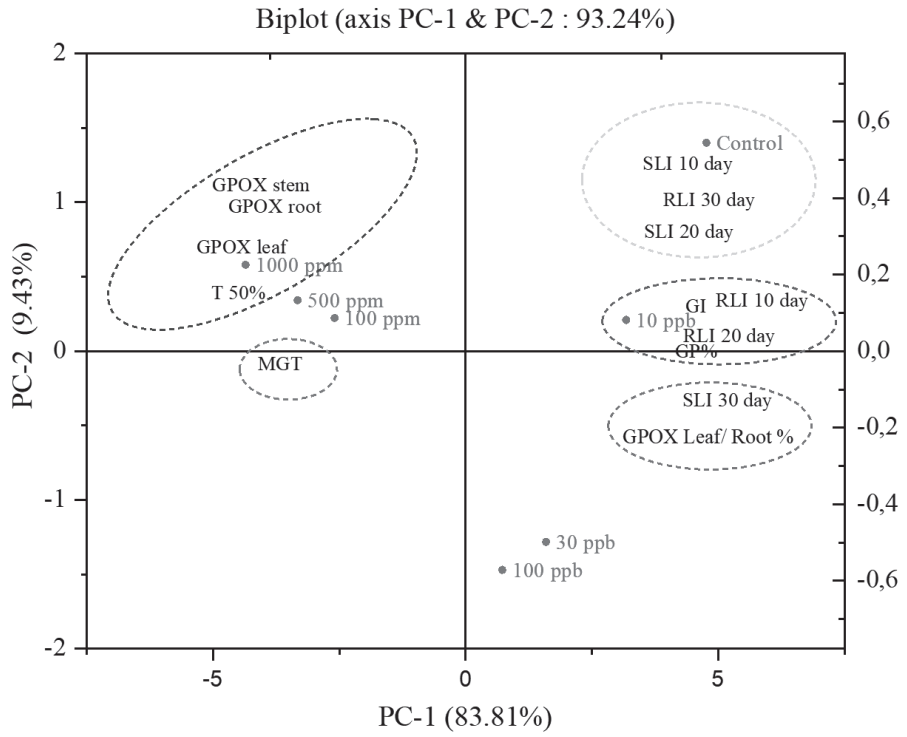


Fig. 3. Biplot of the principal component analysis (PCA) for the variation of germination indices and guaiacol peroxidase activity in *P. australis* seedlings exposed to various cadmium levels. Final germination percentage (GP%), Mean germination time (MGT), Time to 50% of germination (T50%), Germination index (GI), Radical length index (RLI), Shoot length index (SLI), and guaïcol-peroxidase activity in leaf, stem and root (GPOx).

whereas the shortest time to reach 50% of germination was observed in the control group. In accordance with our results, previous studies have demonstrated the negative effects of Cd on the germination of several wetland and aquatic plants, such as *Myriophyllum aquaticum* [40], *Ottelia alismoides* [41], and *Suaeda salsa* [42]. Cd is a non-essential element for plants; it affects and inhibits several physiological processes due to the high solubility, bioavailability, and toxicity at low exposure concentrations. In the present study, the inhibition and the delay of germination by Cd can be related to the disruption of the event chain of germinal metabolism, the impairment of membranes and seed coat integrity, a consequence of reduced water uptake by seed, and a failure in the mobilization of nutrients stored in endosperm tissue. During the germination process, most seeds undergo a specific sequence of events. The major events are imbibition, initiation of respiration, induction of hydrolytic enzymes for the mobilization of stored reserves, and seedling emergence, which results from the enzymatic weakening of the seed coat coupled with the internal force generated by the elongation of the embryonic axis [43]. The seed coat can prevent a strong accumulation of Cd in reserve tissues of heterotrophic germinating seeds, but avoiding metal by coat could not prevent the germination decline [44]. The penetration of Cd into the seed occurs upon imbibition, the testa becomes more permeable over time, and Cd content begins to increase in inner seed tissues [45]. It has been accepted that increased Cd levels in seed tissues may impair amino acids freeing, water and nutrient intake and transport along the embryo axis [46]. Cadmium toxicity can inhibit alpha-amylase activity in seeds, impair reserve carbohydrate and protein hydrolysis, and limit reserve translocation to growing embryonic axes [47]. In addition, Cd causes loss of seed membrane integrity due to membrane lipid peroxidation [44]. In the present study, the seed germination indices GP%, GI, MGT, and T50% indicated that *P. australis* seeds were quite sensitive to the increasing Cd concentration, even though the differences were not significant between all treated groups (Table 1). A slight increase in the order of parts per billion of Cd negatively influenced the rate and speed of germination (e.g., the significant difference between the GI index value in the control group and the group treated with 10 ppb of Cd). The sensitivity of *P. australis* seed to the variations in Cd doses can also be attributed to the low seed mass and nutrient reserve content of this species (in our study, seed weight: mg/100 seeds = 16,77±0,11 mg). According to Haraguchi (2014) [48], *P. australis seed* weight could range between 25 and 11 mg. Although the data for *P. australis* is limited, the vulnerability of the germination process of *P. australis* seeds could be due to the low content of nutrient reserves [49]. According to Steiner et al. (2019) [50], seed size and weight are important indicators of physiological quality since they may affect seed germination and seedling growth, especially under stress conditions. However, the positive or negative correlation between seed size and germination characteristics is species-dependent [51].

## Seedling Length Indices

Compared to the control group, the root and shoot length indices (RLI and SLI) measured at 10, 20, and 30 days showed a significant decrease at all levels of Cd treatment, except in the groups treated with 10 ppb. Our results showed that cadmium's phytotoxicity on early-growth parameters becomes evident with the increase in dose. In groups treated with 100, 500, and 1000 ppm, necrotic roots and yellowing leaves were observed after 20 days of the experiment. The seedlings' morphological structure also revealed an increase in the prevalence of abnormal seedlings, including leaf atrophy, asymmetric development, and twisted leaves, particularly in the 500 and 1000 ppm groups, revealing its toxic effect on the cell cycle and division. Once germination has occurred, two major processes drive plant growth: cell division and cell expansion [52]. Cd-phytotoxicity inhibits seedling growth through different mechanisms: it can affect the cell cycle and division [53]. Cd induces oxidative stress [54]. Can act as a respiratory uncoupler [55]. Cd causes an imbalance in nutrient and water uptake [8]. It also interferes with the uptake and translocation of essential nutrients [56]. In addition, Cd disturbs photosynthesis and seedling growth [9, 57]. Literature provides meager information regarding the toxicity effect of Cd on the early establishment of *P. australis* seedlings. Peng et al. (2010) [38] have reported a decrease in the seedling length of *P. australis* after 10 d of exposure to $Cd^{2+}$, and the Cd-inhibition occurs in a dose-dependent manner. Compared to a control group, the decrease in seedling length was 3% at 1 ppm, 38% at 2 ppm, 47% at 3 ppm, 66% at 4 ppm, and 81% at 5 ppm of $Cd^{2+}$. A similar study observed a significant decrease in shoot and root elongation of *P. australis* seedlings; the seeds were collected from a wildlife reserve and treated with 0.5 and 1 $ug.ml^{-1}$ of Cd for 89 days [39]. However, the tested concentrations in those previous studies were higher than in our study, where 30 and 100 ppb significantly affected root and shoot growth. According to Shafiq et al. (2008) [58], the reason for reduced seedling length in metal treatments could be the reduction in meristematic cells present in this region and some enzymes contained in the cotyledon and endosperms. Cells become active and begin to digest and store food, which is converted into a soluble form and transported to the root, and plumule tips for enzyme amylase, which converts starch into sugar, and protease act on protein. Therefore, when hydrolytic enzyme activities are affected, the food does not reach the root and plumule, affecting the seedling length. In the present study, the effect of Cd toxicity was variable during seedling growth, and the sensitivity of primary root and shoot to Cd was different. The negative effect of Cd on primary root length growth was more pronounced after 30 days than after 10 and 20 days. In contrast, the inhibition effect of Cd on shoot

growth appears to be more significant at day-10 than after 30 and 20 days of the experiment. It could be due to the sensitivity of shoots to Cd in the first days of germination; during this period, the early shoots remain immersed in the medium (direct contact with Cd) until their elongation. After 20 and 30 days, the shoots length increases with the use of nutrients remaining in the cotyledon and water absorption by the root, which becomes functional, reducing the translocation of Cd to the shoot and leaves. The root is considered the first defense line against Cd-phytotoxicity by adsorption in the cell wall and the sequestration of Cd in cells. The roots developed better in the first days due to their lower sensitivity to Cd than the shoots; their structure also plays a significant role in overcoming Cd toxicity. However, after 30 days, the growth in length decreases due to the phototoxic effects of Cd accumulated in the root tissue. Indeed, several studies indicated that *P. australis* reeds accumulated more Cd in roots than in shoots and leaves [21, 22, 39]. Furthermore, Cd accumulation and biochemical detoxification strategies are more enhanced in roots than in leaves of mature *P. australis* plants [22].

## Guaïcol-Peroxidase Activity (GPOx)

In the present study, a gradual increase in the activity of GPOx was observed in Cd-grown seedlings compared to control. Furthermore, a considerable increase in the antioxidant activity of GPOx was found in seedlings that emerged from seeds subjected to high levels of cadmium. The increasing activity of GPOx in *P. australis* seedling organs could be a defensive mechanism, a physiological detoxification process employed to counteract the negative consequences of the oxidative stress induced by excessive Cd absorption. According to Das and Roychoudhury (2014) [14], GPOx is active intracellularly (cytosol, vacuole) in the cell wall and extracellularly; it is considered the key enzyme in removing $H_2O_2$. It is well known that both short-term and chronic Cd exposure increase reactive oxygen species (ROS) and the antioxidant activity of GPOx in mature *P. australis* reeds [59, 60]. The response of GPOx activity in *P. australis* seedlings that emerged from seeds exposed to cadmium has not been investigated to our best knowledge. However, several plants have shown an increase in GPOx activity when exposed to Cd, such as *Phaseolus vulgaris* [61], *Hordeum vulgare* [62], sassafras seedlings [63] and *Oryza sativa* [64]. In contrast, GPOx activity was decreased in other species when exposed to Cd, including *Pisum sativum* [65] and *Brassica napus*. cvs. Zheda 619 [66]. Literature data relating to the impact of Cd on GPOx activity are heterogeneous. The enzymatic antioxidant defense response differs between plant species, stage of development, organ, and duration of exposure, as well as between various stress conditions and their frequencies [67, 15]. In the present study, the leaf/root GPOx activity ratio was >1 in control and all groups treated with low levels of Cd (10, 30, and 100 ppb). In contrast, under high Cd levels (100, 500, and 1000 ppm), the leaf/root GPOx activity ratio was <1 (from 0.4 to 0.6). Based on our findings, we can deduce that high levels of Cd stimulate more GPOx activity in roots and stems than in leaves of *P. australis* seedlings. However, at low Cd levels and no stress conditions, GPOx activity is more stimulated in leaves. *P. australis* is a root Cd-accumulator plant. Therefore, maintaining high GPOx activity in roots and stems under high Cd levels could be a part of a mechanism to prevent the ROS's damaging effects. The GPOx enzyme may also play a role in reducing Cd translocation to leaves, which are more susceptible to Cd toxicity. In their work, Ederli et al. [16] showed that *P. australis* could sequester Cd inside root cortex cells and restrict its translocation to the vascular cylinder and, subsequently, to the stem and leaf. In *P. australis* reeds regenerated from embryogenic callus tissue treated with Cd $(NO_3)_2$, Fediuc and Erdei (2002) [21] found that GPOx activity increases with increasing Cd concentrations in shoots and roots. In our study, the decrease in GPOx activity in leaves exposed to high levels of Cd after a peak induction under 100 ppm could be explained by the fact that, over a certain threshold, the effectiveness of Cd sequestration in roots decreases. Therefore, the ROS generated by Cd accumulation in leaf tissues may have deleterious effects on GPOx enzyme biosynthesis. The increase in the prevalence of abnormal seedlings, including necrotic roots, yellowing, and leaf atrophy, especially in groups treated with 500 and 1000 ppm, observed in the present study, reinforces our assumption. *P. australis* is an excluder plant (Cd root accumulator) with physiological mechanisms enabling it to tolerate Cd toxicity. Therefore, the continuous increase in GPOx activity at high Cd levels in roots could not only prevent the damaging effects of ROS but also reduce Cd translocation via the apoplastic pathway to the leaves, especially since GPOx is known to be involved in the lignification of plant roots under stress. Lignins are cell wall phenolic heteropolymers which result from the oxidative coupling of three monolignols; p-coumaryl, coniferyl, and sinapyl alcohol, in a reaction mediated by peroxidases (EC 1.11.1.7) [68]. Lignin polymers' functional groups (carboxyl, methyl, and hydroxyl) can bind heavy metal ions such as $Cd^{2+}$, reducing their entry into cells [69]. It has been reported that cadmium increases lignification by stimulating guaiacol peroxidase activity in the roots of various plant species, such as *Vicia sativa* [7], *Sedum alfredii* [70] and *Poa pratensis* [71]. The reinforcement of the cell walls by lignification can be interpreted as a response of the plant to form a more effective barrier against excessive amounts of Cd [72]. Under Cd stress, abnormal premature lignification of the epidermis and epidermal cell walls 10-20 mm from the root apex has been observed in *P. australis* reeds [16]. Several studies have indicated that Cd treatment inhibits plant growth, increases the content of lignin, and

up-regulates the activity of GPOx and genes involved in lignin biosynthesis [73, 74, 75]. According to Barceló et al. (2004) [68], lignification may negatively affect root elongation by reducing cell wall extensibility, which minimizes nutrient uptake and plant growth. The negative effect of the GPOx enzyme on root growth probably explains why the leaf/root GPOx activity ratio was >1 in the control and groups treated with low levels of Cd in our study. Under no or low-stress conditions, *P. australis* seedlings could downregulate GPOx activity in the root to preserve optimum growth. Indeed, GPOx is considered one of the key enzymes controlling plant growth, differentiation, and development by regulating auxin levels through auxin catabolism in plants, besides its involvement in ROS removal under stress conditions [76, 77]. In contrast to the current study, Bouchama et al. (2016) [59] revealed that *P. australis* reeds of control and treated groups with 10, 27, and 55 ppb of Cd had a leaf/root GPOx activity ratio <1. Similar results were observed in plants treated with 23 and 230 ppb of Cd by Fediuc and Erdei (2002) [21]. These differential responses are probably due to the age-dependent abiotic stress responses between mature plants and the juvenile seedlings tested in our study. Jiang et al. (2005) [78] indicated that multiple antioxidant enzymes, such as superoxide dismutase, peroxidase, ascorbate peroxidase, and glutathione reductase, appear to be relatively more active during the early stages of leaf development. Due to the pattern of changes in guaiacol peroxidase activity observed in the current study and from literature data, *P. australis* species may have a mechanism to regulate its GPOx activity response not only according to the intensity of the Cd dose and plant organ sensitivity but also according to the stage of seedling development for optimal nutrient uptake and growth.

### The Principal Component Analysis

In order to obtain the best discrimination between the treated groups, a multivariate analysis using a principal component analysis (PCA) was performed to summarize the differential effects of the various Cd levels on the germination characteristics, early seedling growth, and GPOx activity. Our results show a negative correlation between seed germination and seedling growth with the enzymatic activity of GPOx, and germination time. Regarding the dose-response effect on the different variables, the CPA analysis revealed three homogeneous clusters: the first group includes control and 10 ppb of Cd; the second group includes the two concentrations of 30 and 100 ppb; and the third group includes 100, 500, and 1000 ppm concentrations. The increasing cadmium levels in the medium reduced the germination percentage and seedlings' growth, increased the time needed for germination, and stimulated the activity of GPOx in the seedling's organs to varying degrees. In the present study, when the tolerance threshold (Cd ≥100 ppm) is exceeded, the antioxidant activity of GPOx and the defense system of *P. australis* seedlings become ineffective, resulting in wilting and necrosis of the roots, leaves yellowing, and eventually death of the seedlings. It has been shown that the activity of GPOx enzyme increased up to a certain level of Cd stress and then decreased under higher Cd stress while remaining significantly higher than control, such as in *Atriplex halimus* [79], *Gossypium hirsutum* [80], and *Acanthus ilicifolius* [81]. Farooq et al. (2016) [80] study revealed that the enzymatic antioxidant defense response differs in *Gossypium hirsutum* seedlings' organs treated with 1 and 5 µM of $CdCl_2$. While the ratio of leaf/root GPOx activity was >1, the ratio of ascorbate peroxidase (APX), catalase (CAT), and superoxide dismutase (SOD) were >1. These findings could support our hypothesis that the GPOx enzyme does not have an important defense role under low levels of cd-stress, particularly in roots and stems (Cd =10, 30 and 100 ppb), and that the seedling may instead use another defense mechanism.

## Conclusion

Our ecotoxicological bioassay on *P. australis* has shown a significant negative effect of Cd on seed germination, and early seedling growth. The Cd-inhibition of germination characteristics occurred in a dose-dependent manner. However, seedling growth (shoot and root growth) was more sensitive and affected by the low tested Cd levels (10, 30, and 100 ppb) than germination characteristics (germination percentage and time of germination). Furthermore, the effect of Cd toxicity was variable during the seedling growth period, and the sensitivity of primary root and shoot was different. The shoot growth was susceptible during the first ten days of germination, while the root growth was more sensitive after 30 days, where the lowest tested dose of Cd = 10 ppb had significantly inhibited their growth. The tested germination indices, particularly GI, RLI, and SLI, can be considered an effective screening tool for Cd-phytotoxicity in the early establishment stages. In all the seedling's organs, GPOx antioxidant activity response increased gradually as cadmium concentrations increased. In addition, the leaf/root GPOx activity ratio revealed that the GPOx response was dose-and organ-dependent. The leaf/root GPOx activity was >1 in control, and all groups treated with low levels of Cd (10, 30, and 100 ppb) and less than <1 under high Cd levels (100, 500, and 1000 ppm), completely the opposite of what was found by previous studies on older *P. australis* plants. We can deduce that high levels of Cd stimulate more GPOx activity in roots and stems than in leaves of *P. australis* seedlings. However, at low Cd levels and no stress conditions, GPOx activity is more stimulated in leaves. These differential responses could be due to the age-dependent Cd stress response between mature plants and juvenile seedlings. The juvenile *P. australis* seedlings could downregulate GPOx activity in the root to preserve optimum growth under no or low-stress

conditions. The GPOx enzyme is known to be involved in the lignification of roots in plants under cadmium stress. This lignification process can negatively affect root elongation by reducing cell wall extensibility, which in turn inhibits nutrient uptake and plant development. The prevalence of leaf abnormalities and the differential GPOx response observed in the present study could constitute an excellent informative method for monitoring Cd pollution. The study's outcome could contribute to understanding the potential of juvenile seedlings to cope with Cd stress and estimate the potential of this species to spread and establish through seed in contaminated habitats. Furthermore, our results may be useful in further research into the possible use of germination, early seedling growth, and GPOx activity as bioindicators or biomarkers in cadmium-contaminated environments.

## Conflict of Interest

The authors declare no conflict of interest.

## References


1. ESPEJO W., PADILHA J.D.A., KIDD K.A., DORNELES P.R., BARRA R., MALM O., CHIANG G., CELIS J.E. Trophic transfer of cadmium in marine food webs from Western Chilean Patagonia and Antarctica. Mar. Pollut. Bull. **137**, 246, **2018**.
2. RIGBY H., SMITH S.R. The significance of cadmium entering the human food chain via livestock ingestion from the agricultural use of biosolids, with special reference to the UK. Environ. Int. **143**, 105844, **2020**.
3. DONG Q., FANG J., HUANG F., CAI K. Silicon Amendment Reduces Soil Cd Availability and Cd Uptake of Two Pennisetum Species. Int. J. Environ. Res.Public. Health. **16** (9), **2019**.
4. DU J., ZENG J., MING X., HE Q., TAO Q., JIANG M., GAO S., LI X., LEI T., PAN Y., CHEN Q., LIU S.YU X. The presence of zinc reduced cadmium uptake and translocation in Cosmos bipinnatus seedlings under cadmium/zinc combined stress. Plant. Physiol. Biochem. **151**, 223, **2020**.
5. ALVES L.R., PRADO E.R., DE OLIVEIRA R., SANTOS E.F., LEMOS DE SOUZA I., DOS REIS A.R., AZEVEDO R.A., GRATÃO P.L. Mechanisms of cadmium-stress avoidance by selenium in tomato plants. Ecotoxicology. **29** (5), 594, **2020**.
6. GENCHI G., SINICROPI M.S., LAURIA G., CAROCCI A., CATALANO A. The Effects of Cadmium Toxicity. Int. J. Environ. Res.Public.Health. **17** (11), **2020**.
7. RUI H., CHEN C., ZHANG X., SHEN Z., ZHANG F. Cd-induced oxidative stress and lignification in the roots of two Vicia sativa L. varieties with different Cd tolerances. J. Hazard. Mater. **301**, 304, **2016**.
8. NAEEM A., ZAFAR M., KHALID H., ZIA-UR-REHMAN M., AHMAD Z., AYUB M.A., FAROOQ QAYYUM M. Chapter 12 - Cadmium-Induced Imbalance in Nutrient and Water Uptake by Plants. In: Cadmium Toxicity and Tolerance in Plants, HASANUZZAMAN M., PRASAD MNV., FUJITA M (Eds). Academic Press. 299, **2019**.
9. CHOWARDHARA B., BORGOHAIN P., SAHA B., AWASTHI J.P., PANDA S.K. Differential oxidative stress responses in *Brassica juncea* (L.) Czern and Coss cultivars induced by cadmium at germination and early seedling stage. Acta. Physiol. Plant. **42** (7), 105, **2020**.
10. QADIR S., JAMSHIEED S., RASOOL S., ASHRAF M., AKRAM N.A., AHMAD P. Modulation of plant growth and metabolism in cadmium-enriched environments. Rev Environ. Contam. Toxicol. **229**, 51, **2014**.
11. MAŁACHOWSKA-JUTSZ A., GNIDA A. Mechanisms of stress avoidance and tolerance by plants used in phytoremediation of heavy metals. Arch. Environ. Prot. **41** (4), 104, **2015**.
12. SONG Y., JIN L., WANG X. Cadmium absorption and transportation pathways in plants. Int. J. Phytoremediation. **19** (2), 133, **2017**.
13. VIEHWEGER K. How plants cope with heavy metals. Bot. Stud. **55** (1), 35, **2014**.
14. DAS K., ROYCHOUDHURY A. Reactive oxygen species (ROS) and response of antioxidants as ROS-scavengers during environmental stress in plants. Front. Environ. Sci. **2**, **2014**.
15. HASANUZZAMAN M., BHUYAN M., ZULFIQAR F., RAZA A., MOHSIN S.M., MAHMUD J.A., FUJITA M., FOTOPOULOS V. Reactive Oxygen Species and Antioxidant Defense in Plants under Abiotic Stress: Revisiting the Crucial Role of a Universal Defense Regulator. Antioxidants (Basel). **9** (8), **2020**.
16. EDERLI L., REALE L., FERRANTI F., PASQUALINI S. Responses induced by high concentration of cadmium in Phragmites australis roots. Physiol. Plant. **121** (1), 66, **2004**.
17. BACHA H., TEKAYA M., DRINE S., GUASMI F., TOUIL L., ENNEB H., TRIKI T., CHEOUR F., FERCHICHI A. Impact of salt stress on morpho-physiological and biochemical parameters of Solanum lycopersicum cv. Microtom leaves. S.Afr. J.Bot. **108**, 364, **2017**.
18. BAKER A.J.M. Accumulators and excluders -strategies in the response of plants to heavy metals. J.Plant. Nutr. **3** (1-4), 643, **1981**.
19. DALVI A.A., BHALERAO S.A. Response of Plants towards Heavy Metal Toxicity: An overview of Avoidance, Tolerance and Uptake Mechanism. Ann. Plant Sci. **2**, 362, **2013**.
20. SHARMA P., NGO H.H., KHANAL S., LARROCHE C., KIM S.-H., PANDEY A. Efficiency of transporter genes and proteins in hyperaccumulator plants for metals tolerance in wastewater treatment: Sustainable technique for metal detoxification Environ. Technol. Innov. **23**, 101725, **2021**.
21. FEDIUC E., ERDEI L. Physiological and biochemical aspects of cadmium toxicity and protective mechanisms induced in Phragmites australis and Typha latifolia. J. Plant. Physiol. **159** (3), 265, **2002**.
22. ALFADUL S.M.S., AL-FREDAN M.A.A. Effects of Cd, Cu, Pb, and Zn Combinations on Phragmites australis Metabolism, Metal Accumulation and Distribution. Arab. J. Sci. Eng. **38** (1), 11, **2013**.
23. SMITH P. The Book of Seeds: A life size guide to six hundred species from around the world. Ivy Press, **2018.** doi: 10.7208/chicago/9780226362373.001.0001
24. MILKE J., GAŁCZYŃSKA M., WRÓBEL J. The Importance of Biological and Ecological Properties of Phragmites Australis (Cav.) Trin. Ex Steud., in

Phytoremendiation of Aquatic Ecosystems-The Review. Water. **12** (6), 1770, **2020**.
25. BELLO A.O., TAWABINI B.S., KHALIL A.B., BOLAND C.R., SALEH T.A. Phytoremediation of cadmium, lead and nickel contaminated water by Phragmites australis in hydroponic systems. Ecol.Eng. **120**, 126, **2018**.
26. ALBERT A., BRISSON J., BELZILE F., TURGEON J., LAVOIE C. Strategies for a successful plant invasion: the reproduction of Phragmites australis in north-eastern North America. J.Ecol. **103** (6), 1529, **2015**.
27. Environmental Protection Agency (USEPA). Ecological effects test guidelines : Seed germination root elongation toxicity test," OPPTS 850, 4200, EPA 712-C-96-154, Washington DC. **1996**.
28. Organization for Economic Cooperation and Development (OECD). Guideline for the testing of chemicals proposal for updating guideline 208. Terrestrial plant test: seedling emergence and seedling growth test. **2003**.
29. GORAI M., VADEL A.M., NEFFATI M. Seed germination characteristics of Phragmites communis: effects of temperature and salinity. Belg. J. of Bot. **139**, 78, **2006**.
30. GUTERRES J., ROSSATO L., DOLEY D., PUDMENZKY A., BEE C., COBENA V. Assessing germination characteristics of Australian native plant species in metal/ metalloid solution. J. Hazard.Mater. **364**, 173, **2019**.
31. ELLIS R.H. The quantification of ageing and survival in orthodox seeds. Seed Sci. Technol. **9**, 3739, **1981**.
32. FAROOQ M., BASRA S.M.A., AHMAD N., HAFEEZ K. Thermal Hardening: A New Seed Vigor Enhancement Tool in Rice. J.Integr. Plant.Biol. **47** (2), 187, **2005**.
33. Association of Official Seed Analysts (AOSA), Seed vigor testing handbook Contribution No. 32. Association of Official Seed Analysts. **88**, **1983**.
34. LOGGINI B., SCARTAZZA A., BRUGNOLI E.NAVARI-IZZO F. Antioxidative defense system, pigment composition, and photosynthetic efficiency in two wheat cultivars subjected to drought. Plant. physiol. **119** (3), 1091, **1999**.
35. LIN C.C., KAO C.H. NaCl induced changes in ionically bound peroxidase activity in roots of rice seedlings. Plant and Soil. **216** (1), 147, **1999**.
36. FIELDING J.L., HALL J. A biochemical and cytochemical study of peroxidase activity in roots of Pisum sativum: II. Distribution of enzymes in relation to root development. J. Exp Bot. **29** (4), 983, **1978**.
37. ARNOLD R.L.B., FENNER M., EDWARDS P.J.Changes in Germinability, ABA Content and ABA Embryonic Sensitivity in Developing Seeds of Sorghum bicolor (L.) Moench. Induced by Water Stress During Grain Filling. New. Phytol. **118** (2), 339, **1991**.
38. PENG H., GENG W., YONG-QUAN W., MAO-TENG L., JUN X., LONG-JIANG Y. Effect of heavy metal stress on emerging plants community constructions in wetland. Water. Sci. Technol. **62** (10), 2459, **2010**.
39. YE Z.H., BAKER A.J.M., WONG M.H., WILLIS A.J. Zinc, Lead and Cadmium Tolerance, Uptake and Accumulation by the Common Reed, Phragmites australis(Cav.) Trin. ex Steudel. Ann. Bot. **80** (3), 363, **1997**.
40. WANG L., GAO Y., WANG X., QIN Z., LIU B., ZHANG X., WANG G. Warming enhances the cadmium toxicity on macrophyte Myriophyllum aquaticum (Vell.) Verd. seedlings. Environ.Pollut. **268**, 115912, **2021**.
41. CAO Y., LIU H., SUN J., LIU Y., JIANG H.S., WANG W., LI W. Responses of propagule germination and sexual reproduction of submerged macrophytes exposed to cadmium. Chemosphere. **219**, 436, **2019**.
42. LIU S., YANG C., XIE W., XIA C., FAN P. The Effects of Cadmium on Germination and Seedling Growth of Suaeda salsa. Procedia. Environ. Sci. **16**, 293, **2012**.
43. HAUVERMALE A.L., STEBER C.M. GA signaling is essential for the embryo-to-seedling transition during Arabidopsis seed germination, a ghost story. Plant Signal. Behav. **15** (1), 1705028, **2020**.
44. RAHOUI S., CHAOUI A., EL FERJANI E. Membrane damage and solute leakage from germinating pea seed under cadmium stress. J. Hazard. Mater. **178** (1-3), 1128, **2010**.
45. SFAXI-BOUSBIH A., CHAOUI A., EL FERJANI E. Cadmium impairs mineral and carbohydrate mobilization during the germination of bean seeds. Ecotoxicol. Environ. Saf. **73** (6), 1123, **2010**.
46. JAOUANI K., KARMOUS I., OSTROWSKI M., FERJANI E.E., JAKUBOWSKA A.CHAOUI A. Cadmium effects on embryo growth of pea seeds during germination: Investigation of the mechanisms of interference of the heavy metal with protein mobilization-related factors. J. Plant Physiol. **226**, 64, **2018**.
47. SUMERA A., FAHAD S., ZAIB-UN N., UZMA U., MUHAMMA D.A., NAILA A. Effect of cadmium stress on seed germination, plant growth and hydrolyzing enzymes activities in mungbean seedlings. J. Seed Sci. **43**, e202143042, **2021**.
48. HARAGUCHI A. Effects of Salinity on Germination, Seedling Growth and Ecological Properties of Phragmites australis Communities in the Estuary of the Chikugogawa River, Southwestern Japan.Am. J.Plant.Sci. **5**, 584, **2014**.
49. BRISSON J. Roseau commun: le Dr Jekill et Mr Hyde du monde végétal Institut de recherche en biologie végétale, Québec, Canada, **71**, **2014**.
50. STEINER F., ZUFFO A.M., BUSCH A., SOUSA T.D.O., ZOZ T. Does seed size affect the germination rate and seedling growth of peanut under salinity and water stress? Pesqui. Agropecu.Trop. **49**, **2019**.
51. TUMPA K., VIDAKOVIĆ A., DRVODELIĆ D., ŠANGO M., IDŽOJTIĆ M., PERKOVIĆ I., POLJAK I. The Effect of Seed Size on Germination and Seedling Growth in Sweet Chestnut (Castanea sativa Mill.). Forests. **12** (7), 858, **2021**.
52. HUYBRECHTS M., CUYPERS A., DECKERS J., IVEN V., VANDIONANT S., JOZEFCZAK M., HENDRIX S. Cadmium and Plant Development: An Agony from Seed to Seed. Int. J. Mol.Sci. **20** (16), **2019**.
53. CUI W., WANG H., SONG J., CAO X., ROGERS H.J., FRANCIS D., JIA C., SUN L., HOU M., YANG Y., TAI P., LIU W. Cell cycle arrest mediated by Cd-induced DNA damage in Arabidopsis root tips. Ecotoxicol. Environ. Saf. **145**, 569, **2017**
54. MURADOGLU F., GUNDOGDU M., ERCISLI S., ENCU T., BALTA F., JAAFAR H.Z.E., ZIA-UL-HAQ M. Cadmium toxicity affects chlorophyll a and b content, antioxidant enzyme activities and mineral nutrient accumulation in strawberry. Biological Research. **48** (1), 11, **2015**.
55. MANQUIÁN-CERDA K., ESCUDEY M., ZÚÑIGA G., ARANCIBIA-MIRANDA N., MOLINA M., CRUCES E. Effect of cadmium on phenolic compounds, antioxidant enzyme activity and oxidative stress in blueberry (*Vaccinium corymbosum* L.) plantlets grown in vitro. Ecotoxicol.Environ.Saf. **133**, 316, **2016**.
56. ANJUM N.A., KHAN N.A., SOFO A., BAIER M., KIZEK R. Editorial: Redox Homeostasis Managers in Plants under Environmental Stresses. Fronti.Environ.Sci. **4**, **2016**.

57. MORINA F., KÜPPER H. Direct inhibition of photosynthesis by Cd dominates over inhibition caused by micronutrient deficiency in the Cd/Zn hyperaccumulator Arabidopsis halleri. Plant Physiol. Biochem. **155**, 252, **2020**.
58. SHAFIQ M., IQBAL M., ATHAR M. Effect of lead and cadmium on germination and seedling growth of Leucaena leucocephala. J.Appl.Sci.Environ.Manage. **12** (3), **2008**.
59. BOUCHAMA K., ROUABHI R., DJEBAR M.R. Behavior of Phragmites australis (CAV.) Trin. Ex Steud used in phytoremediation of wastewater contaminated by cadmium. Desalin. Water. Treat. **57** (12), 5325, **2016**.
60. KOZŁOWSKA M., JÓŹWIAK-ŻUREK A., SZPAKOWSKA B., GOLINSKI P. Biological aspects of cadmium and lead uptake by Phragmites australis (Cav. Trin ex steudel) in natural water ecosystems. J.Elem. **14**, **2009**.
61. GUTIÉRREZ-MARTÍNEZ P., TORRES-MORÁN M.I., ROMERO-PUERTAS M., CASAS-SOLÍS J., ZARAZÚA P., SANDOVAL-PINTO E., RAMÍREZ-HERNÁNDEZ B. Assessment of antioxidant enzymes in leaves and roots of Phaseolus vulgaris plants under cadmium stress. Biotecnia. **22**, 110, **2020**.
62. JÓCSÁK I., MALGWI I., RABNECZ G., SZEGŐ A., VARGA-VISI É., VÉGVÁRI G., PÓNYA Z. Effect of cadmium stress on certain physiological parameters, antioxidative enzyme activities and biophoton emission of leaves in barley (Hordeum vulgare L.) seedlings. PLoS One. **15** (11), e0240470, **2020**.
63. ZHAO H., GUAN J., LIANG Q., ZHANG X., HU H., ZHANG J. Effects of cadmium stress on growth and physiological characteristics of sassafras seedlings. Sci. Rep. **11** (1), 9913, **2021**.
64. LIU H., ZHANG C., WANG J., ZHOU C., FENG H., MAHAJAN M.D., HAN X. Influence and interaction of iron and cadmium on photosynthesis and antioxidative enzymes in two rice cultivars. Chemosphere. **171**, 240, **2017**.
65. GŁOWACKA K., ŹRÓBEK-SOKOLNIK A., OKORSKI A., NAJDZION J. The Effect of Cadmium on the Activity of Stress-Related Enzymes and the Ultrastructure of Pea Roots. Plants (Basel). **8** (10), **2019**.
66. ALI B., DENG X., HU X., GILL R.A., ALI S., WANG S., ZHOU W. Deteriorative Effects of Cadmium Stress on Antioxidant System and Cellular Structure in Germinating Seeds of Brassica napus L. J. Agric. Sci. Technol. **17** (1), 63, **2015**.
67. BOARETTO L.F., CARVALHO G., BORGO L., CRESTE S., LANDELL M.G.A., MAZZAFERA P., AZEVEDO R.A. Water stress reveals differential antioxidant responses of tolerant and non-tolerant sugarcane genotypes. Plant. Physiol. Biochem. **74**, 165, **2014**.
68. BARCELÓ A.R., GÓMEZ ROS L.V., GABALDÓN C., LÓPEZ-SERRANO M., POMAR F., CARRIÓN J.S., PEDREÑO M.A. Basic peroxidases: The gateway for lignin evolution? Phytochem. Rev. **3** (1), 61, **2004**.
69. GUO X., ZHANG S., SHAN X.Q. Adsorption of metal ions on lignin. J. of Hazard. Mater. **151** (1), 134, **2008**.
70. GUO X., LUO J., DU Y., LI J., LIU Y., LIANG Y.LI T. Coordination between root cell wall thickening and pectin modification is involved in cadmium accumulation in Sedum alfredii. Environ. Pollut. **268**, 115665, **2021**.
71. WANG Y., CUI T., NIU K., MA H. Comparison and Characterization of Oxidation Resistance and Carbohydrate Content in Cd-Tolerant and -Sensitive Kentucky Bluegrass under Cd Stress. Agronomy. **11** (11), 2358, **2021**.
72. LOIX C., HUYBRECHTS M., VANGRONSVELD J., GIELEN M., KEUNEN E., CUYPERS A. Reciprocal Interactions between Cadmium-Induced Cell Wall Responses and Oxidative Stress in Plants. Front. Plant. Sci. **8**, 1867, **2017**.
73. GUTSCH A., SERGEANT K., KEUNEN E., PRINSEN E., GUERRIERO G., RENAUT J., HAUSMAN J.-F., CUYPERS A. Does long-term cadmium exposure influence the composition of pectic polysaccharides in the cell wall of Medicago sativa stems? BMC Plant. Biol. **19** (1), 271, **2019**.
74. SANTORO D.F., SICILIA A., TESTA G., COSENTINO S.L., LO PIERO A.R. Global leaf and root transcriptome in response to cadmium reveals tolerance mechanisms in Arundo donax L. BMC Genomics. **23** (1), 427, **2022**.
75. YANG Y.J., CHENG L.M., LIU Z.H. Rapid effect of cadmium on lignin biosynthesis in soybean roots. Plant. Sci. **172** (3), 632, **2007**.
76. ANGELICA R.D., LEONOR A.G.Z. Phenoloxidases activity in root system and their importance in the phytoremediation of organic contaminants. J. Environ. Chem.Ecotoxicol. **4** (3), 35, **2012**.
77. SHIGETO J., TSUTSUMI Y. Diverse functions and reactions of class III peroxidases. New Phytol. **209** (4), 1395, **2016**.
78. JIANG C.D., LI P.M., GAO H.Y., ZOU Q., JIANG G.M., LI L.H. Enhanced photoprotection at the early stages of leaf expansion in field-grown soybean plants. Plant Sci. **168** (4), 911, **2005**.
79. MESNOUA M., MATEOS-NARANJO E., BARCIA-PIEDRAS J.M., PÉREZ-ROMERO J.A., LOTMANI B., REDONDO-GÓMEZ S. Physiological and biochemical mechanisms preventing Cd-toxicity in the hyperaccumulator *Atriplex halimus* L. Plant Physiol. Biochem. **106**, 30, **2016**.
80. FAROOQ M.A., ALI S., HAMEED A., BHARWANA S.A., RIZWAN M., ISHAQUE W., FARID M., MAHMOOD K., IQBAL Z. Cadmium stress in cotton seedlings: Physiological, photosynthesis and oxidative damages alleviated by glycinebetaine. S. Afr. J. Bot. **104**, 61, **2016**.
81. SHACKIRA A.M., PUTHUR J.T. Enhanced phytostabilization of cadmium by a halophyte-Acanthus ilicifolius L. Int. J. Phytoremediation. **19** (4), 319, **2017**.