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Influence of Lead on *In vitro* Seed Germination and Early Radicle Development of *Acacia auriculiformis* Cunn. Ex Benth Species

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Authors' contributions

This work was carried out in collaboration between all authors. Author AZ wrote the protocol, performed the statistical analysis, managed the analyses of the study and wrote the first draft of the manuscript. Author HO consulted on the study approach. Authors MHI and MM designed the study and managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Aims: To determine the impact of Pb on seed germination and early seedling development in *A. auriculiformis* species.

Place and Duration of Study: The study was carried out in Department of Biology, University Putra Malaysia, between April 2016 and February 2017.

Methodology: The concentrations of lead from 0 to 4 g/L (interval of 0.5 g/L) were used. Seeds were germinated *in vitro* condition. Different parameters were measured including germination percentage, seedling vigour index, tolerance index, germination index, mean germination time and relative injury rate.

Results: The noticeable finding of this study reveals that *A. auriculiformis* seeds have the ability to

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germination and resist Pb toxicity up to 1.5 g/L. Increasing Pb concentration from 1.5 to 3.5 g/L decreased the germination percentage from 57% to 4% respectively.

Conclusion: *Acacia auriculiformis* seeds germination in a high level of Pb (up to 1.5 g/L) indicated species resistance which probably can be used as Pb hyperaccumulator agent in areas or sites contaminated with this metal.

Keywords: Lead pollution; germination percentage; embryo morphology; mean germination time; phytoremediation.

1. INTRODUCTION

Fast paced of Industrialization and unmanaged human activities have caused the global contamination of biotope and biocoenosis mainly by heavy metals pollution, resulting in a severe environmental hazard [1]. The major sources of heavy metals pollution are due to human activities such as electroplating, tanning, wood preservation, chemical industries mining waste, petroleum, household waste, the spreading of sludge on agricultural soils and some industrial processes [2]. Once the metal is absorbed into the soil, it persists, accumulates and then invades the food chain with a serious potential to diminish animal, plant and human health [3]. One of the most persistent heavy metal in the environment is lead (Pb), the second most harmful substance after arsenic [4].

Lead is a major heavy metal distributed widely in polluted sites [5]. It comes mostly from paints, cable covering, fertilisers and pesticides, and metal production [6]. The issue of Pb in a contaminated agricultural soil has been a question of great interest in a wide range of fields [7]. Currently, ex-situ decontamination on Pb-polluted soil using physicochemical techniques is labour intensive, expensive, and affects the soil's biological properties [8]. As an alternative, the use of plants to decontaminate soils, known as phytoremediation could offer an environment-friendly solution to soil remediation and potentially cost-effective [9]. Unfortunately, the high concentration of Pb intervenes in the inhibition of various plant physiological process and development; including photosynthesis, mineral nutrition, sugar transport, seedling growth, and seed germination [10] which disturb the effectiveness of phytoremediation. Among all stages in plants life cycle, germination of the seed is a highly sensitive physiological process.

Seed germination has a pivotal role in the plant propagation and it is regulated by several hormonal interactions and environmental factors

[11]. The effect of Pb on seed germination has been the subject of many studies regarding its high sensitivity to metal pollution [12]. However, research has consistently shown that an unwanted exposure to Pb contributes detrimental effects on seed germination as explained by a lack of some defence mechanisms [13,14]. Although seeds showed an amount of tolerance in the presence of all heavy metals; the percentage of germinated seeds depends on the metal concentration and the type of species [15]. Recent studies have shown that *Acacia* species have the ability to tolerate and accumulate heavy metals in a different part of the plant [16,17].

Acacia auriculiformis A-Cunn ex-Benth or locally known as Ear Leaf Acacia is a fast-growing and valuable tree species; it belongs to the Fabaceae family, which is known as a heavy metals hyperaccumulator species family [18]. Its economic impact is predominantly positive and it is one of the most favoured trees in degraded sites [19]. It is only recently its invasive potential has been noted [20]. In using this plant as a phytoremediation species, the effect of Pb on the germination and early seedling development in *A. auriculiformis* has to be investigated. Hence, this study was undertaken to emphasise the seed germination pattern and early seedling development in different Pb concentrations by using *A. auriculiformis* to contribute a basis for phytoremediation to reduce the risks associated with Pb contamination.

2. MATERIALS AND METHODS

2.1 Materials Preparation

Acacia auriculiformis seeds collected from University Putra Malaysia and species was confirmed by plant taxonomist; Prof Dr. Rusea Go. Seeds were soaked in hot water (80°C) for 12 h to break seeds dormancy [20] prior surface sterilisation using 4% solution of sodium hypochlorite and washed 5 times with sterilised water, to avoid fungal contamination [21].

Lead sulphate ($PbSO_4$) was used to prepare desired Pb concentration solutions. Eight levels of Pb concentrations (from 0 to 4.0 g/L interval of 0.5 g/L) were used in the experiment.

2.2 Experimental Setup

Ten seeds were placed in Petri dishes contained filter paper moistened with 10 ml of distilled water and different Pb concentrations respectively (three repetitions per concentration). The number of seeds germinated was noted until the maximum germination of the control group (distilled water). Seeds were considered as germinated on the first appearance of the radicle. The counting of germinated seeds was holding on until the day where no germination was observed.

2.3 Seed Germination

Several parameters were selected to assess the impact of Pb on *A. auriculiformis* seed germination;

Germination Percentage (GP) = (Number of germinated seeds/ Total number of seeds) [22]

Germination Index (GI) = {(Total number of germinated seeds/ Total number of seeds) x 100} [23]

Seedling Vigor Index (SVI) = (Seedling length (cm) x Germination percentage) [24]

Mean Germination Time (MGT) = $(\sum dn / \sum n)$ [25]

Where

d = The number of days counted from the first day of the germination

n = The number of seeds which germinated on day d.

Relative Pb Injury Rate (RIR) = (GP of the control - GP of the treatment/ GP of the control) [26]

2.4 Radicle Development

Radicle length was measured at the day where no germination was observed in all the treatments. Radicle length and tolerance index (TI) were taken as indicators of Pb phytotoxicity after germination; the radicle length was measured with a ruler starting from the hypocotyl, and the TI was calculated following Wilkins [29]:

Tolerance index (TI) = {(Mean radical length of germinated seed of polluted treatment/ Mean radical length of germinated seed of control) x 100}

2.5 Embryo Development

A histological study was established to examine the impact of Pb concentration on the development of the embryo. Ten seeds were placed in Petri dishes contained filter paper moistened with 10 ml of distilled water and different Pb concentrations (2 g/L and 3.5 g/L), respectively. Samples were collected on day 0 (before imbibition), day 2 (after imbibition), day 4 (for the control treatment), day 8 (for 2 g/L of Pb treatment), and day 16 (for 3.5 g/L of Pb treatment). The histology assay was determined following Jenson [30] and all were cut at the thickness of 8 to 10 μ m with the rotary handle microtome. After removing paraffin and water, the slides were stained with Eosin. The microscopic investigating and photographing of samples were studied by histological observations, using a light microscope.

2.6 Biochemical Response to Pb Stress

Total phenolics contents in the leaf sample were extracted and quantified following Jaafar et al. [27] using Folin-Ciocalteu reagent and absorbance was measured at 725 nm. The results were expressed as mg/g gallic acid equivalent (mg GAE/ g dry sample). The phenolic standard curve was constructed based on series of Gallic acid concentration and the standard curve equation was $y = 0.007x + 0.186$ was obtained.

Lipid peroxidation of plant parts was estimated by the level of malondialdehyde (MDA) production using the thiobarbituric acid method as described by Ibrahim and Jaafar [28]. The MDA content was determinate based on absorbance measured at 532 nm and calculated as μ mol/g of dry weight by using the following formula: $6.45(A_{532} - A_{600}) - 0.56 A_{450}$. The standard curve was created using a series of selected glacial acetic acid concentration.

Starch content was determined spectrophotometrically using a method described by Thayumanavam and Sadasivam (1984); measured based on absorbance at 630nm. Glucose was used in standard curve ($Y = 0.0211x + 0.5219$) and starch content was expressed as mg glucose/g of dry weight. 51846244.

2.7 Statistical Analysis

All the collected data were analysed using the software program SPSS. One-way ANOVA at $p < 0.05$ was used to determine the extent differences between treatments, and the comparison of mean values was performed using Tukey test.

3. RESULTS AND DISCUSSION

In general, any increment in the concentration of Pb was led to a reduction in GP, GI and SVI compared to control (Table 1). For the GP and GI, the difference was not significant up to 1.5 g/L ($P \leq 0.05$) and up to 94.5% reduction of both GP and GI values were observed at the lead concentration of 3.5 g/L. Meanwhile, total inhibition of germination was observed at 4 g/L of lead concentrations. Furthermore, the analysis shows that lead significantly reduced 1.37% SVI in *A. auriculiformis* ($P \leq 0.05$) at 3.5 g/L treatment. Similar negative response pattern was also observed for MGT where *A. auriculiformis* germination has been delayed significantly by almost 10 days (3.5 g/L) compared to control. Lead has also an adverse impact on *A. auriculiformis* seeds strength where increasing Pb concentration from 0.5 to 1.5 and 2.0 g/L increases the RIR from 0.14 to 0.34 and 0.60, respectively. Pb affects 60% of the seed's strength at a concentration of 2.0 g/L, which was the highest.

The application of 1.0, 2.0, and 3.0 g/L of Pb led to a reduction of radicle length (76, 67, and 44% respectively) compared to the control ($P \leq 0.05$) (Fig. 1). Even though reduction in radicle length at 0.5 g/L Pb treatment was not significantly

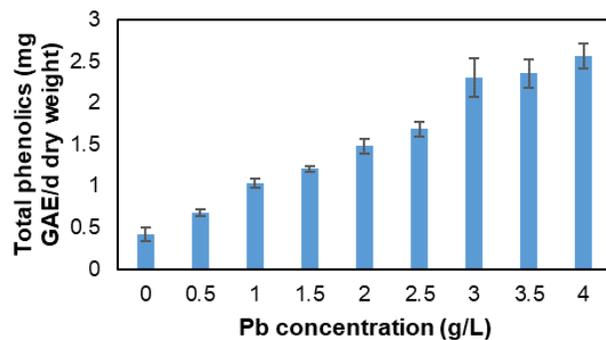
different compared to control, the tolerance index was effectively being reduced about 20%. A significant drop (92%) in radicle length and tolerance index were observed in 3.5 g/L Pb treatment from thus indicated the effect of toxicity of Pb on *A. auriculiformis*.

The highest reduction was approximately 8% was observed at Pb concentration of 3.5 g/L. Moreover, the Pb concentration of 0.5 g/L showed no significant different compared to the control. Similarly, increasing Pb from the control to 3.5 g/L decreased the tolerance index from 100 to 8% respectively ($P \leq 0.05$).

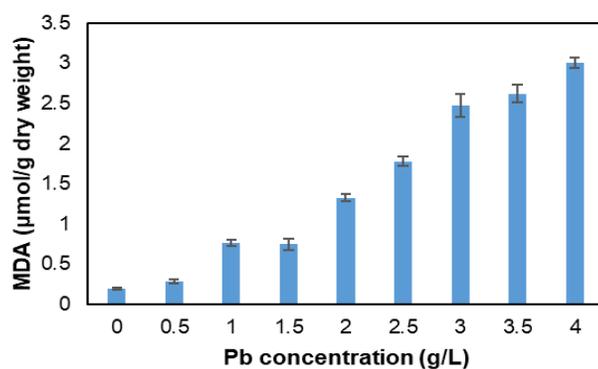
The presence of Pb varied the strength of *A. auriculiformis* germination depending on the concentration of the metal. A significant reduction has been observed in germination potential of *A. auriculiformis* with the increase of Pb over 1.5 g/L compared to the control. The inability of *A. auriculiformis* germination in high Pb percentage (over 3.5 g/L) might be explained by an oxidative damage, membrane alteration, altered sugar, protein metabolisms, and nutrient loss leading to seed toxicity and loss of productivity [31]. There was a clear relationship between Pb toxicity and the damage that has been introduced in *A. auriculiformis* seeds; it has been shown that as much as the concentration of Pb increase, the germination in *A. auriculiformis* seeds decrease, which could be explained by the significant impact that Pb has introduced on the meristematic cells leading to a decrease in the metabolic processes rate of the cell including nucleic acid synthesis, cell division and protein contents and some cotyledons and endosperms enzymes [32]. However, when Pb effects the enzymatic reaction the active meristematic cells

Table 1. Germination percentage, germination index, seedling vigour index and mean germination time of *A. auriculiformis* exposed to a selected different concentration of Pb. Means in the column followed by the same letter do not differ significantly at $p \leq 0.05$

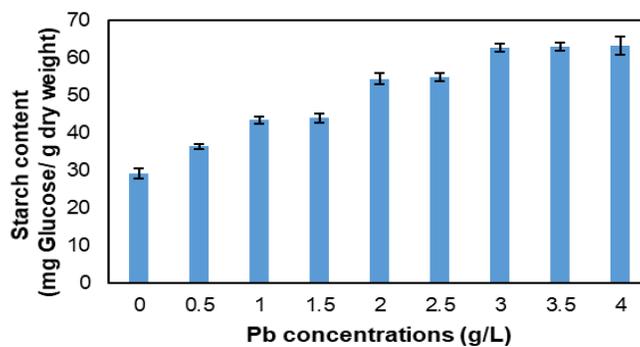
Treatment (g/L)	Germination percentage (GP) (%)	Germination index (GI)	Seedling vigor Index (SVI)	Mean germination time (MGT)	Relative injury rate (RIR)
0.0	60.0 ± 5.77 ^a	0.37 ± 0.036 ^a	315.66 ± 17.60 ^a	4.10 ± 0.90 ^a	-
0.5	50.0 ± 5.77 ^a	0.31 ± 0.019 ^a	261.33 ± 8.00 ^{ab}	4.33 ± 1.45 ^a	0.14 ± 0.038 ^a
1.0	60.0 ± 5.77 ^a	0.37 ± 0.036 ^a	256.00 ± 12.90 ^{ab}	4.63 ± 0.36 ^a	0.14 ± 0.059 ^a
1.5	56.6 ± 8.81 ^a	0.35 ± 0.055 ^a	205.66 ± 20.11 ^b	6.70 ± 0.78 ^{ab}	0.34 ± 0.040 ^{ab}
2.0	23.3 ± 6.66 ^{bc}	0.14 ± 0.041 ^b	83.00 ± 23.51 ^c	8.56 ± 0.43 ^b	0.60 ± 0.051 ^c
2.5	23.3 ± 6.66 ^{bc}	0.14 ± 0.041 ^b	61.00 ± 17.00 ^c	8.56 ± 0.72 ^b	0.62 ± 0.090 ^c
3.0	26.6 ± 3.33 ^b	0.16 ± 0.020 ^b	62.00 ± 2.21 ^c	10.33 ± 0.66 ^{bc}	0.53 ± 0.092 ^{bc}
3.5	3.3 ± 3.33 ^{cd}	0.02 ± 0.020 ^c	4.33 ± 4.33 ^c	14.33 ± 3.33 ^c	0.57 ± 0.077 ^{bc}
4.0	0.0 ± 0.00				



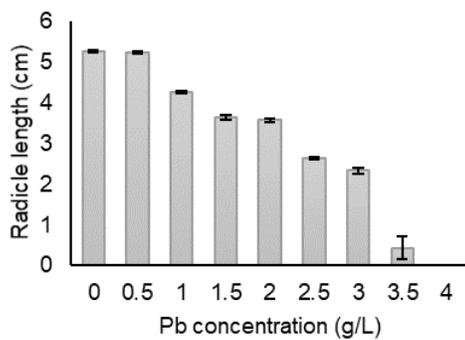
(a)



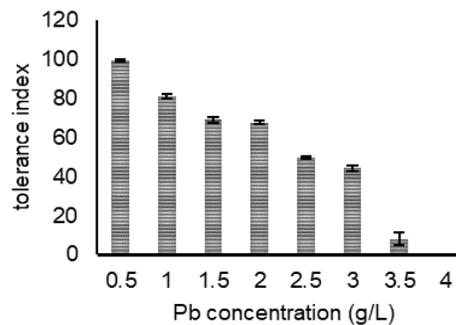
(b)



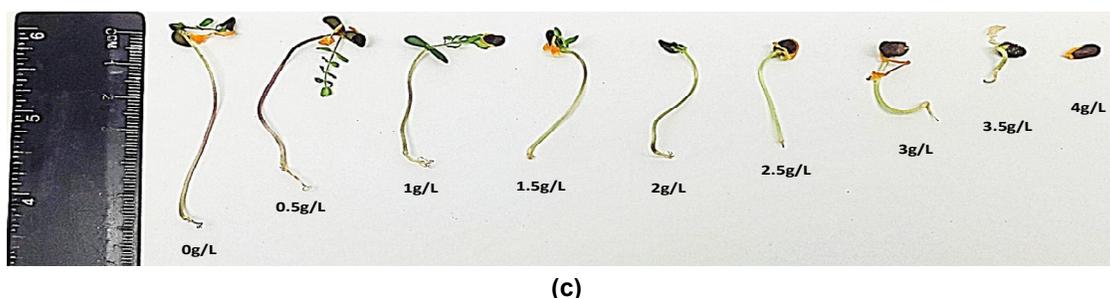
(c)



(a)



(b)



(c)

Fig. 1. Effects of different concentrations of lead on (a) the radicle length; (b) the tolerance index and (c) comparison of radicle morphology of *A. auriculiformis* seedlings

The bar in the graph represents the standard error of the mean \pm SD

take the soluble form and the supplements will not reach the radicle which will reduce its growth [33]. Furthermore, it has been shown that heavy metal toxicity affects the germination by the inhibition of storage food mobilization, degradation of proteolytic activities, disruption of cellular osmoregulation, and reduction in radical formation [34].

Results of histological observation (Fig. 2) showed the impact of Pb treatment on the development of the embryo cells in *A. auriculiformis* seeds. The size of the embryo was equal for all treatments on day 0 and day 2 (before and after imbibition). Comparison on day 4 showed that seed in control treatment was germinated while mitotic cells in seed in the treatment of 2.0 g/L Pb are in place forming a complete embryo. In the meantime, polarizations of mitotic cells in Pb treatment of 3.5 g/L are still in the progress to form an embryo. Complete germination for Pb treatment of 2.0 g/L and 3.0 g/L were achieved at day 8 and 14 respectively. It is clearly obvious that the embryo development was delayed down, and number of mitotic cell division decreased as the concentration of Pb increase.

The Mean Germination Time also was affected by the presence of Pb; the germination rate was related directly to the concentration of metal. It can be seen from the results that as much as the concentration of Pb increase the germination time become longer, which affect its ability to produce normal seedlings. This delay could be a result of the inhibition in the biochemical process believed to be essential for the initiation of germination especially the operation of the oxidative pentose phosphate pathway [35], or may be due to a viability loss because of a decrement in the energy generation by the embryo, which plays a major role in seed

germination and its blockage could affect protein, RNA and DNA synthesis as well as mitosis, since energy is required for these processes to occur [36]. Clemens [37] showed that Pb at toxic concentration can interrupt cell division and RNA replication could clock DNA repair process and another vital physiological process. However, the germination took longer time as much as the concentrations of Pb increase because seeds were developing tolerance by overcoming the germination time delay [38]. Likewise, based on the results, the increasing influx of Pb (up to 2.0 g/L) causes a serious increment in the relative injury rate in *A. auriculiformis* seeds; that can be explained by the decrement of the enzymatic dehydrogenase activity; resulting in a biochemical change can affect the germination process [39].

Biochemical responses of plant towards Pb were shown in phenolic total content, MDA and starch data (Fig. 3). The total content of phenolic compounds in seeds of *A. auriculiformis* under different Pb concentrations was shown in Figure 3a. The total phenolic content of the investigated seed extracts was ranged from 0.416 mg GAE/d dry weight to 2.556 mg GAE/d dry weight of the samples. The methanolic seeds extract of *A. auriculiformis* in the Pb concentration of 3.0, 3.5, and 4.0 g/L had the strongest antioxidant activity against Pb toxicity where it reaches 2.302, 2.351, and 2.556 mg GAE/d respectively.

An enhanced level of lipid peroxidation, as indicated by MDA content, was observed in *A. auriculiformis* seeds in response to increase of Pb concentration (Fig. 3b) indicating an oxidative stress where significant increment of MDA was observed after Pb treatment of 1.0 g/L (50% compared to control) and continually increases at the rate of 1.152 at the same time, the results and statistical analyses indicated that

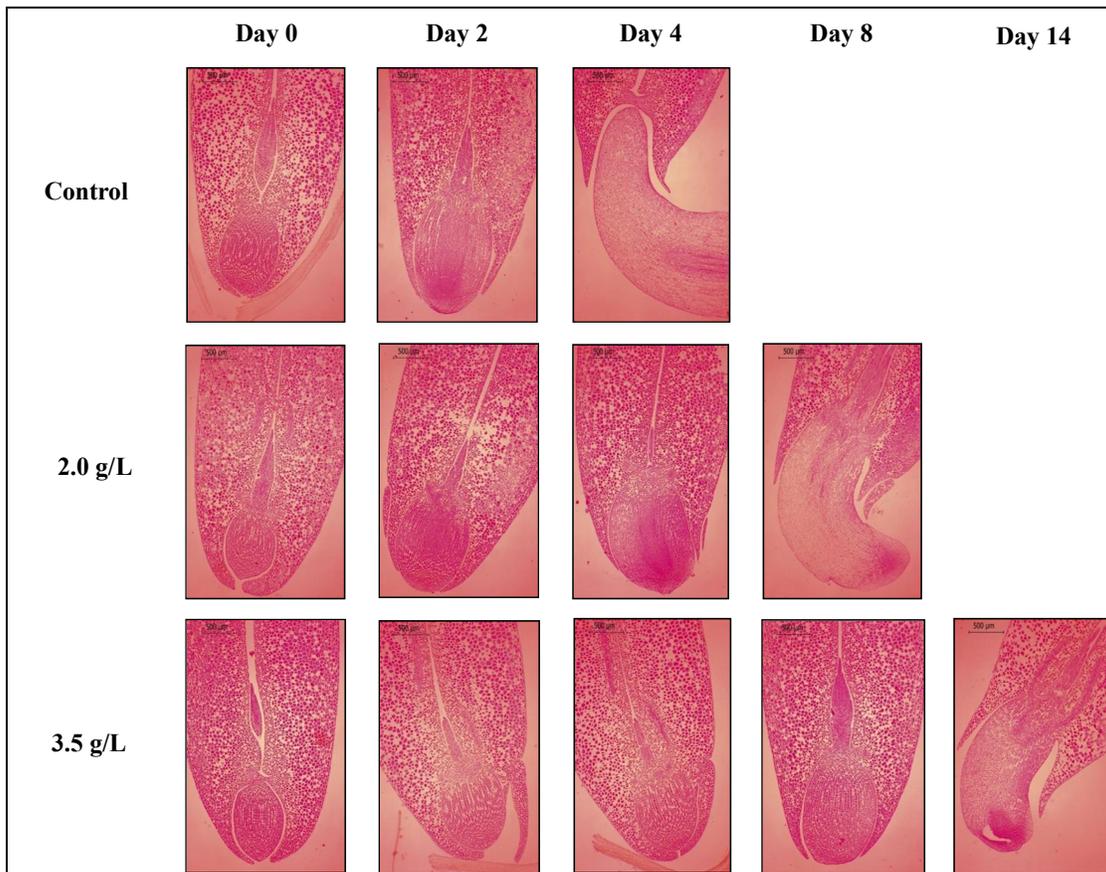


Fig. 2. Comparison in *A. auriculiformis* embryo development and germination in selected Pb treatment

all the Pb concentrations were significantly stimulated the biosynthesis and the accumulation of the starch content in *A. auriculiformis* seeds. In general, with an increment of 1.0 g/L is caused increased in starch content approximately 10 mg glucose/g of dry weight (Fig. 3c). The increment in the MDA and total phenolics content also provide an evidence that Pb contamination induced damage at the cellular level of *A. auriculiformis* seeds as it was explained by a peroxidation of lipid membranes induced by the ROS [40]. Phenolics are aromatic benzene ring compounds with one or more hydroxyl groups produced by plants mainly for protection against stress. The phenolic results indicated that *A. auriculiformis* with the high phenol content had weak chelating activity which exhibited good antioxidant activity. As their free radical scavenging ability is facilitated by their hydroxyl groups, the total phenolic concentration could be used as a basis for rapid screening of antioxidant activity. In the presence of heavy metals, nutrient sources become limited because of the

starch immobilization, that could be resulting from the effects of heavy metals on the enzymes, such as acid phosphatases, proteases and α -amylases, which known to facilitate both seed germination and seedling growth via mobilizing nutrients in the endosperm [41].

Similar results were reported [42] who found that *Phaseolus vulgaris* exposure to lead caused retardation in the germination process. It has also shown that the presence of Pb influences the osmotic balance of seeds, and the entry of metal to the seed may cause damage to the embryo, which could be a reason for the germination delay [43]. Shafiq et al. [44] observed a decrease in the germination of *Leucaena leucocephala* gradually following the increase of Pb at the concentration of 0.025, 0.05, 0.075 and 0.010 g/L. The same trend has been observed by Farooqi et al. [45] who found that seed germination and seedlings vigour index in *Albizia lebbeck* were highly reduced with the increase of Pb concentration. The reduction of

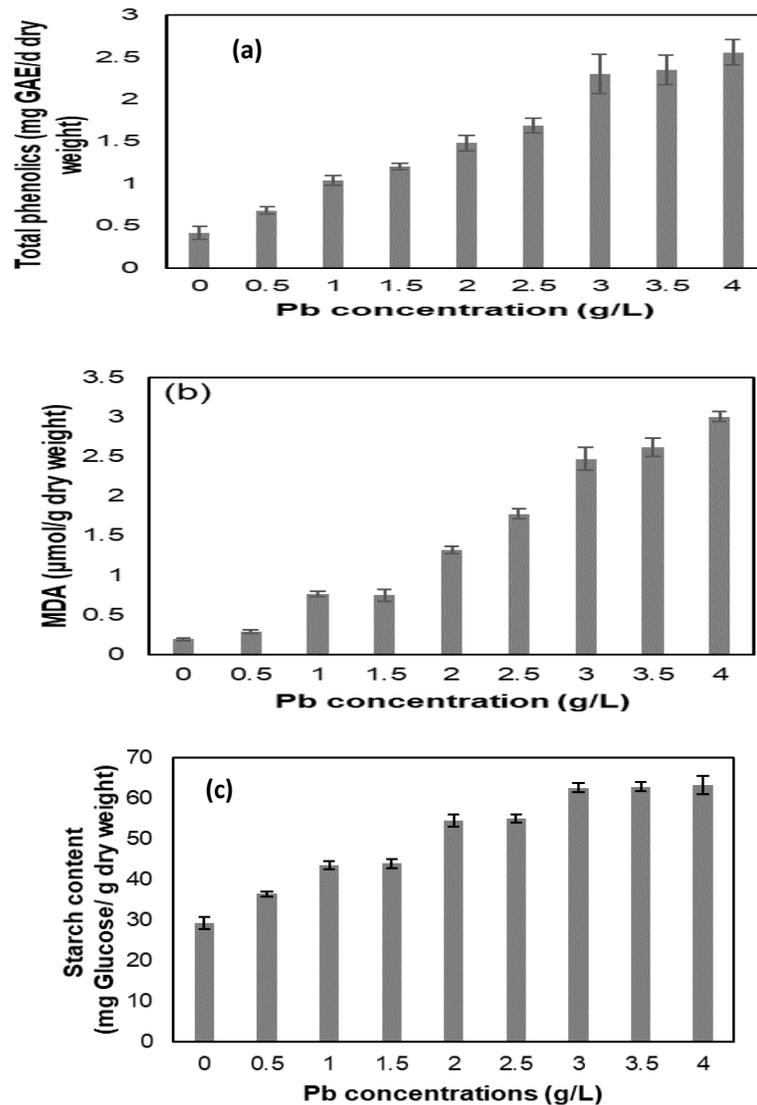


Fig. 3. Biochemical response (a) Total phenolics, (b) Maliondialdehyde (MDA) and (c) Starch content in *A. auriculiformis* seeds when exposed to a selected concentration of Pb.

The bar in the graph represents the standard error of the mean \pm SD

A. auriculiformis germination by Pb might be explained by the inhibition of the activity of acid phosphatases, proteases and α -amylases involved in the hydrolysis of reservations or processes involved in embryo development (such as division cell) and metabolism [46]. The inhibition of metabolism could be explained by the inhibition of the major mobilization of stored reserves in the storage organs (usually the cotyledons or endosperm) which contain substantial quantities of two or more of the major reserves, carbohydrates, oils and proteins [47]. One of the most common reserve carbohydrates

in seeds and an important commercial source in the endosperm is starch, our results showed a gradual increment in the starch content (Fig. 1c) and that could be explained by the inhibition of the α -amylase enzyme which hydrolyse the amylose and amylopectin in the starch [48]. Our results are in agreement with Mihiri et al. [41] who found that Pb reduced 50% of α -amylase in pea seedlings leading to a high starch content. Furthermore, the toxicity of ions can also affect the viability of embryo by accelerating the breakdown of reserved food material of the seed embryo which affects the germination

[49,50,51,52]. Another finding by Kadiriye and Dilek [42] illustrates that the inhibition of germination in *Phaseolus vulgaris* was related to the highly Pb permeability degree of seed coats.

Seed's exposure to high level of Pb known to have stimulating effects on their morphology and physiology by affecting radicles development [31] [50]. Considering the fact that seed's radicle is the first contact with Pb toxicity, Pb penetrates easily to the radicle cortex and affect its elongation [53]. Our results showed that the application of different Pb concentrations introduces reduction in *A. auriculiformis* radicle elongation (Fig. 3c). The same result was reported by Dorogházi et al. [54] who found that seeds treated with a high level of Pb decrease the biological value of the seed as well as the growth of the radicle. That could be explained by the fact that Pb toxicity enhances protein and carbohydrate contents which cause a delay in the radicle emergence, affecting the oxidising ability of the radicle, the activity of peroxidases and polyphenol oxidases, and overall lowering of carbohydrate-metabolising enzymes— α -amylases, β -amylases, acid phosphatases and acid invertases [48].

Previous studies have also reported that the effect of heavy metals on seed germination is related to the type of species, the nature and the concentration of metal [55]. Surprisingly, the noticeable finding of this study, which has never been reported is the great resistance of *A. auriculiformis* seeds to the extremely high concentration of Pb; it was clearly remarkable that there were no differences in the germination percentage and the germination index of *A. auriculiformis* up to 1.5g/L. Dane et al. [56] demonstrated similar results as they found that *Acacia* species including *A. decurrens*, *A. holosericea* and *A. leiocalyx*, showed a high level of tolerance to Pb toxicity. The high concentrations of Pb used appeared to have no effect on *Acacia* species germination. Also, Ali et al. [57] found that *A. victoria* can tolerate Pb toxicity (up to 500 mg/L) and accumulate more than 1000 mg kg/L in leaves and shoots. Furthermore, Masvodza et al. have shown that *A. saligna* and *A. polyacantha* can resist and accumulate different heavy metals including Ni, Cu, Pb and Fe in roots, shoots and leaves which make them suitable plants for phytoremediation.

4. CONCLUSION

This study demonstrates the effect of Pb high concentrations on the germination of *Acacia*

auriculiformis species. In the present investigation, it is concluded that increasing the concentration of lead to high levels has a negative effect on the germination and early seedlings development in *Acacia auriculiformis*. Based on the germination test and tolerance index results, *Acacia auriculiformis* is a very tolerant species which can germinate in a high Pb concentration. With the fast-growing character of this species, it can be used for phytoremediation or reforestation of soils in which the presence of this heavy metal was reported.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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