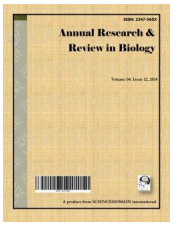

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Assessment of *Acacia auriculiformis* Cunn. Ex Benth. Seed Germination and Growth Resistance towards Arsenic Toxicity

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Authors' contributions

This work was carried with collaboration between all authors. Author AZS conducted all experiments protocol, data collection and drafting the manuscript, Author AZ assisted in designing the planting material. Author AANA consulted on heavy metal analysis and statistics, Author GR involved in plant identification. Author MM designed the study and consulted on the study approach. All authors read and approved the final manuscript

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ABSTRACT

Aim: Assess the ability of *A. auriculiformis* to resist arsenic (As) toxicity during germination and growth.
Place and Duration of Study: The study was conducted in Department of Biology, Universiti Putra Malaysia Serdang, between September 2016 and May 2017.
Methodology: *A. auriculiformis* seeds were germinated in series of As solutions (50 ppm-100 ppm). Each As concentration contained three replicates and each replicate contained ten seeds. After 15 days, germination analysis such as germination percentage, seedling vigor index, relative injury rate and mean germination time were calculated. Meanwhile, another set of *A. auriculiformis*'s seeds were germinated using distilled water and planted in soil treated with different As concentration. After 60 days, the plant morphology, chlorophyll content and growth rate were also recorded.

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Results: The result revealed that, seed germination percentage, seedling vigor index and relative injury rate were significantly reduced at 80-100ppm As concentration, but the mean germination time showed no significant difference between controlled and treated seeds. The growth analysis showed that growth rate and chlorophyll content of treated plants decreased as much as 63.7% and 76.1% respectively. However, all the plants were able to survive up to 100 ppm exposure.
Conclusion: Therefore, it can be concluded that *A. auriculiformis* is capable to resist As contamination during germination and growth, thus showing the potential of species to remove As from soil, as a phytoremediator.

Keywords: Heavy metal; height; resistance; seedling; chlorophyll.

1. INTRODUCTION

Arsenic (As) is the twentieth most abundant element on earth's crust and naturally present in the environment through weather and volcanic activities [1]. The use of insecticides, herbicides and phosphate fertilizers, semi-conductor industries, mining and smelting, industrial processes, coal combustion, timber preservatives will also add up the accumulation of arsenic in the environment especially the soil [2]. According to Agency for Toxic Substances and Disease Registry (ASTDR) [3], permissible level of As in soil must not exceed 40 ppm. However, As contamination has been reported all over the world since 1961 and most of it occurs on groundwater [4].

The growing of agriculture and manufacturing industries has resulted in increased release of a wide range of xenobiotic compound to the environment [5]. Low levels of As exposed to human can stimulate esophageal and abdominal pain, vomiting and also diarrhea [6]. As contamination in drinking water can also lead to cancer and other diseases such as pigmentation changes, hyperkeratosis and muscular weakness. Excess deposition of hazardous waste has become disturbance to the soil thus limiting crop production [7]. Arsenic contamination in soil especially agriculture area did affect plant germination and growth rate, infertility loss, low yield and low quality of yield [8].

There are few remediation techniques available for As such as oxidation, phytoremediation, coagulation-flocculation, adsorption, ion exchange, electrokinetics and membrane technologies but in some cases the technique may produce side product that also has the ability to cause pollution and contamination [2]. An alternative way to overcome this problem is by performing a natural process of phytoremediation through phytoextraction since it was cost-effective and environmental-friendly in terms of absorption of organic and inorganic pollutant or contaminant. Phytoremediation is the use of plants to remove pollutants from the environment [9] and phytoextraction is define as the use of pollutant-accumulating plants to remove metals or organics from soil by concentrating them in the harvestable parts [10]. Phytoextraction needs proper selection of plant species to function as bio-accumulation agent to remove heavy metal from soil. However, there is no species fits for all heavy metal absorption regardless of climates and contamination level [11]. In general, any plant that are able to grow in poor soil, fast growing, utilize heavy metal in their biochemical pathway, wide heavy metal acceptance range and heavy metal resistance during embryo development is an excellence phytoremediation agent [12,13,14]. Several Brassicaceae such as *Alyssum* sp., *Thlaspi* sp. and *B. juncea*; Violaceae such as *Viola calaminaria* and Leguminosae such as *Astragalus racemosus* are known to take up high concentrations of heavy metals and radionuclides [15]. However, the ideal plant species for phytoremediation should have high biomass with high metal accumulation in the shoot tissues [16].

Several other *Acacia* sp. that has been tested to resist As toxicity are such as *A. mangium* and *A. nilotica*, but it was found to highly accumulate a large amount of arsenic on roots and impaired the formation, increasing the number of collapsed cells [17,11]. However, according to a finding in China, *A. auriculiformis* has been used as main species for water and soil conservation and also to improve soil fertility. It was also introduced to South China for ecosystem restoration in barren regions [18]. Until now, there was no report confirmed that *A. auriculiformis* is phytoremediator. Thus, the aim of this study is to evaluate the effectiveness of *A. auriculiformis* for arsenic removal from soil. *Acacia auriculiformis* has an ability to produce

high biomass yield, which adapts well to degraded soil conditions or very poor soil and long dry season [19]. However, further evaluation on effectiveness of *A. auriculiformis* as arsenic hyperaccumulator on the impact on seed germination and seedling growth are essential. Therefore, the objectives were set to assess the impact of arsenic exposure on *A. auriculiformis*'s seed germination and growth.

2. MATERIALS AND METHODS

2.1 Preparation of as Stock and *A. auriculiformis* Seeds

Stock solution of Arsenic was prepared by dissolving 1.0g arsenic trioxide (As_2O_3) in 10 ml of distilled water according to method suggested by Al-Mamun [6].

A. auriculiformis seeds were collected from Universiti Putra Malaysia (UPM) and were identified by Prof Dr Rusea Go from Department of Biology, UPM. According to Malaysian Meteorological Department, during the collection of the seeds (September 2016), Malaysia was experiencing southwest monsoon season with 30°C temperature and monthly average rainfall was at 250mm. Seed sterilization was carried out using 4% (v/v) according to the method of Costa & Sharma [20] before soaked in distilled water for 12h for dormancy breaks purpose [21].

2.2 Germination of *A. auriculiformis* Seeds

Ten *A. auriculiformis* seeds were placed into petri dish containing filter paper moistened with 10 ml of arsenic ranging from 0 to 100 ppm with 10 ppm interval, respectively. All the petri dishes were left at 33°C ±2°C for 15 days and observed every 24 h to count the number of seed germinated. The growth analysis on germination percentage [22], seedling vigor index [23], mean germination time [24] and relative injury rate [22] were calculated based on equations as follows:

Germination percentage (GP) =

$$\frac{\text{Number of germinated seeds}}{\text{Total number of seeds}} \times 100$$

Seedling vigor index (SVI) =

Seedling length x germination percentage

Mean germination time (MGT) = $\frac{\sum dn}{\sum n}$

Where,

d= number of days counted from first day of germination
n= the number of seed germinated on day d

Relative injury rate (RIR) =

$$\frac{\text{GP control} - \text{GP treatment}}{\text{GP control}}$$

2.3 Plant Growth of *A. Auriculiformis* Assessment

Distilled water germinated *A. auriculiformis* seeds were planted in the polybags containing 2 kg of the mixture of top soil and sand (4:1). The germinated seeds were watered with 500 ml of distilled water or As solution with different concentration for the first time, respectively. The plant arranged in Random Block Design (RBD) and was watered with 250 ml distilled water three times per week. All excess water was collected to avoid contamination to the surrounding area. On day 60 also, the leaves morphological appearance was observed and the chlorophyll content of the plant leaves was estimated using SPAD-502Plus Chlorophyll Meter (Konica Minolta).

2.4 Data Analysis

All the data collected from the experiment were then analyzed by one-way Analysis of Variance (ANOVA) using Duncan's multiple range test to analyze mean of more than three means group using SPSS software version 21.

3. RESULTS AND DISCUSSION

3.1 Germination Percentage (%)

Seed germination is one of the most important quantitative parameter in studying the effect of heavy metal on seedling growth because it was a sensitive process due to lack of defence mechanism [25]. Gradual decrement of seedling condition was recorded as As concentration increase (Fig. 1.), as similar to pattern in rice and winter wheat germination after exposed to As [26,27]. Two possible explanations were proposed previously, it was either due to limitation to produce plumule and radicle as As

concentration increase during germination [28] or it may result from interference of As with protease, ribonuclease and leucine aminopeptidase [29].

Both 50 ppm and 60ppm seedlings were able to germinate beyond the safe limit of As at 40 ppm [3]. With reduction of 36-42% on length of seedling compared to control, it was indicated *A. auriculiformis* resistance towards As toxicity at low concentration like *A. fernesiana* [30,31,32] and potentially be explored as As hyperaccumulator agent. The length and size of the seedling were shown significant decreases starting from 70 ppm (mg/L). In the case of excessive arsenic exposure such as in 70 ppm and above, critical malfunction in metabolic processes shall be started leading to shut down of plant defence mechanism and further increment of As concentration may lead to the death seed germination as shown at 100 ppm [33,34]. The hypocotyl of the seedling exposed to arsenic was appeared to be dark red coloured, showing phosphorus (P) deficiency [35]. Arsenic has almost similar properties to P and due to that, it able to substitute P in the phosphate groups of DNA [36] causing failure in photophosphorylation, genetic transfer, the transportation of nutrients, and phospholipid cell membranes which finally leading to drop in seed germination and growing rate [37]. Germination percentage (GP) reflects the reaction rate of plant seeds to their living environment [27] which also estimated the viability of seed population for early seedling growth [38]. After 15 days of As exposure, seed GP was found to be in dwindling trend (Table 1). Even though *A. auriculiformis* seeds showed negative relationship between GP and As exposure, it was still better compared to other plants such as rice reported previously [27] where only 50.2% reductions on GP were shown by *A. auriculiformis* at 50ppm As concentration (above the safe level limit).

3.2 Seedling Vigor Index

Similar decline of pattern is also seen for SVI with significant impact was shown by above 70 ppm exposure. SVI not only calculated to determine the level of activity and performance of seed but also measure the germination capacity and growing tendency of seedling [39]. Decrease in SVI value tells that the performance of the seeds was decreased due to several physical and biochemical process such as imbibition, enzyme activation and reduction in water uptake [34]. The presence of As will accelerate the accumulation of phenolic compound which lead to mucilage excretion, deaths of lateral root buds and root wrinkling [11]. The absence of root in early germination can leads to water deficiency which ultimately causes reduction of enzyme activities to degrade stored material in endosperm [40]. Prior to this, water uptake was decreased and caused failure in seed germination. This was supported by the increasing of relative injury rate (RIR) value (Table 1).

Fig. 1. Acacia auriculiformiss' seedlings length after exposed with different concentration of arsenic

3.3 Relative Injury Rate and Mean Germination Time (days)

RIR indicates that high amount of arsenic exposure do have great impact to the seeds. Increased in RIR value together with increasing of mean germination time (MGT) value indicates that *A. auriculiformis* take longer time to repair itself after As exposure. The highest As exposure caused the seed to have delay in time taken to germinate (6.67 days) while the normal seed only takes 3.98 days to germinate. Even though no significant different ($p<0.05$) between exposed seed and control recorded, the seedling size produced were different for each treatment (Fig. 1). Thus proved that, MGT only indicates the time taken for seed to germinate but not the health status of the seed. At the early stage of exposure 0-24hours, the seeds mitotic activities might have be disturbed with the presence of As [41]. Osborne [42] found that the damaged DNA in germinating seeds was repaired during the lag period in the early stage of hydration and for seed that has been exposed to As, all healing processes on the DNA can be completed within 96-120 hours [35] before it was able to germinate as seen in this study.

3.4 Growth Rates (Cm•Day) and Chlorophyll Content (Spad Unit)

Although Karataglis [43] stated that germination assessment is the best indicator for testing metal toxicity, Kapustka [44] believe that germination is less sensitive and effective compared to other plants end point responses such as plant height, numbers of leaves, fresh weight, dry weight and chlorophyll content were analysed to assess the potential of plant to grow in high As concentration. After 60 days of As exposure, *A. auriculiformis* plant height and leaves size do not show a drastic decreasing pattern (Fig. 2). The mean height for control *A. auriculiformis* plant was 16.97 cm while the mean height for 50 ppm, 60 ppm, 70 ppm, 80 ppm, 90 ppm and 100 ppm exposure were reduced ranging from 33.6% to 48.3% compared to the control plant. In many tolerant plants, As present in root cells and rapidly combined with single molecules to form phytochelatin (PC) [45] and sequestered in the vacuole [46]. The sequestration of As-PC into vacuole was important to complete As detoxification because As-PC found to most stable in acidic environment which is vacuole [47]. This process will prevent As interaction with cellular metabolism and cell disruption. From the results obtained, all the plants treated with As were able to survive and suggested that As-PC formation is taken place in *A. auriculiformis* thus make it resistance towards As toxicity.

However, positive characteristic in the survival in As contaminated soil does not reflect on the growth rate. The growth rate ($cm•day^{-1}$) of exposed plant was lower than the normal plant growth where the control *A. auriculiformis* growth rate was at 0.28 $cm.day^{-1}$ and As exposed plant were ranging from 0.13-0.08 $cm•day^{-1}$. Lower growth rate is the accumulation outcome of decline in physiological activity such as photosynthesis [48]. Ability to sustain the growth progress in any plant is much depending on the effectiveness of the plant to provide sufficient resources. Exposure to As has caused reduction in chlorophyll content (Table 1) which can consequently lead to disruption of photosynthetic machinery [27]. Since *A. auriculiformis* is from Fabaceae family which usually characterized as hyperaccumulator, any absorbed As is not immobilized in the roots but instead moves through xylem to the leaves and other parts of the plants [49]. Due to this, the cellular membranes become damaged and causing electrolyte leakage [50] and effect the photosynthesis process prior to lack of photosynthetic pigments. Protochlorophyllide reductase activity also inhibited due to presence of As [51]. In addition, the decreasing in number of chlorophyll has been reported in spinach [52]. Chlorophyllase activity increase due to the presence of As where it will hydrolyse chlorophyll and reduce the amount of it [53]. According to Seregin [54] excessive accumulation of As in chloroplast will substitute Fe^{2+}, Zn^{2+}, Mg^{2+} and then destroyed the structure and function of chloroplast. Thus, this causes marginal reduction in chlorophyll content. Leaf chlorophyll content is directly associated with the efficiency and capacity of the photosynthetic apparatus and hence provide useful information about photosynthetic potential of *A. auriculiformis* after exposed with As. In the case of 100ppm exposure, 76.1% drop in chlorophyll content caused up to 68% drop in growth rates. This was clear indication of *A. auriculiformis* inability to sustain the significant amount of resources with inefficient photosynthesis.

Presences of As not only reduce the chlorophyll content, it also affect numbers of leaves and chlorosis symptoms of the young leaves (Fig. 2). However, most of the plants recovered from chlorosis as soon as the true leaves appear but the size of the leaves appear slightly impaired.

Table 1. Mean comparison for germination and growth analysis of *A. auriculiformis* after exposed with serial concentration of as

Concentration (ppm)	Germination percentage (%)	Seedling vigor index	Relative injury rate	Mean germination time (days)	Growth rates ($cm \cdot day^{-1}$)	Chlorophyll content (SPAD units)
0	76.7 ± 11.5^{a}	283.7 ± 15.3^{a}	0.0 ± 0.0^{a}	3.9 ± 1.9^{a}	0.2767	48.5 ± 2.6^{a}
50	50.0 ± 10.0^{b}	82.5 ± 6.6^{b}	0.3 ± 0.1^{ab}	4.5 ± 1.1^{a}	0.1131	31.4 ± 2.3^{b}
60	43.3 ± 15.3^{b}	60.6 ± 41.3^{bc}	0.4 ± 0.3^{b}	5.0 ± 1.6^{a}	0.0828	21.3 ± 0.8^{cd}
70	40.0 ± 17.3^{bc}	31.3 ± 9.5^{cd}	0.5 ± 0.1^{bc}	5.2 ± 3.0^{a}	0.0969	18.4 ± 2.7^{cd}
80	30.0 ± 17.3^{bcd}	26.0 ± 4.6^{d}	0.6 ± 0.3^{bc}	4.9 ± 1.9^{a}	0.1348	24.0 ± 2.7^{c}
90	10.0 ± 17.3^{d}	12.3 ± 17.1^{d}	0.9 ± 0.3^{c}	6.0 ± 3.6^{a}	0.1055	16.7 ± 1.4^{de}
100	13.3 ± 15.3^{cd}	11.9 ± 2.3^{d}	0.8 ± 0.2^{c}	6.7 ± 2.5^{a}	0.1004	11.6 ± 1.7^{e}

Values are mean and standard error measurement made on three replicates. Superscripts within the means of each column (a-d) with different letters indicate significant difference

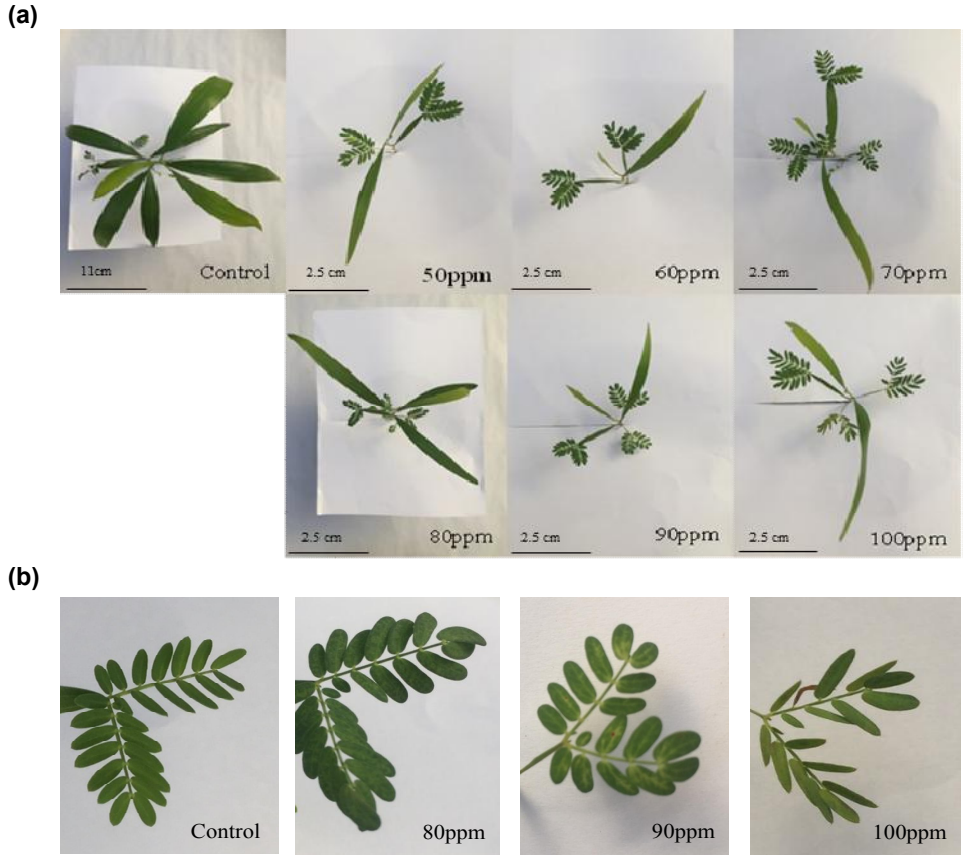

Fig. 2. Development of *A. auriculiformis*'s leaves after 60days exposure to selected As concentration (a) Comparison on the size of leaves (b) Emergence of chlorosis and necrosis

The chlorosis observed in *A. auriculiformis* leaves also indicates chlorophyll degradation [11]. Chlorosis symptoms appeared at three highest concentration of arsenic exposure which could be a proof of increased arsenic concentration in the leaves membrane [55]. Necrosis also happens when As is been translocated to the leaves which reflects to deficiencies of nutrients such as potassium, phosphorus and nitrogen [56]. The ability of As to substitute phosphorus causing the occurrence of necrosis.

4. CONCLUSION

A. auriculiformis seeds were able to germinate up to 80 ppm As level. Although all seed growth analysis shown negative correlation, the *A. auriculiformis* itself is able to survive and grow in As-contaminated soil up to 100ppm. Nevertheless, the growth rate of exposed plants was different compared to the normal. Therefore, it can be concluded that *A. auriculiformis* is resistance and tolerance to As toxicity.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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