

GROWTH, LEAF ANATOMY AND MERCURY UPTAKE IN AZOLLA PINNATA AND AZOLLA FILICULOIDES

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ABSTRACT

Azolla pinnata and *Azolla filiculoides* can be used as heavy metal phytoremediator, but *A. pinnata* is not a hyperaccumulator. Plant response to heavy metals is not only seen from the ability to accumulate heavy metals, but also from the aspects of growth and changes in plant tissue structure. This study aimed to evaluate the development, leaf anatomy and ability to absorb mercury in *Azolla* with non-hyperaccumulator and hyperaccumulator characters. The study was conducted by growing *A. pinnata* and *A. filiculoides* in containers containing 5 L of hydroponic solution that was either mercury-free or polluted with mercury at a concentration of 0.5 ppm. The results showed that *A. filiculoides* showed better tolerance and physiological adaptation to mercury contamination compared to *A. pinnata*, which experienced a decrease in biomass due to low detoxification ability. The detoxification ability of *A. filiculoides* is also supported by the anatomical adaptation in the form of leaf mesophyll thickening. Although *A. pinnata* absorbed more mercury, its inability to overcome toxic effects reflected different adaptation strategies. The ability of *A. filiculoides* to store mercury without disturbing its physiology shows its potential as a promising phytoremediation agent.

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INTRODUCTION

Heavy metal pollution, particularly mercury, is one of the most significant environmental concerns in various aquatic and terrestrial ecosystems. The main sources of mercury pollution are gold mining, industry, and the use of certain chemical fertilisers or pesticides. Mercury enters water bodies through liquid and solid wastes discharged into rivers without treatment (Zou, 2023), precipitated by rain, or dissolved with runoff into waterways (Aleku et al., 2024). Mercury has high toxicity, is stable in the environment and can accumulate in the food chain, making it harmful to living things, including humans (Gworek et al., 2020). The accumulation of mercury in the aquatic environment can reduce the growth, reproduction and survival of aquatic biota (Ibrahim et al., 2025). One way to reduce mercury content in the aquatic environment is through phytoremediation, which is the use of aquatic plants to absorb and neutralise heavy metals (Ma et al., 2021; Sitarska et al., 2023). Plant species selection is an important key in phytoremediation (Amabogha et al., 2023). *Azolla* is known as an aquatic plant that has high potential for phytoremediation of water bodies (Nahar, 2020).

Azolla is a genus of water ferns that symbiotize with the blue alga *Anabaena azollae* in fixing nitrogen. This plant can grow fast, has a high biomass and has been widely used in agriculture as green fertiliser. *Azolla* is also known to have the ability to absorb heavy metals in water, although its accumulation ability depends on the species and environmental conditions (Devi et al., 2024). Some *Azolla* species exhibit both non-hyperaccumulator and hyperaccumulator characteristics. Hyperaccumulator plants are plants that accumulate high amounts of heavy metals in their tissues without showing severe symptoms of toxicity. *Azolla pinnata* is an effective phytoremediator, but is not categorised as a hyperaccumulator (Azza et al., 2011; Dewi et al., 2023). *Azolla pinnata* can be used as a phytoremediator for Cd, Cu, Cr, Fe, Pb, Mn and Zn (Kumar et al., 2020). While *Azolla filiculoides* is a hyperaccumulator (Lestari et al., 2024). *A. filiculoides* can effectively absorb Cr, Pb, Zn, Hg, Cu, Cd, Ag and Ti (Hassanzadeh et al., 2021). These differences are interesting in terms of the accompanying physiological and anatomical changes. Plant response to heavy metals is not only seen from the accumulation of heavy metals themselves, but also from the aspects of growth and changes in plant tissue structure.

Mercury uptake by plants depends on several factors such as plant species, mercury concentration, duration of exposure and environmental conditions (Ma et al., 2021; Yu et al., 2018). In plants, mercury can interfere with photosynthesis, respiration and protein synthesis (Vijay et al., 2025). These effects can be seen in visual symptoms such as chlorosis, necrosis and retarded growth. Previous studies have shown that heavy metals can cause modification of leaf, stem and root structures, such as thickening of cell walls and changes in tissue size (Carrasco-Gil et al., 2013; Hamim et al., 2019; Tauhida et al., 2022). Hyperaccumulator plants develop adaptive mechanisms to avoid metabolic disruption or retarded growth due to the presence of heavy metals. These mechanisms are through a combination of active uptake, rapid transport from root to shoot, detoxification, safe storage in vacuoles and protection system with antioxidants (Leitenmaier & Küpper, 2013; Pasricha et al., 2021; Skuza et al., 2022).

This study aimed to evaluate the growth, leaf anatomy and ability to absorb mercury in *Azolla* characterised as non-hyperaccumulator (*Azolla pinnata*) and hyperaccumulator (*Azolla filiculoides*). By comparing the two groups, this study is expected to identify the most effective *Azolla* species in phytoremediation of heavy metals, especially mercury. This study is not only relevant in the context of science but also has practical implications for the management of polluted environments.

METHODS

The research was conducted using the experimental method. This research was arranged using a Completely Randomised Design with two factors. The first factor was plant species (*Azolla pinnata* and *Azolla filiculoides*). The second factor is the concentration of mercury in the growing medium (0 ppm and 0.5 ppm). The treatment combination was repeated four times.

Procedure

Preparation of test plant

To obtain a sufficient number of test plant, *A. pinnata* and *A. filiculoides* were cultivated for two weeks in a container measuring 28 x 20 cm and 12 cm in height, containing 5 L of hydroponic solution. The fertiliser dose used was half of the usual fertiliser dose. The hydroponic fertiliser used was AM Mix GoodPlant brand.

Phytoremediation process design

Sixteen containers were prepared. Each container was filled with 5 L of hydroponic solution and aerated with aerator. For the treatment of mercury contamination at 0.5 ppm, 3.4 mg of HgCl₂ was added. The 5 L water limit was marked using a permanent marker. A total of 20 *A. pinnata* or *A. filiculoides* resulting from breeding in the hydroponic system were transferred to each container. The amount of water container was maintained every two days by adding water up to the predetermined water level. The position of each container was randomly changed every two days. The process was carried out for four weeks.

Observed parameters

Mesophyll thickness of *Azolla* leaves

Microscopic preparations of leaf anatomy were made using the paraffin method. Leaf samples were put into vials and fixed by soaking in 70% alcohol for 24 hours. The samples were re-soaked using alcohol with a concentration of 80% and 90% for 30 minutes. Furthermore, the samples were soaked with 100% alcohol for 60 minutes. The alcohol in the samples was removed by immersing them in an alcohol:xylol solution in the ratio of 3:1, 1:1 for 30 minutes and 100% xylol solution for 60 minutes. The samples were immersed in xylol:paraffin solution in the ratio of 1:9 and incubated at 57°C for 24 hours. After that, the samples were immersed in pure paraffin and incubated at 57°C for 24 hours. Samples were removed and embedded in paraffin blocks and left for one hour. Sample slicing was performed by using a rotary microtome. The slices were placed on a glass slide. Paraffin on the sample was removed by heating the glass slide at 45°C using a hot plate. The sample was glued to the glass slide using a mixture of glycerine and albumin. After the sample was glued, the glass slide was dipped in xylol for six minutes, alcohol:xylol in the ratio of 1:3, 1:1, and 3:1 for three minutes. This was followed by immersing the glass slides in 100% alcohol for six minutes, 90%, 80% and 70% alcohol for three minutes. The samples were stained with 1% safranin in 70% alcohol for one hour. After staining, the samples were immersed in 80%, 95%, and 100% alcohol for one minute, alcohol:xylol in the ratio of 3:1, 1:1, 1:3 and xylol for three minutes. The sample on the glass slide was dabbed with glycerine and covered with a cover glass. The glass slide and cover glass were glued with transparent nail polish. Microscopic specimen preparations were observed under a binocular light microscope with a magnification of 10x ocular lens and 10x objective lens. Images on the microscope were documented using a camera connected to Optilab Viewer 2.2 software. Mesophyll thickness was measured using Image Raster 3.

Plant dry weight

Plant dry weight is obtained by weighing all plants in each container that has been dried at 65°C until the weight stabilises.

Mercury content in plants

Mercury content in *A. pinnata* and *A. filiculoides* was measured only in plants treated with 0.5 ppm mercury in the growth medium. Before the mercury content was measured by using AAS, the dried plant tissues were digested using a combination of Acid (HNO₃ and H₂SO₄).

Data analysis

Plant growth (dry weight) and leaf anatomy (mesophyll thickness) data were analysed using Two-Way Anova with JASP (Jeffrey's Amazing Statistic Program) 0.18.3.0 software. If the treatment had a significant effect, the Tukey test was carried out with $P < 0.05$. Comparison of the ability of *A. pinnata* and *A. filiculoides* to absorb mercury was conducted using a T-test.

RESULTS AND DISCUSSION

Plant dry weight is one of the most parameters in measuring in plant growth and plant health (Kiri, 2023; Malik et al., 2012). The results showed that plant dry weight was not influenced by plant species but was influenced by mercury in the growing medium. *Azolla pinnata* showed a decrease in dry weight when grown in mercury-contaminated media. This is due to *A. pinnata*'s inability to tolerate or detoxify mercury. In contrast, the dry weight of *Azolla filiculoides* did not experience significant changes despite being grown in mercury-contaminated media. This indicated that *A. filiculoides* has a high tolerance to mercury exposure. *A. filiculoides* responds to heavy metals by producing metallothionein, which detoxifies and maintains homeostasis of heavy metal in plants (Schor-Fumbarov et al., 2005). Additionally, it contains heavy metal-tolerant microorganisms that can support plant adaptation to heavy metal pollution (Banach et al., 2020), and modify anatomical and reproductive structure (Jonoubi & Karimi Limanjoubi, 2024).

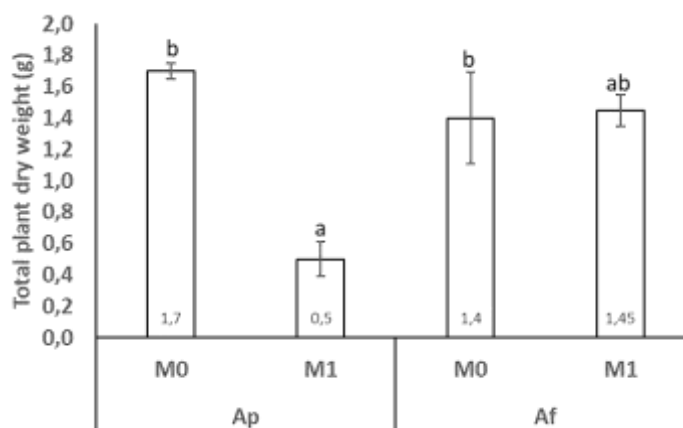


Figure 1. Total plant dry weight of *Azolla pinnata* (Ap) and *Azolla filiculoides* (Af) in growing media that was either mercury free (M0) or polluted with mercury at a concentration of 0.5 ppm (M1). Values are means and standard error (SE) of four replicates of each treatment. Bar for each treatment with the same letter are not significantly different ($P < 0.05$).

The difference in resistance between the two species was also reflected in the anatomical response of the leaves. In *A. pinnata*, the presence of mercury in the growth medium did not significantly affect the mesophyll thickness of the leaf. The leaf mesophyll of *A. pinnata* remained stable, indicating no change in the internal structure of the leaf in response to mercury contamination. In contrast, *A. filiculoides* showed an increase in mesophyll thickness when grown in polluted media. This mesophyll thickening reflects structural changes to increase physiological resistance to environmental stress. With increasing mesophyll thickness, there is more space for heavy metal storage (Mussina et al., 2018; Vasilevskaya & Struzhko, 2020), and the amount of heavy metal per gram of plant dry weight decreases (Ruiz et al., 2022). In addition, (Jonoubi & Karimi Limanjoubi, 2024) reported that *A. filiculoides* grown on heavy metal-contaminated media will have smaller epidermal cells, a thicker stem cortex, and a modified root structure.

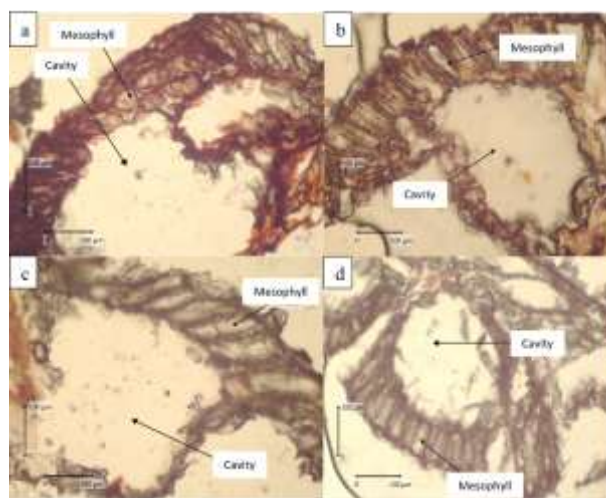


Figure 2. Longitudinal axial section of leaves from mercury-polluted *A. pinnata* (a), non-mercury-polluted *A. pinnata* (b), mercury-polluted *A. filiculoides* (c), and non-mercury-polluted *A. filiculoides* (d)

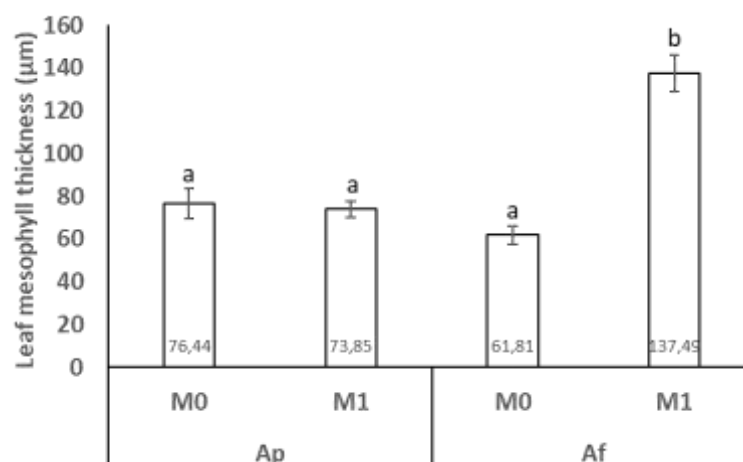


Figure 3. Leaf mesophyll thickness. For further explanation see Fig. 1.

One of the most interesting results was the mercury content in plant tissues. Although statistically there were no significant difference between two species, the average mercury content in *A. pinnata* was higher on a dry weight basis. This was surprising because *A.*

filiculoides is known as a hyperaccumulator (Lestari et al., 2024) while *A. pinnata* is a non-hyperaccumulator plant (Azza et al., 2011; Dewi et al., 2023). This result suggests that *A. pinnata* absorbs more mercury but is unable to control its distribution and detoxification, resulting in reduced growth. In contrast, *A. filiculoides* is thought to be able to store mercury in certain parts without affecting overall growth. This difference is related to different physiological strategies in dealing with heavy metals. *A. pinnata* absorbs more mercury but is more susceptible to its toxic effects. While *A. filiculoides* absorbs mercury efficiently while maintaining its physiological functions, *A. filiculoides*, as a hyperaccumulator, has internal defence mechanisms that allow mercury to be stored in a non-toxic form.

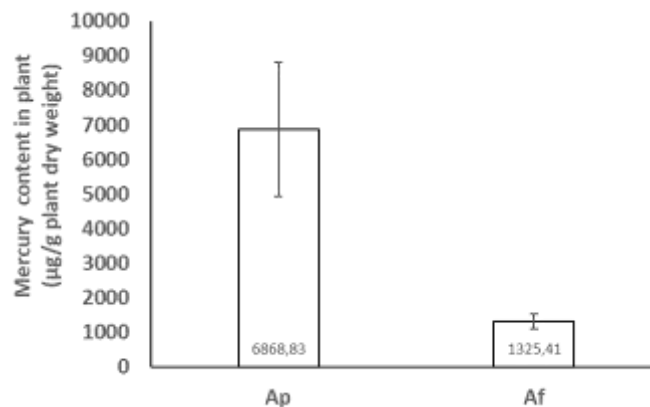


Figure 4. Mercury content in *Azolla pinnata* (Ap) and *Azolla filiculoides* (Af). Values are means and standard error (SE) of four replicates of each treatment

Overall, the results showed that *A. filiculoides* has an advantage in term of tolerance and adaptation to mercury contamination compared to *A. pinnata*. Only *A. filiculoides* was able to maintain growth and showed clear leaf anatomy adaptation. This makes *A. filiculoides* a better candidate for phytoremediation of mercury-contaminated waters. This study also provides a deeper understanding of the difference in adaptation strategies in hyperaccumulator and non-hyperaccumulator plants. Further research to explore the biochemical mechanisms underlying the differences in response between hyperaccumulator and non-hyperaccumulator plants is essential. Research can also be extended to examine the effect of other types of heavy metals on both species. This information is very valuable for determining environmental policies based on biological solution, so that the results of this study can contribute to efforts to preserve the environment through an ecological approach.

CONCLUSION

The results showed that *Azolla filiculoides* has better tolerance and adaptation to mercury contamination compared to *Azolla pinnata*, which experienced a decrease in dry weight due to inability to detoxify the heavy metal. Physiological adaptation in the form of mercury detoxification and anatomical adaptation in the form of mesophyll tissue thickening allow *A. filiculoides* to maintain its growth despite being in a mercury-exposed environment. Although *A. pinnata* absorbed more mercury, the inability to withstand the toxic effects showed a distinctive difference in adaptation strategies between hyperaccumulator and non-hyperaccumulator plants. The ability to store mercury without disrupting physiological function, *A. filiculoides* has great potential as a phytoremediation agent and is important to be further investigated to support ecological solutions for environment conservation.

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