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Effects of Beneficial Bacterial Inoculation on Arsenic Hyperaccumulation Ability of *Pteris vittata* under Planthouse Conditions

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ABSTRACT

The widespread problem of arsenic buildup in soil is often addressed through phytoremediation techniques. *Pteris vittata*, an herbaceous fern known as the Chinese ladder brake, exhibits exceptional arsenic hyperaccumulation, storing over 27,000 mg of arsenic per kilogram in its aboveground biomass as dry weight. Planting *P. vittata* in arsenic-contaminated areas emerges as a promising strategy, facilitating the fern's absorption and accumulation of arsenic from the soil and, consequently, mitigating environmental arsenic levels. This study was conducted to assess the impact of two bacterial strains, *Bacillus* sp. 3P20 (CCB-MBL 5013) and *Enterobacter* sp. 3U4 (CCB-MBL 5014), on arsenic hyperaccumulation by *P. vittata*. The total arsenic content in both soil and plant samples was quantified using inductively coupled plasma-optical emission spectrometry. The results showed a significant difference ($P < 0.0001$) between *P. vittata* inoculated with *Bacillus* sp. 3P20 and spiked with 200 and 500 mg/L arsenic, having a total arsenic content of 240 and 255.25 mg/kg, respectively, compared to the control (un-inoculated), which has 143 mg/kg. Additionally, there was a significant difference ($P < 0.0001$) between *P. vittata* inoculated with *Enterobacter* sp. 3U4 and spiked with 200 and 500 mg/L arsenic. Although no significant increase in the leaf greenness value of the plant was observed in the first and fourth weeks a noteworthy

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increase was recorded after the eighth week of transplanting. These indicate that the bacterial strains promoted plant growth and significantly enhanced the efficiency of arsenic hyperaccumulation by the fern.

Keywords: Arsenic, beneficial bacteria, contamination, hyperaccumulation, *Pteris vittata*, soil

INTRODUCTION

Arsenic pollution is a global threat to plants, animals, and human health. Existing solutions are costly and ineffective (Alka et al., 2020). The widespread occurrence of arsenic pollution across different regions worldwide has raised significant concern. The high costs, disruptive effects, and potential generation of harmful by-products associated with traditional remediation methods have led to a notable shift towards exploring eco-friendly and sustainable approaches to address the problem of arsenic contamination (Manzoor et al., 2019). Bioremediation, using natural agents like microorganisms and plants, offers a promising, affordable, and eco-friendly alternative to traditional methods for effectively cleaning up polluted areas (Alka et al., 2020).

Arsenic-contaminated soils can be remediated by phytoremediation technology, an economical and environmentally friendly soil remediation technique (Manzoor et al., 2019). The method is a unique, cost-efficient, ecologically beneficial, and environmentally friendly soil remediation process. It utilizes hyper-accumulator plants, e.g., *P. vittata*, to remove metal pollutants from the soil.

Progress in arsenic removal from contaminated soils and water through phytoremediation has notably advanced with the identification of the hyperaccumulator *P. vittata*. *Pteris vittata*, commonly called Chinese brake fern, is an herbaceous plant native to China that can accumulate high arsenic in its fronds. It is the first identified arsenic-hyperaccumulator to store significant arsenic levels in its aboveground biomass. *Pteris vittata* has gained significant recognition for its exceptional ability to hyper-accumulate arsenic (Setyawan et al., 2021). It has the potential for phytoextraction of arsenic from polluted soils into harvestable parts (Popov et al., 2021). Its ability to store large quantities of arsenic in its plant tissue makes it a promising choice for phytoremediation in arsenic-contaminated soils (Setyawan et al., 2021).

Regardless of arsenic concentrations, species types, growth media, and treatment times, most arsenic accumulated in *P. vittata* is concentrated in the fronds, demonstrating efficient arsenic transfer from roots to fronds. The amounts of arsenic in the growth media and exposure duration correlate with the arsenic levels in the roots and fronds. The amount of arsenic in the fronds declined with the plant's aging. *Pteris vittata* can accumulate as much as 27,000 mg/kg of arsenic, with detrimental effects on plants becoming evident at approximately 10,000 mg/kg dry weight (J. Wang et al., 2002; Vandana et al., 2020). Arsenic is mainly kept in pinnae and rhizomes in the fronds of *P. vittata* (Tiwari et al., 2016). However,

it was absent in the cell walls, rhizoids, or reproductive regions. In contrast, non-accumulator plants typically experience adverse effects at 5-100 mg/kg dry weight levels of arsenic (Vandana et al., 2020). The concentration of arsenic was highest in the apical pinnae apex and gradually declined in the pinnae at lower places in the same frond. It was also more significant at the edge in single pinnae (Bui, 2017). Arsenic is taken up primarily by *P. vittata* and other plants as As(V), and they are more tolerant to it than As(III) (Yan et al., 2019; Yang et al., 2022).

Recent research indicates that the ability of *P. vittata* to hyper-accumulate arsenic is not solely a result of its inherent traits; instead, it is influenced significantly by the interactions with microorganisms in the rhizosphere. The rhizosphere is a dynamic environment where microorganisms flourish and engage in complex molecular communication with the plant. Soil bacteria are considered a preferred choice for phytoremediation research due to their ability to enhance host plants' growth and their resistance to heavy metals. Their ability to flourish in metal-polluted environments and support plant growth while eliminating heavy metals from the soil are significant factors in their high regard for phytoremediation studies (Tirry et al., 2018).

This study explores the relationship between locally isolated arsenic-tolerant bacteria *Bacillus* sp. 3P20 (CCB-MBL 5013) and *Enterobacter* sp. 3U4 (CCB-MBL 5014), and *P. vittata* in the processes of arsenic hyperaccumulation and plant growth promotion. The use of beneficial bacteria in phytoremediation shows promise as an eco-friendly and sustainable method to enhance arsenic removal from contaminated soils (Setyawan et al., 2021). A better understanding of the association between *P. vittata* and these microorganisms could lead to innovative and cost-effective strategies to tackle arsenic pollution, ultimately promoting a safe and healthier environment.

MATERIALS AND METHODS

Selection of Bacterial Isolates

One hundred and twenty-four (124) bacterial isolates were subjected to qualitative screening to assess their capacity for promoting plant growth through the production of plant growth-promoting (PGP) substances such as indole-3-acetic acid (IAA), siderophore, 1-aminocyclopropane-1-carboxylate (ACC) deaminase, and mineral solubilization, including phosphate, potassium, and silicate. The ten most promising strains demonstrating arsenic tolerance and PGP traits were selected for quantitative analysis. These PGP traits were then evaluated under arsenic stress across concentrations ranging from 50 to 2000 mg/L of As (III). *Bacillus* sp. 3P20 and *Enterobacter* sp. 3U4 were selected for further investigation to determine their impact on the growth of *P. vittata* and their potential role in bioremediation efficiency (Muazu, 2024).

Exposure of *Bacillus* sp. 3P20 and *Enterobacter* sp. 3U4 to Arsenic Stress

The isolates were treated with 500 mg/L of sodium arsenite ($NaAsO_2$) and then observed under a scanning electron microscope (SEM, Leo Supra 50 VP, Germany) and transmission electron microscope (TEM, ZEISS Libra 120, Germany).

SEM and TEM Sample Preparations

The sample for SEM was prepared using the hexamethyldisilazane (HMDS) method. A 24-hr bacterial culture was centrifuged at 1,000–2,000 x *g* for 15 min, and the pellet was resuspended in McDowell-Trump fixative prepared in 0.1 M phosphate buffer (pH 7.2, Sigma-Aldrich, USA), for overnight fixation. The resuspended sample was then centrifuged, and the supernatant was discarded. The pellet was then transferred to an Eppendorf tube and resuspended in 0.1 M phosphate buffer (Buffer wash 1). The process was repeated using the same buffer (Buffer wash 2). Then, the sample was centrifuged, and the pellet was resuspended in 1% osmium tetroxide (Polysciences, USA), prepared in the phosphate buffer above for at least 2 hr (post-fixation). The resuspended sample was then centrifuged, and the pellet was resuspended in distilled water (post-fix wash 1), and the process was repeated (post-fix wash 2). The sample was centrifuged, and the supernatant was discarded. The sample was dehydrated in various ethanol concentrations (Sigma-Aldrich, USA): 50% ethanol (10 min), 75% ethanol (10 min), 95% ethanol (10 min), and 100% ethanol (2 x 10 min). Dehydrated cells were immersed in 1–2 ml of HMDS (ALFA Chem, USA), for 10 min. The HMDS was decanted from the sample tube, and the vial with the cells was left in a desiccator to air-dry at room temperature. The dried cells were then mounted on an SEM specimen stub with double-sided sticky tape. The specimens were coated with gold and viewed in the SEM (Leo Supra 50 VP, Germany).

For TEM sample preparation, the procedure was the same as that for SEM up to the post-wash 2 step above. After that, the sample was centrifuged, and the supernatant was discarded. Then, the tube containing the fixed pellet cells was placed in a water bath at 45°C for 15–30 min. An agar solution of 3% (Oxoid, United Kingdom) was prepared by dissolving the agar in boiling distilled water. The solution was then poured into a still-molten tube and placed in the water bath at 45°C, maintaining the agar in liquid form. The warm agar was transferred to the tube containing the pellet using a warm pipette after the temperature of both the agar and the pellet had equilibrated to 45°C; the mixture was then stirred up to break the pellet into small blocks and suspended in the agar. The agar with the suspended pellet blocks was then immediately poured onto a clean glass slide and allowed to solidify within 1–2 min. The solidified agar containing the cells was cut into small cubes of 1 mm^3 with a sharp razor blade and then placed in a vial containing 50% ethanol (Sigma-Aldrich, USA). The cubes were then processed by dehydration in various ethanol concentrations: 50% ethanol (15 min), 75% ethanol (15 min), 95% ethanol (2 x 15 min), 100% ethanol (2 x 30 min),

and acetone (Bendosen, Malaysia) (2 x 10 min). Followed by infiltrating resin using the mixture of acetone: spurs resin (Sigma-Aldrich, USA) mix (1:1) in a rotator for 15–30 min. Then, it was infiltrated in spur's mix overnight, after which the resin was changed and allowed to stay in the rotator for 48 hr. Then, it was embedded and cured in an oven at 60°C for 12–48 hr. The cubes were then cut using an ultramicrotome, placed on a copper grid, stained, and viewed under TEM.

Transplanting of *P. vittata* and Soil Sample Collection

Healthy *P. vittata*, which grows naturally in the field and are approximately of the same size (16 cm in height) and weight, were collected from the Universiti Sains Malaysia (USM) main campus and immediately transported to the plant house aseptically and then carefully rinsed with distilled water to remove dirt and soil remnants. The plants were selected, transplanted into polybags containing soils, and kept for one week of acclimatization. The plants were grown in a plant house with temperatures ranging from 22°C at night to 30°C during the day (Abou-Shanab et al., 2020; Q. Wang et al., 2011).

The soil sample was collected from the main campus of USM at latitude 05^0 21.341'N and longitude 10^0 018.048'E, at an elevation of 31 m. The soil was taken from the top layer (0–20 cm), where *P. vittata* grows naturally (Abou-Shanab et al., 2020). The soil was mixed in a sizable container, air-dried, crushed, and sieved with a 2-mm sieve. A total of 3 kg of soil was transferred into separate polybags, each measuring 18 cm in diameter and 13 cm in length (Abou-Shanab et al., 2020). The polybags were then grouped into three groups. The first group served as the control and contained 3 kg of untreated soil (Bui, 2017). The second group contained the same soil but was enriched with a 200 mg/L solution of sodium arsenite ($NaAsO_2$, Sigma-Aldrich, USA), while the last group was spiked with 500 mg/L of $NaAsO_2$ (Abou-Shanab et al., 2020). The bags were arranged in a randomized block design on the benches in the plant house for the experiment (Abou-Shanab et al., 2020; Bui, 2017).

Inoculation of Bacterial Isolates

Twenty (20) ml of the 24-hr bacterial culture of *Bacillus* sp. 3P20 and *Enterobacter* 3U4 (OD600; 1.2) were inoculated into the corresponding plastic bag during the first and second week of transplanting. The setup was maintained and watered daily in the plant house for the eighth week, and the plant was uprooted after the eighth week of transplanting for arsenic content analysis. The arsenic content of *P. vittata* and the soil samples from the sampling site (natural mineral soils) was initially measured and recorded. Inductively coupled plasma optical emission spectroscopy (ICP-OES, Thermo-Fisher Scientific, USA), was employed to determine the concentrations of total and extractable metals in the soil samples (Abou-Shanab et al., 2020; Lampis et al., 2015; Q. Wang et al., 2011). The analysis was conducted by ALS Technichem (M) Sdn. Bhd. (Malaysia).

Determination of the Leaf Chlorophyll Content of the Plant

The leaf chlorophyll content of the plants was evaluated at the first, fourth, and eighth week of transplanting non-destructively using a SPAD (Soil Plant Analysis Development) 502 chlorophyll meter (Konica Minolta, USA). The device uses light absorption to measure the leaf greenness of the plant, and the values were recorded accordingly (Nacoon et al., 2020). The *P. vittata* was harvested after the eighth week of transplanting. The roots of the plants were washed with clean running tap water to remove soils that adhered to the roots, and the plant samples were dried in an oven at 80°C for 3 days (Nacoon et al., 2020). Drying the samples in an oven at a high temperature helps to remove any moisture and preserve the samples for further analysis or storage (Q. Wang et al., 2011). The shoot and root dry weights were determined after harvesting the plants. The *P. vittata* samples were then ground using a mortar and pestle, and each was transferred into a Ziploc plastic bag and labeled accordingly. The samples were sent to the ALS Technichem (M) Sdn. Bhd. (Malaysia) to determine the total arsenic content.

Detection of Plant and Soil Arsenic Content

The sample was prepared by digestion in an acidic solution with four replications each, and the digested sample was aspirated into ICP-OES. The intensity of characteristic light emitted by each excited element was observed to be proportional to its concentration. The arsenic decontamination was measured by evaluating arsenic levels in plant tissues after the experiment and comparing them with the initial and final soil arsenic concentrations to evaluate the arsenic uptake by *P. vittata* and the effects of the bacterial inoculation on the hyperaccumulation ability (Muazu, 2024).

Assessment of the Efficiency of Phytoremediation

The efficiency of phytoremediation by the plant was determined by evaluating the bioconcentration factor (BCF). The following formula was used to calculate: BCF = C harvested tissue / C soil, where "C harvested tissue" denotes the metal concentration in various plant tissues, and "C soil" represents the metal concentration in soil measured in mg/kg (Debela et al., 2022).

Data Analysis

The data obtained from this research were analyzed through analysis of variance (ANOVA) to determine if there were significant differences among treatments, using GraphPad Prism statistical software version 9.0 (Inc., USA). One-way and two-way ANOVA were used for the analysis, followed by Tukey's multiple comparison tests. The statistical differences were considered significant at $P < 0.05$.

RESULTS

SEM and TEM Analysis

The SEM images of the bacterial isolates grown without arsenic (control) and those

grown in 500 mg/L arsenic (III) revealed no significant changes in the treated cells of *Bacillus* sp. 3P20 compared to the untreated cells, indicating that the cells were unaffected by the exposure to 500 mg/L arsenic (Figure 1).

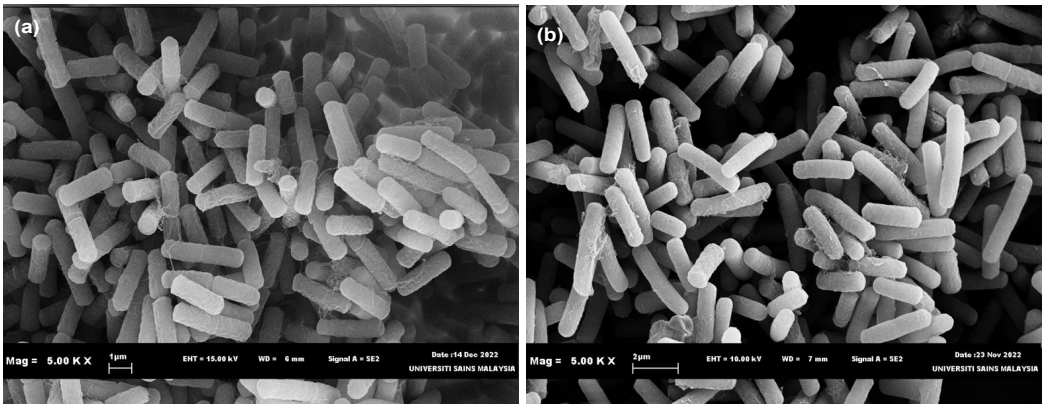

Figure 1. Scanning electron microscopy of *Bacillus* sp. 3P20. (a) Untreated (control) cells without the arsenic treatment; (b) Cells treated with 500 mg/L of arsenic

Similarly, there were no significant changes in the morphology of *Enterobacter* sp. 3U4 cells treated with 500 mg/L As(III) compared to the control (Figure 2).

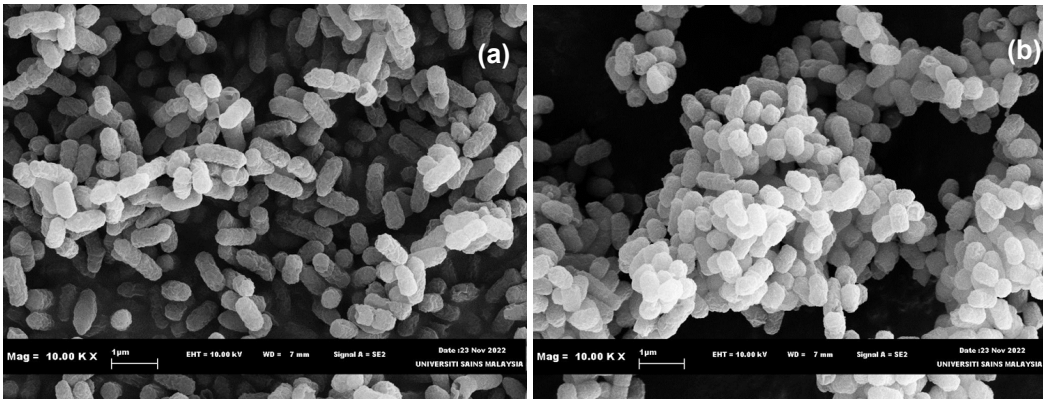

Figure 2. Scanning electron microscopy of *Enterobacter* sp. 3U4. (a) Untreated (control) cells without the arsenic treatment; (b) Cells treated with 500 mg/L arsenic

TEM was used to investigate the intracellular accumulation of arsenic. The TEM results for the treated and untreated (control) cells are presented in Figure 3 for *Bacillus* sp. 3P20 and Figure 4 for *Enterobacter* sp. 3U4. There were no observable differences between treated and untreated cells.

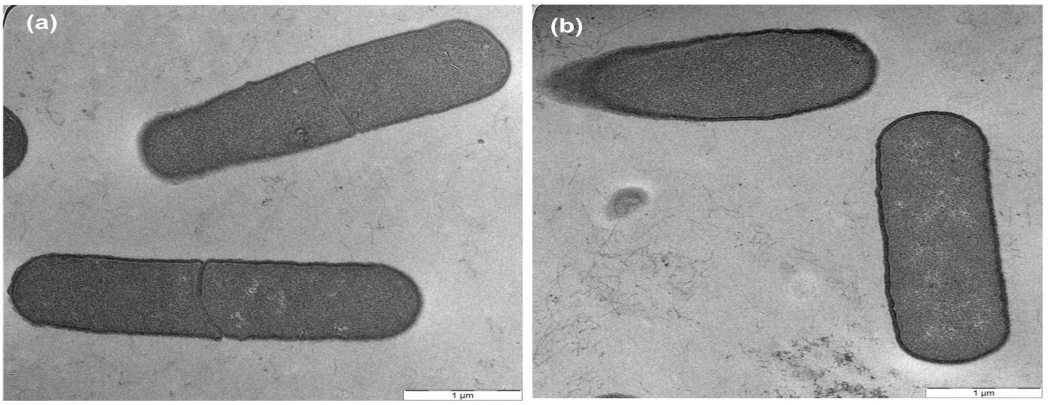

Figure 3. Transmission electron microscope of *Bacillus* sp.: (a) without arsenic exposure, the bacterial cells showed paired cells; (b) the bacterial cells exposed to 500 mg/L arsenic (sodium arsenite), indicating that the cells were unaffected by the treatment

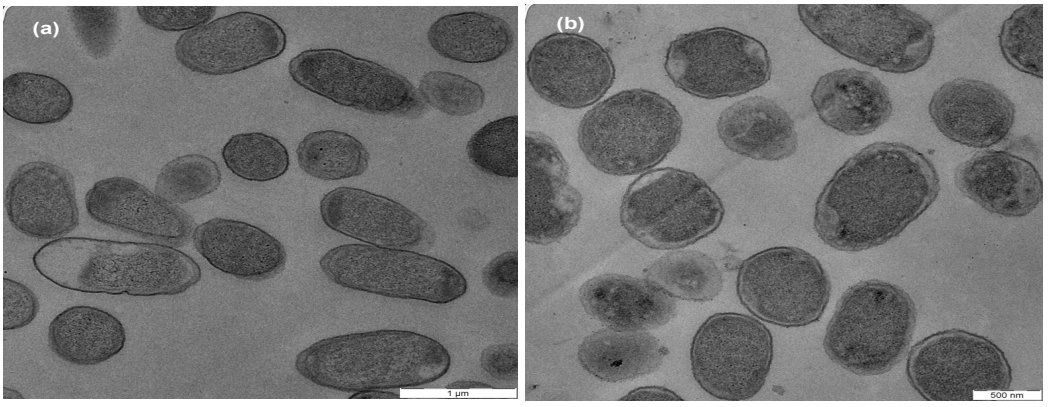

Figure 4. Transmission electron microscope of *Enterobacter* sp.: (a) the cells without arsenic exposure; (b) cells exposed to 500 mg/L of arsenic, indicating that the cells were unaffected by the arsenic treatment

Total Arsenic Contents in Plant (*P. vittata*) and Soil Samples before Bacterial Inoculation

The total arsenic contents of plant and soil samples were determined and recorded prior to the commencement of the experiment as the initial arsenic concentration. The result shows that the total arsenic content of *P. vittata* in the samples ranged from 122 to 143 mg/kg, and that of the soil samples ranged between 5 to 6 mg/kg.

Total Arsenic Contents in *P. vittata* after Bacterial Inoculation

From the result obtained, the total arsenic content in the frond of *P. vittata* after the experiment was observed to be higher in the plants inoculated with *Enterobacter* sp. 3U4 and spiked with 500 mg/L arsenic solution (499.5 mg), followed by the plant inoculated with 200 mg/L arsenic (239.5 mg/kg). There was a significant difference ($P < 0.0001$) between the two treatments, and the rate of accumulation increases with

increasing concentrations of arsenic. The result showed a significant difference ($P < 0.0001$) between the *P. vittata* inoculated with *Bacillus* sp. 3P20 and spiked with 200 and 500 mg/L arsenic, having a total arsenic content of 240 and 255.25 mg/kg, respectively, compared to the control (un-inoculated), which has 143 mg/kg. Although there was an increase in the arsenic content in the plants inoculated with 3P20 and spiked with 200 and 500 mg/L arsenic, no significant difference was observed across treatments. In contrast, there was a significant difference ($P < 0.0001$) in the total arsenic content of *P. vittata* inoculated with *Enterobacter* sp. 3U4 and spiked with 200 mg/L and those spiked with 500 mg/L arsenic with the same isolate (Figure 5).

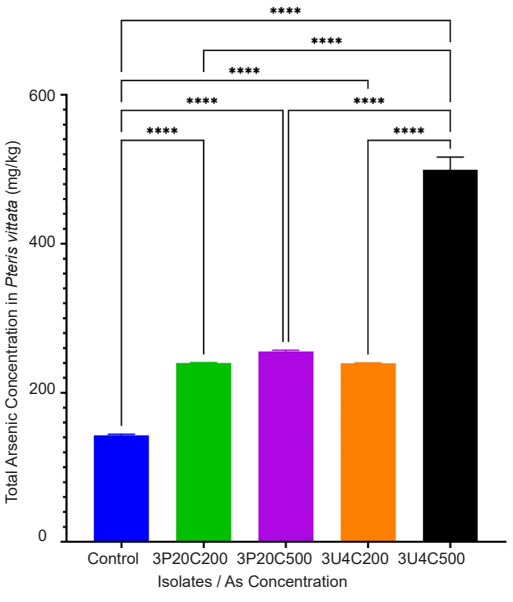

Figure 5. Total arsenic content of *Pteris vittata* after the eighth week of transplanting

Note. Data were analyzed using a one-way analysis of variance followed by Tukey's multiple comparisons tests. Data represents mean ± standard error of the mean (SEM); ****$P < 0.0001$ compared to control. 3P20 (*Bacillus* sp.) and 3U4 (*Enterobacter* sp.) are the bacterial isolates while C200 and C500 indicate 200 and 500 mg/L of arsenic concentrations, respectively

Shoot and Root Dry Weights of *P. vittata*

After harvesting the plants, the dry shoot and root weights of *P. vittata* were taken. The results showed a significant decrease ($F_{4,15} = 81.33$, $P < 0.0001$) in the shoot dry weights of the *P. vittata* inoculated with *Bacillus* sp. 3P20 and spiked with 200 and 500 mg/L arsenic (2.27 and 2.53 g, respectively) compared to the control (3.35 g) at $P < 0.05$. Similarly, no significant difference was observed in *P. vittata* inoculated with *Enterobacter* sp. 3U4 and spiked with 200 mg/L of arsenic with a dry shoot weight of 3.2 g, but there was a significant decrease in the shoot dry weights of *P. vittata* inoculated with *Enterobacter* sp. 3U4 and spiked with 500 mg/L arsenic with the dry shoot weights (2.78 g), compared to the control shoot dry weight (3.35 g) (Figure 6a). The one-way ANOVA result revealed a significant increase ($F_{4,15} = 21.85$, $P < 0.0001$) in the root dry weights of *P. vittata* inoculated with all the isolates at different levels of arsenic. The root dry weight of the plant inoculated with *Bacillus* sp. 3P20 and spiked with 200 and 500 mg/L were observed to be 1.49 and 1.86 g, respectively, compared to the control (1.42 g). The root dry weights of *P. vittata* inoculated with *Enterobacter* sp. 3U4 and spiked with 200 and 500 mg/L arsenic were observed to be 1.74 and 1.80 g, respectively. It showed a significant increase ($P < 0.0001$) in the root dry weight of the plant compared to the control (1.42 g) (Figure 6b).

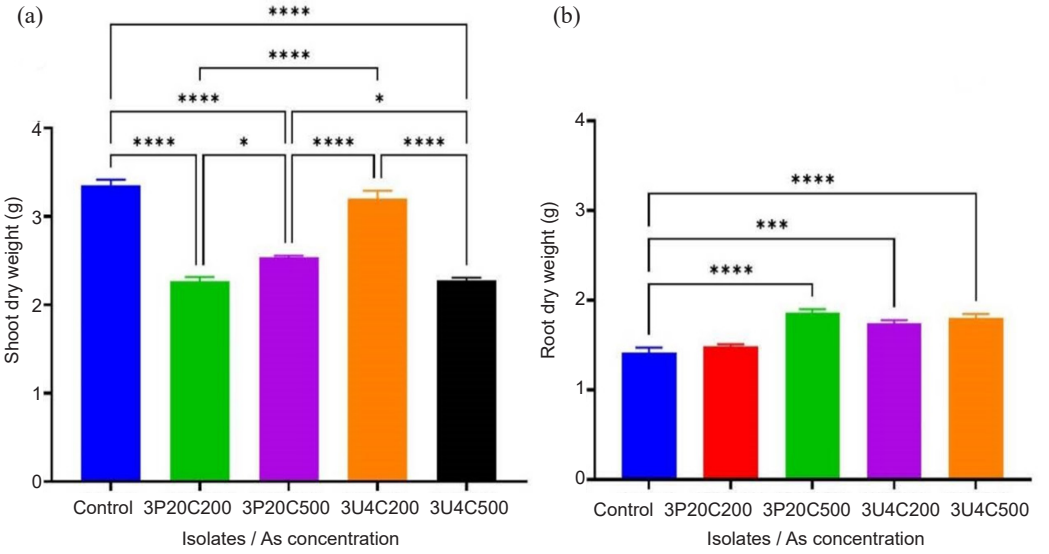

Figure 6. Shoots and roots dry weights of *Pteris vittata* after harvesting in the eighth week. (a) Shoot dry weight and (b) root dry weight

Note. Data were analyzed using a one-way analysis of variance followed by Tukey's multiple comparisons tests. Data represents mean ± standard error of the mean (SEM); *$P < 0.05$, ****$P < 0.0001$ compared to control; 3P20 (*Bacillus* sp.) and 3U4 (*Enterobacter* sp.) are the bacterial isolates while C200 and C500 indicate 200 and 500 mg/L of arsenic concentrations, respectively

Chlorophyll Content (SPAD Values) of the *P. vittata*

The chlorophyll contents of the *P. vittata* were measured by non-destructive means (SPAD meter). The results of the SPAD values at the first, fourth and eighth week of transplanting are presented in Figures 7a-7c. The result showed there was no significant difference between the chlorophyll content of the plants inoculated with *Bacillus* sp. (3P20) and *Enterobacter* sp. (3U4) compared to the control at both the first and fourth week of transplanting (Figures 7a-7c). At the eighth week of transplanting, a two-way ANOVA indicated that there was a significant increase ($P < 0.005$) in the SPAD values of the *P. vittata* inoculated with 3P20 at 200 mg/L (34.23) and 500 mg/L arsenic (36.67) compared to the control plants (30.11) (Figure 7). On the other hand, there was a significant increase ($P < 0.05$) in the SPAD values of the *P. vittata* inoculated with *Enterobacter* sp. 3U4 and spiked with 200 mg/L arsenic (36.45). However, there was no significant difference in the plant inoculated with the same isolate and spiked with 500 mg/L of arsenic (32.93) compared to the control (33.49). The results showed that the isolates significantly increased the chlorophyll content of the plants at the eighth week of transplanting ($P < 0.05$) (Figure 7). It could be due to the production of phytohormones and mineral solubilization, which are among the factors that help plant growth and development.

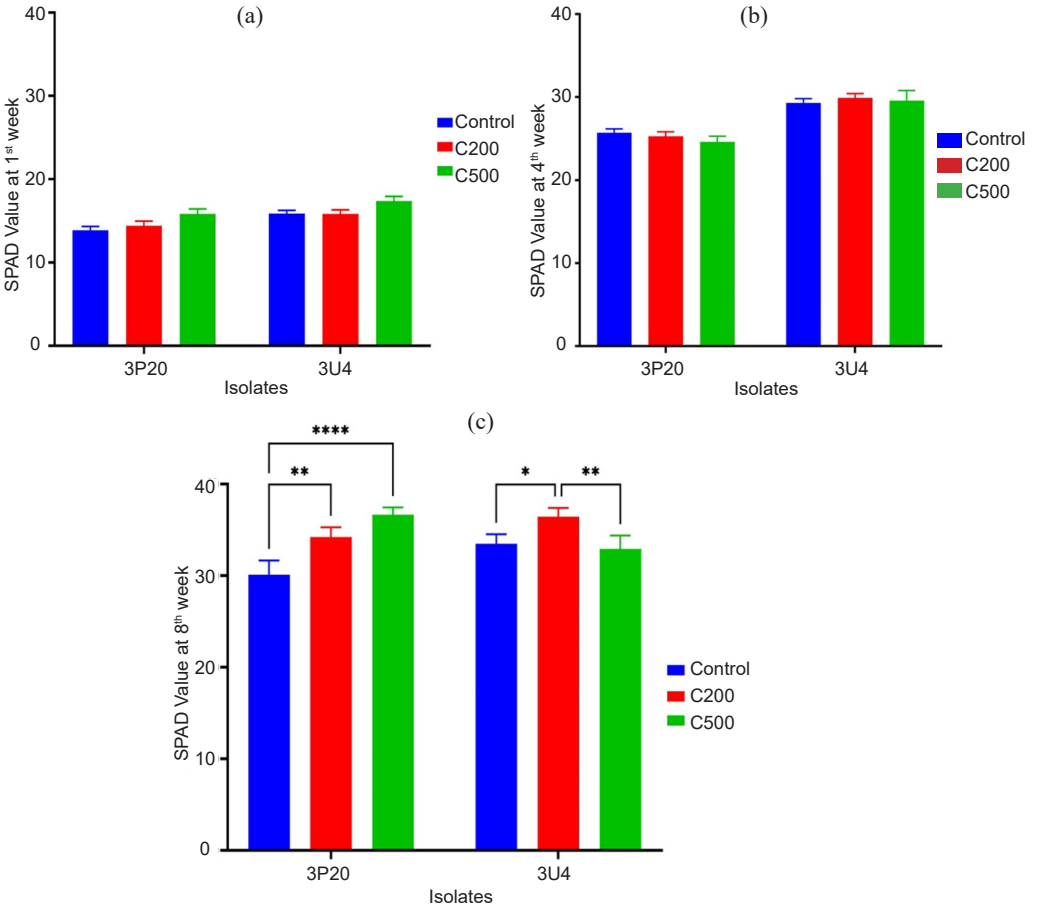

Figure 7. Chlorophyll content of *Pteris vittata* (a) first week, (b) fourth week, and (c) eighth week of transplanting, respectively

Note. Data were analyzed using a two-way analysis of variance followed by Tukey's multiple comparisons tests. Data represent mean ± standard error of the mean (SEM); *$P < 0.05$, **$P < 0.005$, ****$P < 0.0001$ compared to the control; 3P20 (*Bacillus* sp.) and 3U4 (*Enterobacter* sp.) are the bacterial isolates while C200 and C500 indicate 200 and 500 mg/L of arsenic concentrations, respectively

Bioconcentration Factor of Arsenic in *P. vittata*

The BCF of arsenic in *P. vittata* was determined as presented below.

The BCF of arsenic in *P. vittata* inoculated with *Enterobacter* sp. 3U4 and spiked with 500 mg/L arsenic solution $= \frac{499.5\ \text{mg/kg}}{5\ \text{mg/kg}} = 99.9$ ·

While BCF of arsenic in *P. vittata* inoculated with *Enterobacter* sp. 3U4 and spiked with 200 mg/L arsenic = $\frac{239.5\ \text{mg/kg}}{5\ \text{mg/kg}} = 47.9$ ·

The BCF of arsenic in *P. vittata* inoculated with *Bacillus* sp. 3P20 and spiked with 500 mg/L arsenic solution $= \frac{255.25\ \text{mg/kg}}{5\ \text{mg/kg}} = 51.05$ and BCF of arsenic

in *P. vittata* inoculated with *Bacillus* sp. 3P20 and spiked with 200 mg/L arsenic $= \frac{240\ \text{mg/kg}}{5\ \text{mg/kg}} = 48.$

DISCUSSION

The SEM and TEM analyses revealed no significant changes in the morphology of the *Bacillus* sp. 3P20 and *Enterobacter* sp. 3U4 cells when exposed to 500 mg/L of arsenic (As (III)). TEM was used to investigate the intracellular accumulation of arsenic. TEM results showed no significant changes between the control and the cells treated with 500 mg/L arsenic from this study. Ghosh et al. (2020) reported that the electron microscopy images displayed noticeable alterations in the morphology of *Bacillus* sp. strain IIIJ3-1 when exposed to arsenic stress in aerobic and anaerobic conditions. In their research, the cells treated with arsenic showed a reduction in size and a rugged and folded cell surface compared to the control cells (Ghosh et al., 2020). The TEM images of *Bacillus* sp. 3P20 and *Enterobacter* sp. 3U4 showed no noticeable changes in the bacterial cells treated with 500 mg/L of arsenic compared to the control (untreated) cells. It was probably due to the ability of the bacteria to tolerate high concentrations of the toxic metal (up to 1,000 mg/L (*Bacillus* sp.) and 900 mg/L (*Enterobacter* sp.).

Arsenite-oxidizing bacteria can transform the toxic form of arsenite into the less harmful form of arsenate, while arsenate-reducing bacteria can convert arsenate back into arsenite (Liao et al., 2011). However, in both instances, a significant quantity of the converted arsenate and arsenite remains trapped within the bacterial cells instead of being released. The TEM study provided additional evidence by confirming the accumulation of arsenic inside the bacterial cells. Exposure to high-level arsenic can negatively impact the cell wall and cytoplasmic membrane of bacteria, resulting in compromised structural integrity and disrupted cellular functions.

Using arsenic-tolerant plant growth-promoting rhizobacteria (PGPR) in the bioremediation of arsenic-contaminated soils can be a promising strategy to mitigate the adverse effects of metal contamination on plants and the soil environment. These bacteria can assist in the growth and survival of plants in contaminated soils and improve the efficiency of phytoremediation. In arsenic-contaminated soils, phytohormones-producing microorganisms may stimulate plant growth and development (Lu et al., 2022; Q. Wang et al., 2019). The efficiency of arsenic phytoextraction can be enhanced by introducing naturally occurring bacteria with growth-promoting properties and arsenic-tolerant ability. It is especially beneficial for hyperaccumulator plant species such as *P. vittata*. These bacteria possess the ability to convert As (V) to As (III), leading to an overall improvement in the effectiveness of the arsenic remediation process (Lampis et al., 2015).

The inoculation of *Bacillus* sp. 3P20 and *Enterobacter* sp. 3U4 in the soils influenced the ability of *P. vittata* to remove arsenic from the spiked soils. The bacteria also improved the phytoextraction ability

of *P. vittata*. Several hyperaccumulating plant species inoculated with rhizosphere or endophytic bacterial strains exhibited enhanced plant growth and biomass output (Sessitsch et al., 2013). A *Bacillus subtilis* strain demonstrated the ability to boost the growth of plants, leading to increased nickel accumulation. This improvement was likely due to the strain's IAA production ability, potentially promoting plant growth. Similarly, a chromium-resistant *Pseudomonas* strain that also produces IAA showed the ability to enhance the growth of *Brassica juncea* and, as a result, increased the extraction of trace elements.

Bacillus sp. 3P20 and *Enterobacter* sp. 3U4 produces plant growth-promoting substances such as phytohormones siderophores and solubilizes phosphate, potassium, and silicate, thereby promoting the growth and development of *P. vittata* even under arsenic stresses. The outcomes of this study showed that *Bacillus* sp. 3P20 and *Enterobacter* sp. 3U4 could survive well in arsenic-contaminated soils and significantly enhance plant growth. Based on the results of the plant biomass, there was a decrease in the shoot dry weights of *P. vittata* despite the inoculation of the isolates, which could be due to the arsenic accumulation in the fronds of the plants. The excessive accumulation of arsenic hinders plant growth and biomass production by disrupting essential physiological processes like photosynthesis, respiration, and nutrient absorption (Han et al., 2020). Higher arsenic concentrations in its fronds are linked to reduced shoot dry weight (Kong et al., 2017). On the other hand, there was a significant increase in the root dry weights of the plants compared to the control (untreated), indicating an efficient translocation of the arsenic metal from the roots to the shoots because of bacterial inoculation.

The result showed a significant increase in the chlorophyll content of the plants at the eighth week of transplanting, which could be due to the production of phytohormones and mineral solubilization by the two bacterial strains, which are part of the factors that help plant growth and development. An essential condition for plant growth-promoting bacteria is the efficient colonization of rhizosphere soils by these bacteria (Q. Wang et al., 2018). *Pteris vittata* can withstand a total arsenic level of up to 5,000 mg/kg without experiencing any injury; as a result, the mechanism behind its resistance is of significant interest. Recent findings indicate enhanced ROS metabolism is a crucial physiological mechanism for arsenic resistance (Yan et al., 2019). The works of other researchers support these findings. Lampis et al. (2015) reported that a carefully selected mixture of bacteria, known for enhancing plant growth and aiding in arsenic transport, can improve arsenic extraction from contaminated environments. This technique has been successfully employed with the hyperaccumulator fern species *P. vittata*. The introduction of *Agrobacterium radiobacter* (strain D14) into *Populus deltoides* could enhance the tree's ability to tolerate arsenic, stimulate its growth, improve its absorption efficiency, and facilitate arsenic movement within the

plant (Q. Wang et al., 2011). When grown in arsenic-rich soil, *P. vittata* can extract arsenic through phytoextraction (Antenozio et al., 2021).

The capacity of *P. vittata* to endure and accumulate high levels of arsenic may be attributed, at least in part, to the presence of arsenic-tolerant bacteria that reside in symbiosis with the plant (Abou-Shanab et al., 2020; Cai et al., 2019). In a similar research, Xu et al. (2016) reported that arsenic-resistant endophytes could potentially facilitate plant growth in *P. vittata*, consequently enhancing phytoremediation efficiency in sites contaminated with arsenic. Diverse viewpoints exist concerning the precise location of arsenic conversion and the transportation of arsenite, which is the form of arsenic that moves from the roots to the fronds. It has been proposed that arsenate, rather than arsenite, is the primary form of arsenic transported from the roots to the fronds, and the conversion of arsenate primarily occurs in the fronds (Vandana et al., 2020). Q. Wang et al. (2018) reported that the survival of the greens growing in the contaminated soils was boosted by the inoculation of *Bacillus megaterium* bacteria (strain H3). Greens inoculated with strain H3 had an increase in edible tissue biomass compared to the controls. Bacteria can reduce the accumulation of metals in plant tissues and immobilize them in the soil. However, the specific impact of bacteria from the same genus on metal accumulation and immobilization variations remains unclear. It is evident that different types of PGPR assist in the remediation of soil contaminated with trace metals through diverse mechanisms (Guo et al., 2020).

Plant-microbe interactions play a significant role in plant development, transport, and uptake of nutrients in the rhizosphere. Many studies have demonstrated that the microbiota in a plant's environment directly affects how well it responds to local environmental stress (Upadhyay et al., 2018). Several bacterial genera, including *Acidovorax*, *Alcaligenes*, *Bacillus*, *Mycobacterium*, *Paenibacillus*, *Pseudomonas*, and *Rhodococcus*, have been frequently used in phytoremediation (Sharma, 2021). Bacteria have proven useful in phytoremediation by increasing metal bioavailability by synthesizing organic acids, carbohydrates, and plant growth stimulants. Thus, the effect of phytoremediation might be influenced by all of the elements that influence plant root development and productivity, such as light, temperature, humidity, and soil properties (Liu et al., 2017; Sharma, 2021). The effects of phytoremediation by bacteria, primarily *Bacillus* and *Pseudomonas* species, are directly influenced by plant development and biomass. It can improve phytoremediation by promoting plant growth (Sharma et al., 2021). The combined inoculations of local bacteria can boost arsenic accumulation and *P. vittata* biomass, lowering the soil's arsenic content (Antenozio et al., 2021).

Most of the rhizosphere bacteria identified from arsenic-rich and natural mineral soils can both oxidize As (III) to As (V) or reduce As (V) to As (III). The inoculation of *Bacillus* sp. 3P20 and *Enterobacter* sp. 3U4 in the soils influenced the ability of *P. vittata* to remove arsenic from the spiked soils. The microbes

supplied PGP substances such as IAA, phytohormones that promote plant growth, and siderophores that increase iron uptake and are observed to solubilize phosphate and potassium. The bacteria also improved the phytoextraction ability of *P. vittata*. Several hyperaccumulating plant species inoculated with rhizosphere or endophytic bacterial strains exhibited enhanced plant growth and biomass output (Sessitsch et al., 2013).

The BCF of arsenic in *P. vittata* across treatments was greater than 1, indicating its suitability for arsenic bioremediation. Plants with a BCF value exceeding one are viable candidates for phytoextraction (Debela et al., 2022).

CONCLUSION

The inoculation of *Bacillus* sp. 3P20 and *Enterobacter* sp. 3U4 in the soils spiked with 200 and 500 mg/L arsenic significantly influences the arsenic hyperaccumulation ability of *P. vittata*. The results indicate a significant increase in the growth of the plants, and the rate of hyperaccumulation of the arsenic metal increases in inoculated plants compared to the control (untreated) *P. vittata*. Based on the result, the isolates play a significant role in arsenic biotransformation, its hyperaccumulation by *P. vittata*, and its survival and growth in the contaminated soils.

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REFERENCES

Abou-Shanab, R. A. I., Mathai, P. P., Santelli, C., & Sadowsky, M. J. (2020). Indigenous soil bacteria and the hyperaccumulator *Pteris vittata* mediate phytoremediation of soil contaminated with arsenic species. *Ecotoxicology and Environmental Safety*, *195*, 110458. https://doi.org/10.1016/j.ecoenv.2020.110458

Alka, S., Shahir, S., Ibrahim, N., Chai, T.-T., Bahari, Z. M., & Manan, F. A. (2020). The role of plant growth promoting bacteria on arsenic removal: A review of existing perspectives. *Environmental Technology and Innovation*, *17*, 100602. https://doi.org/10.1016/j.eti.2020.100602

Antenozio, M. L., Giannelli, G., Marabottini, R., Brunetti, P., Allevato, E., Marzi, D., Capobianco, G., Bonifazi, G., Serranti, S., Visioli, G., Stazi, S. R., & Cardarelli, M. (2021). Phytoextraction efficiency of *Pteris vittata* grown on a naturally As-rich soil and characterization of As-resistant rhizosphere bacteria. *Scientific Reports*, *11*, 6794. https://doi.org/10.1038/s41598-021-86076-7

Bui, T. K. A. (2017). Phytoremediation potential of *Pteris vittata* L. and *Eleusine indica* L. through field study and greenhouse experiments. *Journal of Vietnamese Environment*, *8*(3), 156–160. https://doi.org/10.13141/jve.vol8.no3.pp156-160

Cai, C., Lanman, N. A., Withers, K. A., DeLeon, A. M., Wu, Q., Gribskov, M., Salt, D. E., & Banks, J. A. (2019). Three genes define a bacterial-like arsenic tolerance mechanism in the arsenic hyperaccumulating fern *Pteris vittata*. *Current Biology*, *29*(10), 1625-1633.e3. https://doi.org/10.1016/j.cub.2019.04.029

Debela, A. S., Dawit, M., Tekere, M., & Itanna, F. (2022). Phytoremediation of soils contaminated by lead and cadmium in Ethiopia, using Endod (*Phytolacca dodecandra* L). *International*

Journal of Phytoremediation, *24*(13), 1339–1349. https://doi.org/10.1080/15226514.2021.2025336

Ghosh, S., Mohapatra, B., Satyanarayana, T., & Sar, P. (2020). Molecular and taxonomic characterization of arsenic (As) transforming *Bacillus* sp. strain IIIJ3-1 isolated from As-contaminated groundwater of Brahmaputra river basin, India. *BMC Microbiology*, *20*, 256. https://doi.org/10.1186/s12866-020-01893-6

Guo, J., Muhammad, H., Lv, X., Wei, T., Ren, X., Jia, H., Atif, S., & Hua, L. (2020). Chemosphere Prospects and applications of plant growth promoting rhizobacteria to mitigate soil metal contamination: A review. *Chemosphere*, *246*, 125823. https://doi.org/10.1016/j.chemosphere.2020.125823

Han, Y.-H., Jia, M.-R., Wang, S.-S., Deng, J.-C., Shi, X.-X., Chen, D.-L., Chen, Y., & Ma, L. Q. (2020). Arsenic accumulation and distribution in *Pteris vittata* fronds of different maturity: Impacts of soil As concentrations. *Science of the Total Environment*, *715*, 135298. https://doi.org/10.1016/j.scitotenv.2019.135298

Kong, Z., Deng, Z., Glick, B. R., Wei, G., & Chou, M. (2017). A nodule endophytic plant growth-promoting *Pseudomonas* and its effects on growth, nodulation and metal uptake in *Medicago lupulina* under copper stress. *Annals of Microbiology*, *67*, 49–58. https://doi.org/10.1007/s13213-016-1235-1

Lampis, S., Santi, C., Ciurli, A., Andreolli, M., & Vallini, G. (2015). Promotion of arsenic phytoextraction efficiency in the fern *Pteris vittata* by the inoculation of As-resistant bacteria: A soil bioremediation perspective. *Frontiers in Plant Science*, *6*, 80. https://doi.org/10.3389/fpls.2015.00080

Liao, V. H.-C., Chu, Y.-J., Su, Y.-C., Hsiao, S.-Y., Wei, C.-C., Liu, C.-W., Liao, C.-M., Shen, W.-C., & Chang, F.-J. (2011). Arsenite-oxidizing and arsenate-reducing bacteria associated with arsenic-rich groundwater in Taiwan. *Journal of Contaminant Hydrology*, *123*(1–2), 20–29. https://doi.org/10.1016/j.jconhyd.2010.12.003

Liu, S.-H., Zeng, G.-M., Niu, Q.-Y., Liu, Y., Zhou, L., Jiang, L.-H., Tan, X.-F., Xu, P., Zhang, C., & Cheng, M. (2017). Bioremediation mechanisms of combined pollution of PAHs and heavy metals by bacteria and fungi: A mini review. *Bioresource Technology*, *224*, 25–33. https://doi.org/10.1016/j.biortech.2016.11.095

Lu, Y., Liao, S., Ding, Y., He, Y., Gao, Z., Song, D., Tian, W., & Zhang, X. (2022). Effect of *Stevia rebaudiana* Bertoni residue on the arsenic phytoextraction efficiency of *Pteris vittata* L. *Journal of Hazardous Materials*, *421*, 126678. https://doi.org/10.1016/j.jhazmat.2021.126678

Manzoor, M., Abid, R., Rathinasabapathi, B., De Oliveira, L. M., da Silva, E., Deng, F., Rensing, C., Arshad, M., Gul, I., Xiang, P., & Ma, L. Q. (2019). Metal tolerance of arsenic-resistant bacteria and their ability to promote plant growth of *Pteris vittata* in Pb-contaminated soil. *Science of the Total Environment*, *660*, 18–24. https://doi.org/10.1016/j.scitotenv.2019.01.013

Muazu A. S. (2024). *Bacterial population of* Pteris vittata *with potentials for bioremediation of arsenic rich soils and plant growth promotion* [Unpublished Doctoral thesis]. Universiti Sains Malaysia.

Nacoon, S., Jogloy, S., Riddech, N., Mongkolthanaruk, W., Kuyper, T. W., & Boonlue, S. (2020). Interaction between phosphate solubilizing bacteria and arbuscular mycorrhizal fungi on growth promotion and tuber inulin content of *Helianthus tuberosus* L. *Scientific Reports*, *10*, 4916. https://doi.org/10.1038/s41598-020-61846-x

Popov, M., Zemanová, V., Sácký, J., Pavlík, M., Leonhardt, T., Matoušek, T., Kaňa, A., Pavlíková, D., & Kotrba, P. (2021). Arsenic accumulation and speciation in two cultivars of *Pteris cretica* L. and characterization of arsenate reductase Pc*ACR2* and arsenite transporter Pc*ACR3* genes in the hyperaccumulating cv. Albo-

lineata. *Ecotoxicology and Environmental Safety*, *216*, 112196. https://doi.org/10.1016/j.ecoenv.2021.112196

Sessitsch, A., Kuffner, M., Kidd, P., Vangronsveld, J., Wenzel, W. W., Fallmann, K., & Puschenreiter, M. (2013). The role of plant-associated bacteria in the mobilization and phytoextraction of trace elements in contaminated soils. *Soil Biology and Biochemistry*, *60*, 182–194. https://doi.org/10.1016/j.soilbio.2013.01.012

Setyawan, H. B., Yulianto, R., Santoso, W. D., & Suryandari, N. (2021). Fern plant (*Pteris vittata*) as a phytoremediator of arsenic heavy metal and its effect to the growth and quality of Kale (*Ipomea reptans* Poir). In *IOP Conference Series: Earth and Environmental Science* (Vol. 637, No. 1, p. 012066). IOP Publishing. https://doi.org/10.1088/1755-1315/637/1/012066

Sharma, P. (2021). Efficiency of bacteria and bacterial assisted phytoremediation of heavy metals: An update. *Bioresource Technology*, *328*, 124835. https://doi.org/10.1016/j.biortech.2021.124835

Sharma, P., Tripathi, S., Chaturvedi, P., Chaurasia, D., & Chandra, R. (2021). Newly isolated *Bacillus* sp. PS-6 assisted phytoremediation of heavy metals using *Phragmites communis*: Potential application in wastewater treatment. *Bioresource Technology*, *320*(Part B), 124353. https://doi.org/10.1016/j.biortech.2020.124353

Tirry, N., Tahri Joutey, N., Sayel, H., Kouchou, A., Bahafid, W., Asri, M., & El Ghachtouli, N. (2018). Screening of plant growth promoting traits in heavy metals resistant bacteria: Prospects in phytoremediation. *Journal of Genetic Engineering and Biotechnology*, *16*(2), 613–619. https://doi.org/10.1016/j.jgeb.2018.06.004

Tiwari, S., Sarangi, B. K., & Thul, S. T. (2016). Identification of arsenic resistant endophytic bacteria from *Pteris vittata* roots and characterization for arsenic remediation application. *Journal of Environmental Management*, *180*, 359–365. https://doi.org/10.1016/j.jenvman.2016.05.029

Upadhyay, M. K., Yadav, P., Shukla, A., & Srivastava, S. (2018). Utilizing the potential of microorganisms for managing arsenic contamination: A feasible and sustainable approach. *Frontiers in Environmental Science*, *6*, 24. https://doi.org/10.3389/fenvs.2018.00024

Vandana, U. K., Gulzar, A. B. M., Singha, L. P., Bhattacharjee, A., Mazumder, P. B., & Pandey, P. (2020). Hyperaccumulation of arsenic by *Pteris vittata*, a potential strategy for phytoremediation of arsenic-contaminated soil. *Environmental Sustainability*, *3*, 169–178. https://doi.org/10.1007/s42398-020-00106-0

Wang, J., Zhao, F. J., Meharg, A. A., Raab, A., Feldmann, J., & Mcgrath, S. P. (2002). Mechanisms of arsenic hyperaccumulation in *Pteris vittata*. Uptake kinetics, interactions with phosphate, and arsenic speciation. *Plant Physiology*, *130*(3), 1552-1561. https://doi.org/10.1104/pp.008185

Wang, Q., Ma, L., Zhou, Q., Chen, B., Zhang, X., Wu, Y., Pan, F., Huang, L., Yang, X., & Feng, Y. (2019). Inoculation of plant growth promoting bacteria from hyperaccumulator facilitated non-host root development and provided promising agents for elevated phytoremediation efficiency. *Chemosphere*, *234*, 769–776. https://doi.org/10.1016/j.chemosphere.2019.06.132

Wang, Q., Xiong, D., Zhao, P., Yu, X., Tu, B., & Wang, G. (2011). Effect of applying an arsenic-resistant and plant growth-promoting rhizobacterium to enhance soil arsenic phytoremediation by *Populus deltoides* LH05-17. *Journal of Applied Microbiology*, *111*(5), 1065–1074. https://doi.org/10.1111/j.1365-2672.2011.05142.x

Wang, Q., Zhang, W.-J., He, L.-Y., & Sheng, X.-F. (2018). Increased biomass and quality and reduced heavy metal accumulation of edible tissues of vegetables in the presence of Cd-tolerant and immobilizing *Bacillus megaterium* H3. *Ecotoxicology and Environmental Safety*, *148*, 269–274. https://doi.org/10.1016/j.ecoenv.2017.10.036

Xu, J.-Y., Han, Y.-H., Chen, Y., Zhu, L.-J., & Ma, L. Q. (2016). Arsenic transformation and plant growth promotion characteristics of As-resistant endophytic bacteria from As-hyperaccumulator *Pteris vittata*. *Chemosphere*, *144*, 1233–1240. https://doi.org/10.1016/j.chemosphere.2015.09.102

Yan, H., Gao, Y., Wu, L., Wang, L., Zhang, T., Dai, C., Xu, W., Feng, L., Ma, M., Zhu, Y.-G., & He, Z. (2019). Potential use of the *Pteris vittata* arsenic hyperaccumulation-regulation network for phytoremediation. *Journal of Hazardous Materials*, *368*, 386–396. https://doi.org/10.1016/j.jhazmat.2019.01.072

Yang, C., Han, N., Inoue, C., Yang, Y.-L., Nojiri, H., Ho, Y.-N., & Chien, M.-F. (2022). Rhizospheric plant-microbe synergistic interactions achieve efficient arsenic phytoextraction by *Pteris vittata*. *Journal of Hazardous Materials*, *434*, 128870. https://doi.org/10.1016/j.jhazmat.2022.128870