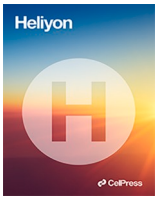
Heliyon 10 (2024) e33078

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon



Research article

Improving sunflower growth and arsenic bioremediation in polluted environments: Insights from ecotoxicology and sustainable mitigation approaches

Muhammad Qadir [a], Anwar Hussain [a,**], Mohib Shah [a], Muhammad Hamayun [a], Asma A. Al-Huqail [b], Amjad Iqbal [c,*], Sajid Ali [d,***]

[a] Department of Botany, Garden Campus, Abdul Wali Khan University Mardan, Khyber Pakhtunkhwa Pakistan
[b] Department of Botany and Microbiology, College of Science, King Saud University, Riyadh, 11451, Saudi Arabia
[c] Department of Food Science & Technology, Garden Campus, Abdul Wali Khan University Mardan, Khyber Pakhtunkhwa Pakistan
[d] Department of Horticulture and Life Science, Yeungnam University, Republic of Korea

ARTICLE INFO

Keywords:
Antioxidants
Arsenic contamination
Biotransformation
Hydroponics
Plant growth promoting rhizobacteria

ABSTRACT

The issue of arsenic (As) contamination in the environment has become a critical concern, impacting both human health and ecological equilibrium. Addressing this challenge requires a comprehensive strategy encompassing water treatment technologies, regulatory measures for industrial effluents, and the implementation of sustainable agricultural practices. In this study, diverse strategies were explored to enhance As accumulation in the presence of *Acinetobacter bouvetii* while safeguarding the host from the toxic effects of arsenate exposure. The sunflower seedlings associated with *A. bouvetii* demonstrated a favorable relative growth rate (RGR) and net assimilation rate (NAR) even less than 100 ppm of As stress. Remarkably, the NAR and RGR of *A. bouvetii*-associated seedlings outperformed those of control seedlings cultivated without *A. bouvetii* in As-free conditions. Additionally, a markedly greater buildup of bio-transformed As was observed in *A. bouvetii*-associated seedlings ($P = 0.05$). An intriguing observation was the normal levels of reactive oxygen species (ROS) in *A. bouvetii*-associated seedlings, along with elevated activities of key enzymatic antioxidants like catalases (CAT), ascorbate peroxidase (APX), superoxide dismutase (SOD), and peroxidases (POD), along with non-enzymatic antioxidants (phenols and flavonoids). This coordinated antioxidant defense system likely contributed to the improved survival and growth of the host plant species amidst As stress. *A. bouvetii* not only augmented the growth of the host plants but also facilitated the uptake of bio-transformed As in the contaminated medium. The rhizobacterium's modulation of various biochemical and physiological parameters indicates its role in ensuring the better survival and progression of the host plants under As stress.

* Corresponding author.
** Corresponding author.
*** Corresponding author.
E-mail addresses: drhussain@awkum.edu.pk (A. Hussain), amjadiqbal@awkum.edu.pk (A. Iqbal), drsajid@yu.ac.kr (S. Ali).

https://doi.org/10.1016/j.heliyon.2024.e33078
Received 14 January 2024; Received in revised form 25 May 2024; Accepted 13 June 2024
Available online 14 June 2024

1. Introduction

The contamination of agricultural lands with arsenic (As) poses significant concerns for humans, animals, and plant species alike. Over the past few decades, As has made its way into soils through a combination of natural or geogenic processes as well as human-induced or anthropogenic activities [1,2]. Elevated levels of As in agricultural soil have a profound impact on various aspects of plant proliferation and maturation. This includes the inhibition of root growth, disruption of plant cell functions, impairment of reproduction, reduction in yield, and adverse effects on the health of consumers who consume the harvested produce [3,4]. The acceptable threshold for As-contaminated water and food is 10 μg/L, which is in line with the standards of the World Health Organization [5]. Research has indicated that As^{+3} is the most hazardous form, followed by As^{+5}. Inorganic As in varying oxidation states exhibits greater toxicity compared to its organic counterpart. Elemental As is considered the least toxic form [6].

Agricultural soil contamination is primarily attributed to the practice of irrigating with water that is contaminated with As [7]. Moreover, the utilization of pesticides, fertilizers, industrial activities, disposal of metals, recycling and waste processing, mining, and smelting can contribute substantial amounts of As to agricultural soils [8]. To lessen the ramifications of As, various techniques such as solidification and stabilization have been proposed for soil decontamination. However, these techniques necessitate careful control and long-term monitoring [9,10]. Recently, attention has been drawn to certain plant species known as hyperaccumulators that can accumulate elevated levels of As. Nonetheless, a major challenge in utilizing these plants in the field is their reduced biomass and slower growth rate. However, the association with rhizobacteria has shown to assist the host plants in alleviating such stressful conditions, resulting in increased plant mass and thereby enhancing bioremediation potential [11]. Plant-microbe-assisted bioremediation stands out as a financially viable, innovative, and resourceful strategy for reclaiming contaminated soil while also optimizing the progress indicators of the host plant through the collaboration with associated microorganisms [12]. Various mechanisms have been elucidated in the context of plant-microbe associations, aimed at soil reclamation and stress alleviation. These mechanisms encompass metal exclusion, biosorption, precipitation, removal, as well as intra- or extracellular accumulation [13]. Several bacterial species (*Pantoea* sp., *Agrobacterium tumefaciens, Agrobacterium* sp., *Alcaligenes faecalis, Bacillus arsenooxydans*, *Enterobacter* sp., *Comamonas* sp., *Microbacterium lacticum*, *Pseudomonas arsenitoxidans* and *Pseudomonas* sp.) have been identified for their ability to disrupt the biochemical cycle of As. These bacteria achieve this by transforming As into states characterized by distinct solubility, mobility, bioavailability, and toxicity properties [14].

Among several microbes, Gram-negative, aerobic *Acinetobacter bouvetii* stands significant due to its robust ability to form resilient biofilms [15]. This feature could make areas affected by pollution less vulnerable to metals because this microbe helps to keep metals in one place and reduces the chance of them moving around. Similar to *Pantoea conspicua*, *A. bouvetii* has various abilities that may reduce the damaging repercussions of metal ions, helping to clean up polluted environments [16]. The integration of *A. bouvetii* into innovative bioremediation strategies holds significant promise as ongoing research advances, offering an eco-conscious and sustainable solution to rectifying heavy metal-contaminated sites. This approach not only safeguards ecological equilibrium but also safeguards human health.

In conjunction with *A. bouvetii*, the sunflower plant, *H. annuus*, a member of the Asteraceae family, exhibits remarkable hyperaccumulator properties [17]. This plant has demonstrated the capacity to accumulate substantial quantities of heavy metals from both soil and water sources. The sunflower's potential for phytoremediation is noteworthy, owing to its exceptional ability to absorb and sequester various heavy metals like lead, zinc, chromium, As, and cadmium [18]. The plant's hyperaccumulation ability stems from its extensive root system and efficient pathways for metal translocation [19]. Consequently, it provides a sustainable and environmentally beneficial approach for restoring habitats marred by metal pollution.

The overarching objective of this study was to investigate whether this particular bacterium in the soil around plant roots could help improve the removal of As and also boost the growth of sunflowers. In this connection we aimed to: (i) assess the ability of the bacteria isolated from the soil around plant roots in contaminated areas to tolerate As; (ii) introduce the chosen rhizobacteria to *H. annuus* by adding them to a Hoagland solution that contains As supplementation; (iii) measure the ability of the rhizobacteria-associated and non-associated sunflower seedlings to take up As through their root systems; (iv) evaluate the biochemical and physiological reactions of sunflower seedlings linked with the chosen microbes, as they accumulate more As while also safeguarding the host from the harmful effects of the As.

2. Materials and methods

2.1. Selection of as tolerant plant growth rhizobacteria from bacterial culture collection

For the study, a total of 8 rhizobacterial strains with known abilities to promote plant growth were chosen. These strains are named MT1, MT6, MT8 *Acinetobactor bouvetii, Pantoea agglomerans Acinetobacter beijerinckii (C5), Raoultella planticola (C9)* and *Pseudocitrobacter anthropi*. These strains were obtained from the soil surrounding plants such as *Malvastrum tricuspidatum*, *Parthenium hysterophorus*, and *Chlorophytum comosum* L. that were cultivated in areas near Premier sugar mill and Khyber tobacco company, where the soil is known to be contaminated with metals [11,20]. The strains were exposed to varying levels of As, ranging from 100 to 1200 ppm, in the form of sodium arsenate salt (Na_3AsO_4) to see how well they could grow in the presence of As. The strains that showed the ability to grow under higher As concentrations were chosen for further study. The strain that demonstrated higher tolerance to As was then used for the experiment involving *H. annuus* (sunflower).

2.2. Exploring the biosorption capacity of heat-inactivated bacteria for as

The strain was cultured in a liquid medium (Luria Bertani broth) and shaken for 24 h at 120 rpm and 28 °C. The cells were collected by using a centrifuge at a speed of 5600 rcf for 10 min after the cultures had been left overnight. After rinsing the cells with sterilized distilled water, they were heat-treated using an autoclave at a temperature of 121 °C under a pressure of 15 lbs for a duration of 15 min to inactivate the enzymes and ensure their deactivation. Upon completion of the heat treatment, the cells were incubated overnight in L.B broth enriched with various levels (100, 300, 500, 900, and 1200 ppm) of As using sodium arsenate salt (Na_3AsO_4) as the source of As. The temperature during this incubation was set at 28 °C [21]. To investigate the biotransformation of As, the same culture conditions as described earlier were employed to cultivate live cells at a temperature of 28 °C for a duration of 24 h. The amounts of arsenate and arsenite in the culture media were quantified using the Community Bureau of References (BCR) protocol [22].

2.3. Hydroponic experiments

Healthy and consistent *H. annuus* seeds were cleansed of contamination by immersing them in a 0.1 % $HgCl_2$ solution for a duration of 5 min. To eliminate any remaining traces of $HgCl_2$, the seeds underwent five thorough rinses using autoclaved distilled water. Following this, the seeds were germinated in sterilized autoclaved sand. Once the seedlings reached a two-leaf stage, they were transferred to holders placed in plastic pots designed to hold 500 mL of Hoagland's medium. These pots were filled with a 0.5X Hoagland's nutrient solution [23]. A suspension of the bacterial strain with a concentration of 10^{-6} cells/mL was introduced into the pots using a sterile 50 mL injection. One group of pots served as the control without any bacterial inoculation. The plants in both the control group and the group with the rhizobacterial strain were further divided into subgroups, each subjected to varying levels of As (0, 25, 50, and 100 ppm). Arrangement of the pots adhered to a randomized complete block design. This experimental setup included a combination of two factors: As levels (with the four mentioned concentrations) and the presence or absence of the rhizobacterial strain inoculation. Five pots were assigned to each treatment, and within each pot, there were four seedlings.

The seedlings were cultivated within a controlled environment in a plant growth chamber (LabTech; LGC-5101 G), where conditions were carefully upheld to 28.2 °C temperature, 68 % humidity, a 13-h photoperiod, and a light intensity of 35000 Lux in a plant growth chamber (LabTech; LGC-5101 G). Following a 14-day period in the experiment, the plants were collected, and the RGR (Equation (1)) [24] and NAR (Equation (2)) [25] of the seedlings were assessed. Additionally, at the conclusion of the experiment, the rhizobacterium colonization in the roots was evaluated as follows:

$$RGR = \frac{\ln W2 \ - \ln W1}{t\,2 \ - t\,1} \tag{1}$$

ln = natural log, W1 and W2 are seedlings dry weights after t1 and t2 time.

$$NAR = \frac{W\,2 \ - W\,1}{t\,2 \ - t\,1} \times \frac{In\,L\,2 \ - In\,L\,1}{L\,2 \ - L\,1} \tag{2}$$

W2 and W1 denote seedlings dry weights after t1 and t2 (times), lInL1 and InL2 denote natural logs of A1 and A2 (leaf areas) after t1 and t2 (times).

Pooled data obtained from three repeated experiments were statistically analyzed.

2.4. Metal uptake and bioaccumulation

2.4.1. Extraction of as

The plant materials and root exudates underwent the same processing method as the soil samples, exploiting a modified BCR extraction protocol. The goal was to determine the As content in both the plant biomass and the exudates from the roots. The ordered extraction process encompassed three stages.

2.4.2. Phase 1: acid soluble

The roots and shoots of air-dried seedlings were treated separately. For each 5 g of plant material, 20 mL of a solution containing 0.11 mol per liter of acetic acid was blended in. The mixture was placed in a shaker at 25 °C and 120 rpm for 24 h. After this, the samples were subjected to centrifugal force at 504 rcf for 3 min, and the resulting pellet was retained for subsequent processing. Regarding the root exudates, 20 mL of the exudates were concentrated to 2 mL and subjected to the same treatment as the plant biomass.

2.4.3. Phase 2: reducible

A newly prepared solution of 0.5 M NH_2OH–HCl (20 mL) was combined with the pellet obtained from phase 1. The pH was tuned to 1.5 with the addition of nitric acid, and the mixture was swirled at 25 °C for 16 h.

2.4.4. Phase 3: oxidizable

Next, 10 mL of 30 % hydrogen peroxide was combined with the residues from stage 2. After shaking the mixture, the pH was then tuned to 2. The mixture was desiccated using a rotary evaporator, and the resulting product was re-dissolved in 25 mL of 1 M

ammonium acetate. This solution was then swirled for an additional 16 h.

2.5. Sample analysis

To quantify the presence of As in the samples, an atomic absorption spectrometry system with a heated quartz tube and a continuous flow hydride generator (HG-2, PerkinElmer Model 1100 spectrometer, USA) was employed. The measurement of As content in the samples was carried out using an acetylene/air flame and recorded using this setup.

2.6. Bio-concentration factor of as

To ascertain the bio-concentration of As in sunflower seedlings, the following formula (Equation (3)) was used.

$$BCF = \frac{Metal\ concentration\ in\ biomass}{Metal\ concentration\ added\ to\ the\ media} \quad (3)$$

2.7. Exudates collection

Sunflower seedlings were collected from hydroponic cultures following a two-week growth period. The roots of the seedlings were rinsed with sterilized distilled water. To gather root exudates, the cleaned roots of the seedlings were immersed in test tubes filled with sterile deionized water for a duration of 6 h [26]. Root exudates were subsequently obtained by individually centrifuging the contents of each test tube for a duration of 10 min at a speed of 3000 rpm.

2.8. Indole 3-acetic acid determination

To ascertain the concentration of IAA, fresh sunflower leaves and their root exudates were processed. The sunflower leaves were first powdered into a fine consistency using liquid nitrogen. A total of 10 g of this powder was integrated with 20 mL of absolute methanol. The mixture was vigorously swirled, and the solid debris was separated by centrifuging at 5600 rcf for 10 min. A colorimetric technique utilizing Salkowski reagent was then used to quantify the IAA present in the resulting supernatant [27]. For the root exudates, 10 mL of the sample was used and processed in the same manner as mentioned for the leaves sample.

2.9. Total soluble sugar and lipids

For the leaves of the host plant, about 100 mg of leaves were crushed within 1 mL of a mixture containing equal volumes of chloroform, methanol and water to extract the sugars. As for the root exudates, 0.1 mL of the sample was directly supplemented to the extraction mixture, which was 1 mL of the same mixture (MCW). The samples were mixed using a vortex and subsequently left in a water bath at 50 °C for 30 min. After this, the mixture underwent centrifugation at 14,000 rpm for 5 min at ambient temperature to obtain the supernatant. The acquired supernatant was delicately moved to a 15 mL falcon tube. To determine the sugar content, the phenol-sulfuric acid methodology was adopted. In a test tube, 0.1 mL of the supernatant was blended together with 10 μL of 80 % phenol. Afterward, 1 mL of fuming H_2SO_4 was introduced into the mixture, and the mixture was left undisturbed at ambient temperature for 10 min. Subsequent to vigorous stirring for 30 s and incubating for 20 min at ambient temperature, the absorbance value was measured at 490 nm using a double beam spectrophotometer, specifically the Lambda 25 model by Parkin Elmer [28].

To extract lipids, 0.2 g of sunflower seedling leaves were directly placed into a solution consisting of methanol and chloroform in a 1:2 vol ratio. After filtration, the filtered solution was vigorously shaken briefly, and then 0.8 mL of a 0.73 % NaCl solution was supplemented. The lipids that collected at the bottom were quantified utilizing the technique explained by Byreddy, Gupta [29]. To begin, the sample was moved to a conical flask, which was connected to a condenser through which air flux passed. The flask was then placed on a water bath and subjected to heat at a temperature of 50–60 °C for a duration of 5 h. Once the water had evaporated, 5 mL of concentrated sulfuric acid was introduced to 0.2 mL of the sample, and this mixture was heated for a further 10 min. After allowing the mixture to cool down, 2.4 mL of the phosphoric-vanillin reagent was added. Following an incubation period of 30 min at ambient temperature, the light absorption at 490 nm was assessed and registered.

2.10. Estimation of antioxidants

2.10.1. Nonenzymatic

For the extraction of total flavonoids, fresh leaves weighing 0.5 g were crushed and blended with 80 % ethanol (5 mL). This mixture was left overnight on a shaker. In the case of root exudates, 0.5 mL of the exudates was combined with 5 mL of 80 % ethanol. Following this, the extract was subjected to centrifugation at a temperature of 25 °C for a duration of 15 min at a speed of 10,000 rpm. The resultant flavonoid-rich liquid, or supernatant, was collected and kept at a temperature of 4 °C for further investigation. To measure the total flavonoids, the $AlCl_3$ method was exercised. Specifically, 0.5 mL of the extract was mixed with 80 % methanol (4.5 mL), 10 % $AlCl_3$ (0.1 mL), and CH_3CO_2K (0.1 mL). After 30 min of incubation at room temperature, the components were mixed thoroughly. The optical density of this mixture was then measured at 415 nm. Quercetin was used as a standard for creating a standard curve [30].

For the extraction of phenolics, 1 g of seedlings was crushed (or 1 mL of root exudates was used) and mixed with 16 mL of ethanol.

This mixture was then incubated at a high temperature of 80 °C for a period of 3 h. After this incubation, any solid debris was removed by subjecting the mixture to centrifugation at 5600 rcf and room temperature for 10 min. The resulting supernatant was further clarified by passing it through Whatman No. 42 filter paper. To concentrate the filtrate, it was reduced to a volume of 1 mL under vacuum conditions at a temperature of 40 °C using a rotary evaporator. The concentrated filtrate was then diluted with deionized water (10 mL) and stored at a temperature of 4 °C for further analysis [31]. The phenolic contents were finally measured at 650 nm as described by Malik and Singh [32].

To measure proline in plant samples and root exudates, the Bates [33] method was employed. Approximately 0.1 g of plant sample were crushed (or 0.1 mL of root exudates was mixed) with 4 mL of a solution containing 3 % sulfo-salicylic acid. This mixture was then subjected to centrifugation at 5600 rcf for 5 min. From the collected supernatant (2 mL), an acid ninhydrin solution was added, and the resulting mixture was heated for 1 h at a temperature of 100 °C. After cooling, proline was extracted from the mixture using 4 mL of toluene through a separatory funnel. The absorbance of the solution was measured at 520 nm, and a blank measurement was taken for comparison.

For the estimation of glutathione (GSH) activity, an enzyme extract from the seedlings was mixed with 300 μL of NADPH, 300 μL of oxidized glutathione (GSSG), 300 μL of EDTA, and 1.8 mL of phosphate buffer. The oxidation of NADPH was then monitored by measuring the absorbance at 340 nm at 1-min intervals [34].

2.10.2. Enzymatic

To prepare the enzyme extract for superoxide dismutase (SOD), a buffer solution was created by mixing 50 mM KH_2PO_4 at a pH of 7.8 with other components including 1 % Triton X-100, 100 μM EDTA, 2 % Polyvinylpyrrolidone, and a protease inhibitor cocktail (Roche, Mannheim, Germany). This buffer was used to homogenize 100 mg of fresh leaves, and the resulting mixture was subjected to sonication at 4 °C using a Leela-Sonic-50 sonicator. After sonication, the mixture was filtered through 2.0 μm polycarbonate filters (Osmonics, USA). The filtrate was then centrifuged at 4 °C and 5600 rcf for 20 min. The clear liquid above the sediment was collected as the enzyme extract for SOD activity determination. To measure SOD activity, the inhibition of nitro-blue tetrazolium (NBT) by the enzyme extract was assessed. A reaction mixture was prepared comprising 100 μL of crude plant extract, 50 mM sodium phosphate buffer (pH 7.6), 12 mM L-methionine, 50 mM sodium carbonate, 10 μM riboflavin, 1 mM EDTA, and 50 μM NBT in sterilized distilled water. The reaction mixture was subjected to white light for 15 min to kick off the SOD reaction. The absorbance of the mixture was then observed at a wavelength of 560 nm [35].

Catalase enzyme activity (CAT) was measured by computing the rate at which it breakdowns hydrogen peroxide (H_2O_2). Frozen leaves (0.5 g) were mashed using liquid nitrogen, and the enzyme was isolated in 10 mL of a buffer solution holding 50 mM sodium phosphate (pH 7.0). The phosphate buffer was complemented with 1 mM EDTA-Na_2 and 2 % (w/v) polyvinylpyrrolidine-40 (PVP-40). The enzyme extract was attained by centrifuging the mixture at 4 °C and 5600 rcf for 15 min. To assess CAT activity, a fraction of the collected supernatant (0.1 mL) was mixed with 3 % (v/v) hydrogen peroxide (H_2O_2) and 0.1 mM EDTA. The alteration in optical density was then noted at a wavelength of 240 nm. This permitted the calculation of the rate at which the catalase enzyme cleaves hydrogen peroxide [36].

The activity of ascorbic acid oxidase (APX) was detected using hydrogen peroxide (H_2O_2) as a substrate. In a reaction mixture of 1 mL (prepared as previously mentioned), there was 0.1 mM H_2O_2, 0.5 mM ascorbic acid, 50 mM phosphate buffer (pH 7.0) and 200 μL of enzyme extract. The reaction mixture was permitted to advance, and the change in absorbance was evaluated at a wavelength of 290 nm. This measurement permitted for the calculation of APX activity built on the reaction of ascorbic acid with H_2O_2 [37].

The peroxidase activity was quantified concurring to the protocol of Pütter [38]. In a cuvette, a mixture of 3 mL of 0.1 M phosphate buffer, 0.05 mL of 20 mM guaiacol, 0.03 mL of 12.3 mM (0.04 %) hydrogen peroxide solution, and 0.1 mL of enzyme extract (collected as described earlier) was combined. The mixture was vigorously shaken for a brief period. The time required for the optical density of the mixture to increase by 0.1 at 436 nm was recorded and utilized for further calculations using Equation (4) to determine the peroxidase activity.

$$\text{Enzyme activity} = \left(\frac{500}{\Delta t}\right) \times \left(\frac{1}{1000}\right) \times \left(\frac{TV}{VU}\right) \times \left(\frac{1}{f\ wt}\right) \tag{4}$$

TV, total volume of the extract; VU, volume used; Δt, time change in minute and f wt, leaf weight (g)

The approach outlined by Szabo, Idiţoiu [39] employed to compute the free radical-scavenging activity using 1, 1-diphenyl-2-picrylhydrazyl (DPPH) as the free radical source. Approximately 0.1 g of fresh leaves were ground and mixed with 1 mL of methanol. To prepare the DPPH working solution, 0.004 g of DPPH was dissolved in 100 mL of methanol. Next, 1 mL of the DPPH solution was added to 0.5 mL of the sample, mixed vigorously, and kept in the dark for 30 min. The reduction in color intensity was measured at 517 nm. The calculation of free radical scavenging activity was based on the following Equation (5):

$$\%DPPH = \left(\frac{1 - AE}{AD}\right) \times 100 \tag{5}$$

Whereas,

AE denotes OD of sample containing DPPH + leaves extract and AD denotes OD of blank DPPH.

2.10.3. Lignin in roots of host

The total lignin in sunflower roots was quantified using the modified Kolson protocol as described by Moreira-Vilar, Siqueira-Soares

[40]. The plant material was vigorously shaken in 72 % H_2SO_4 for 7 min at 47 °C to aid in digestion. Following digestion, the samples were subjected to autoclaving at 121 °C for a duration of 30 min. The soluble lignin was separated from insoluble lignin through filtration, and the absorbance was measured at 280 nm and 215 nm. Lignin contents were determined using the following Equation (6):

$$S = \frac{4.53(A215 - A280)}{300} \quad (6)$$

The above equation is a collective of the following two equations:

$$A280 = 0.68\ F + 18\ S \text{ and } A215 = 0.15\ F + 70\ S$$

Whereas,

A_{280} = O.D at 280 nm,

A_{215} = O.D at 215 nm,

F=The furfural (g),

S=The soluble lignin (g).

The values 0.68, 0.15, 18, and 70 represent the molar absorptivity of furfural and soluble lignin at 280 and 215 nm, respectively. The insoluble residues were subjected to a 550 °C temperature for 4 h to burn off organic matter, and the resulting ash content was determined. Lignin content was calculated from the insoluble residues by subtracting the weight of the dry mass from the total ash weight. The total lignin content was determined by combining both insoluble and soluble lignin measurements and was expressed as milligrams per gram of cell wall.

2.10.4. Malonaldehyde estimation

Sunflower leaves (0.2 g) were crushed and mixed with 2 mL of 0.6 % thiobarbituric acid. The mixture was then subjected to centrifugation at 8064 rcf for 10 min. The resulting supernatant was heated at 100 °C for 15 min in a water bath and then cooled for 10 min at room temperature. After cooling, the sample was centrifuged once again at 8064 rcf for 10 min. The obtained supernatant was used for measuring optical density (OD) readings at wavelengths of 450 nm, 532 nm, and 600 nm [41].

2.10.5. Electrolyte leakage

Fresh leaves from sunflower seedlings were carefully washed with deionized water to remove any electrolytes adhering to the surface. These washed leaves were then placed in sealed vials containing 10 mL of deionized water. The vials with the leaves were placed in a shaker operating at 100 rpm and maintained at a temperature of 25 °C. After incubating for 24 h, the initial electrical conductivity of the samples (L1) was measured. Subsequently, the samples were exposed to autoclaving at 120 °C for 20 min, and the conductivity (L2) was measured once again. The degree of electrolyte leakage was assessed using the following formula (Equation (7)) and expressed as the percentage of electrolyte leakage [42].

$$\%EL = \frac{L1}{L2} \times 100 \quad (7)$$

2.10.6. Reactive oxygen species (ROS) accumulation

To assess the accumulation of reactive oxygen species (ROS) through the 3,3′-diaminobenzidine (DAB) staining method, fresh leaves from the plants were immersed in a solution containing DAB (10 mL) placed in a plate. The plate was then placed in a shaking incubator and left to incubate for 4–5 h at a speed of 80–100 revolutions per minute (rpm). After this incubation, any excess DAB stain and chlorophyll were removed by boiling the leaves in a mixture of acetic acid, ethanol, and glycerol (in a ratio of 1:3:1 v/v). Finally, the leaves that had been stained with DAB were observed under a light microscope at a magnification of 400X [43].

2.11. Statistical analysis

The means of various groups were compared using Analysis of Variance (ANOVA), followed by subsequent Posthoc analysis, which included Duncan's Multiple Range Test (DMRT). A significance level of 0.05 was employed to establish statistical significance. The

Table-1
Screening of rhizobacterial isolate with different concentration of arsenic in the form of Sodium arsenate salt.

S.No.	Strain name	100 ppm	300 ppm	500 ppm	900 ppm	1200 ppm
1	MT1	+	–	–	–	–
2	MT6	+	+	–	–	–
3	MT8	+	–	–	–	–
4	*Acinetobacter bouvetii*	+	+	+	+	–
5	*Raoultella planticola* (C9)	+	+	–	–	–
6	C4	+	–	–	–	–
7	*Acinetobacter beijerinckii* (C5)	+	+	+	–	–
8	*Pseudocitrobacter anthropi*	+	–	–	–	–

Resistant (+) and susceptible (–).

statistical techniques were executed performing the analysis employing the SPSS software for Windows (Version 21). Additionally, GraphPad Prism (Version 5.03) was employed to transform the data into graphical representations.

3. Results

3.1. Selection of potent bacterial strain

Among the selected group of 8 strains, it is important to highlight those strains MT1, MT8, C4, and *Pseudocitrobacter anthropi* demonstrated remarkable resilience by thriving in the presence of arsenate supplementation at a concentration of 100 ppm. On the other hand, strain MT6 and *Raoultella planticola* (C9) exhibited an even higher tolerance, enduring arsenate concentrations of up to 300 ppm. Notably, the strain *Acinetobacter beijerinckii* (C5) displayed robust tolerance, enduring arsenate levels as high as 500 ppm. Particularly impressive was the strain *Acinetobacter bouvetii*, which exhibited an exceptional level of tolerance, surviving in arsenate concentrations as high as 900 ppm (Table 1).

3.2. A. bouvetii growth in as contaminated medium

In this research, an increase in bacterial growth was noted in the media supplemented with As. The growth of *A. bouvetii* reached its maximum at 24 h in the media containing 900 ppm of As. However, it's important to highlight that beyond this point, the growth of the strain sharply decreased, indicating the strain's sensitivity to higher concentrations of arsenate (Fig. 1a).

3.3. Biosorption and biotransformation of as

The non-living bacterial cells showed the ability to adsorb the metal (Fig. 1b). These bacterial cells effectively absorbed As within 24 h, resulting in minimal amounts remaining in the media that was initially supplemented with 100 ppm of As. Moreover, the trend of biosorption by *A. bouvetii* increased with higher concentrations of arsenate in the culture media, reaching its highest point at 900 ppm. However, there was a noticeable decrease in biosorption in the broth containing 1200 ppm of arsenate (Fig. 1b).

A. bouvetii demonstrated the ability to convert As^{+5} to As^{+3} through biotransformation (Fig. 1c). Within 24 h, *A. bouvetii* converted over 50 % of As^{+5} to As^{+3} in media with arsenate concentrations of up to 500 ppm. Additionally, as the metal concentration increased to 900 ppm, even larger amounts of arsenate were transformed into arsenite. However, in media with 1200 ppm of As, the biotransformation capability notably decreased (Fig. 1c).

3.4. As uptake and accumulation in A. bouvetii associated sunflower seedlings

The roots of sunflower seedlings associated with *A. bouvetii* accumulated a greater amount of As specifically 82.41 μg/g and 37.91 μg/g of roots respectively, compared to seedlings without *A. bouvetii*, at a metal concentration of 100 ppm (Fig. 2a). This heightened uptake and accumulation of As in both treatments can be attributed to the elevated presence of As in the growth media. However, in *A. bouvetii*-free seedlings, the majority of the metal existed in the form of arsenite (>95 %), whereas in *A. bouvetii*-associated seedlings, notably higher amounts of arsenate were present, suggesting a considerable deviation (Fig. 2a).

The bioconcentration factor (BCF) in both *A. bouvetii*-associated and *A. bouvetii*-free sunflower seedlings was not significant ($p = 0.05$) when cultivated in media containing 25 ppm of As, and in both cases, the BCF value approached 1 (Fig. 2b). However, there was a noticeable decrease in BCF ($P = 0.05$) in *A. bouvetii*-free seedlings cultivated in media with 50 and 100 ppm of As. On the other hand, the BCF of *A. bouvetii*-associated seedlings when grown in media containing 100 ppm of As was 0.82 (Fig. 2b).

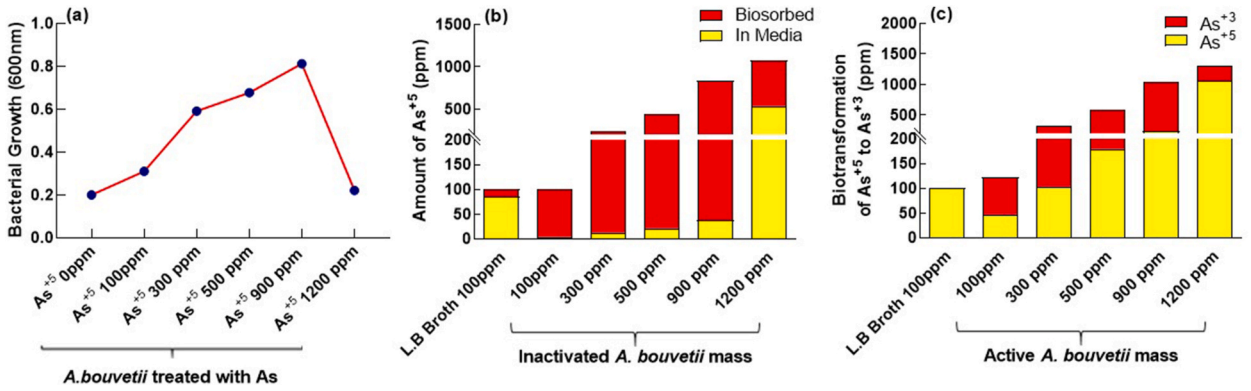


Fig. 1. Influence of varying arsenic concentrations on the capacity of *A. bouvetii* to a) produce biomass b) biosorb As^{+5} and c) biotransformation of As^{+5} to As^{+3}.

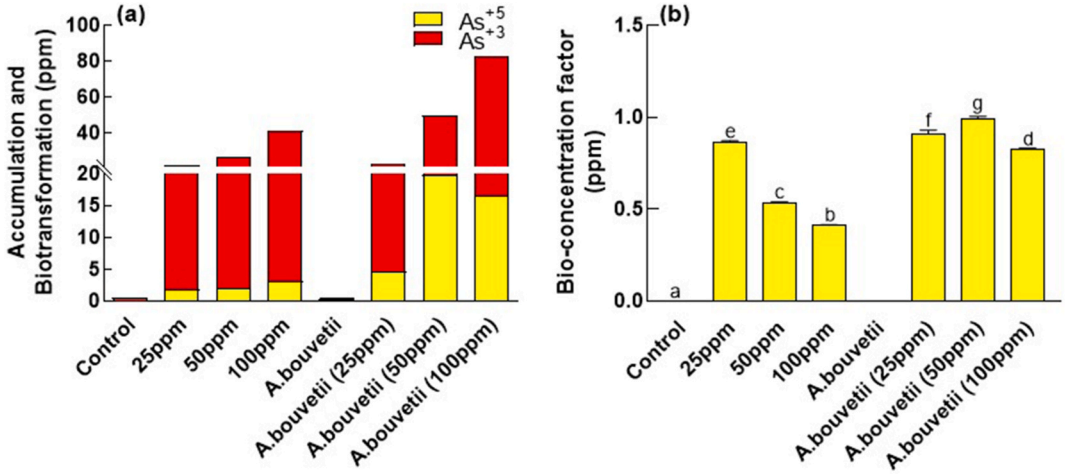


Fig. 2. Accumulation/biotransformation (a), and bio-concentration (b) of arsenic in *A. bouvetii*-associated sunflower seedlings. The data presented here represent the mean ± standard error of 12 replicates, and the letters indicate significance levels among different treatments ($p < 0.05$).

3.5. Host microbe interaction ameliorate as stress and rescuing host growth attributes

3.5.1. NAR and RGR

The NAR of a plant represents the amount of biomass that gets incorporated into its cells. When sunflowers were exposed to As, it had a substantial negative impact on the NAR, resulting in a 74 % reduction at 100 ppm of As, as shown in Fig. 3a. However, when *A. bouvetii* was introduced to the Hoagland's media having host plants, the stress caused by As was alleviated, leading to a significant improvement in the NAR of the sunflower seedlings. Specifically, seedlings associated with *A. bouvetii* exhibited notably higher NAR ($P = 0.05$) when encountering 25 ppm of As compared to both stressed and unstressed seedlings (Fig. 3a).

The RGR of sunflower seedlings not associated with *A. bouvetii* showed a clear decrease in media containing As, and this decrease became more pronounced as the metal concentration increased, as shown in Fig. 3b. However, the presence of *A. bouvetii* had a striking advantage on RGR in the sunflower seedlings ($P = 0.05$). In fact, the RGR of sunflower seedlings associated with *A. bouvetii* under As stress was even higher than that of the unstressed control seedlings (Fig. 3b).

3.5.2. Modulating host IAA production

When cultivated hydroponically, *H. annuus* seedlings produced and released indole acetic acid (IAA). Leaves were the primary contributors to IAA synthesis, accounting for 85.87 % of the total, while roots released a smaller portion (14.12 %) into the surrounding liquid media, as shown in Fig. 4a. The exposure to As stress led to an unfavorable influence on the plant's endogenous pool of

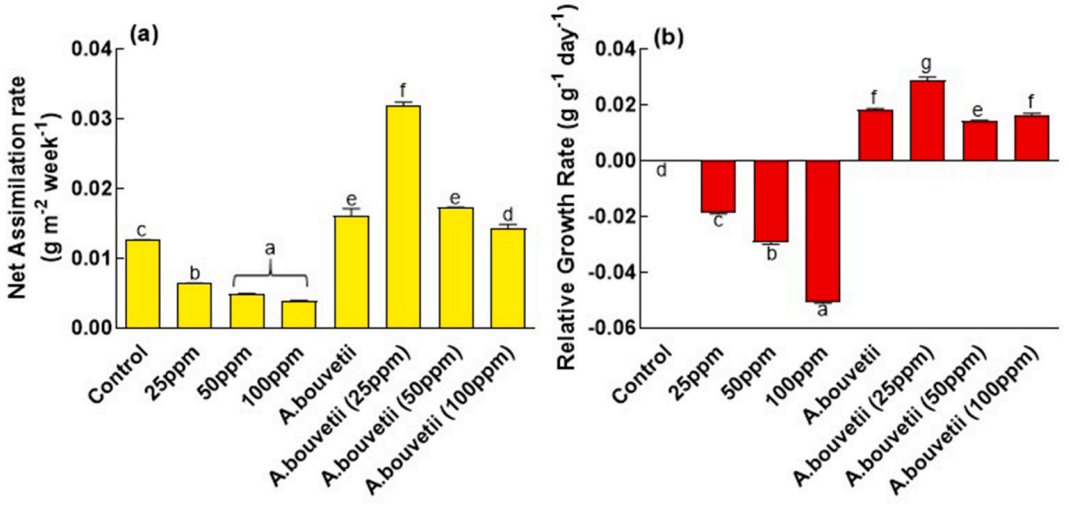


Fig. 3. Effect of arsenic and *A. bouvetii* on the a) NAR and b) RGR of the host seedling. The data presented here represent the mean ± standard error of 12 replicates, and the letters indicate significance levels among different treatments ($p < 0.05$).

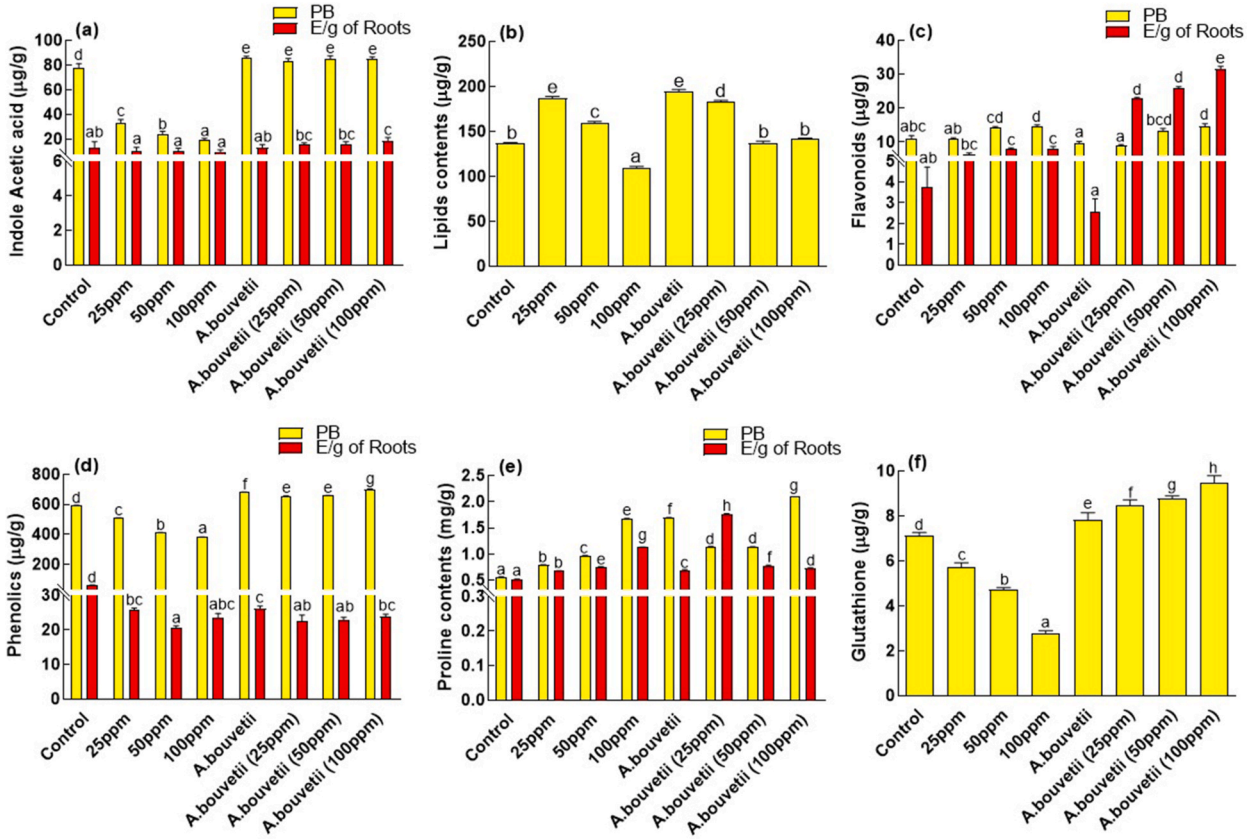


Fig. 4. Effect of different levels of arsenic and *A. bouvetii* on the endogenous and exogenous a) IAA, b) lipids, c) flavonoids, d) phenolic and e) proline of hydroponically grown sunflower seedlings. In the figure PB stands for plant biomass and E/g of roots stands for exudates per gram of roots. The data presented here represent the mean ± standard error of 12 replicates, and the letters indicate significance levels among different treatments ($p < 0.05$).

IAA, with its levels decreasing as the concentration of As increased. For instance, in media with a concentration of 100 ppm of As, the level of endogenous IAA decreased to 24.87 % of the control. Interestingly, the concentration of released IAA remained consistent across different concentrations of As. In seedlings associated with *A. bouvetii,* the IAA concentration was notably elevated in comparison to seedlings without the bacterial association. However, the amount of IAA released by the roots remained unchanged. Importantly, exposure to higher levels of As did not affect IAA synthesis in *A. bouvetii* associated seedlings (Fig. 4a).

3.5.3. Total lipids

In *A. bouvetii* free seedlings, the lipid concentration increased in the presence of medium supplemented with 25 and 50 ppm of As. Nonetheless, there existed a noticeable and statistically significant ($P = 0.05$) decline in lipid content in the medium supplemented with 100 ppm of As (Fig. 4b). On the other hand, *A. bouvetii* associated seedlings exhibited a different pattern. The lipid contents of these seedlings significantly ($P = 0.05$) increased upon exposure to 0 and 25 ppm of As, whereas under 50 and 100 ppm of As-induced stress, the lipid production returned to normal levels (Fig. 4b).

3.6. Non-enzymatic antioxidants

3.6.1. Flavonoids

In *A. bouvetii* free seedlings, the total flavonoid content exhibited a significant increase ($P = 0.05$) with higher concentrations of As in the medium. Both the endogenous and released flavonoid amounts were higher in comparison to the unstressed seedlings (Fig. 4c). Similarly, the endogenous flavonoid levels in *A. bouvetii* associated seedlings were similar to those in *A. bouvetii* free seedlings. However, *A. bouvetii* associated seedlings showed a significant ($P = 0.05$) increase in the release of flavonoids into the medium when treated with As (Fig. 4c).

3.6.2. Phenolics

In *A. bouvetii* free seedlings, the accumulation of phenolic compounds significantly ($P = 0.05$) decreased in media supplemented with As, in contrast to the control seedlings (Fig. 4d). Conversely, in *A. bouvetii* associated seedlings, the endogenous phenolic content

was 18.05 % higher than that in *A. bouvetii* free seedlings. Moreover, a substantial decline of 90.88 % was observed in the amount of released phenolics by symbiotic seedlings in comparison to non-symbiotic seedlings when grown in As-free media. However, under As stress, the amount of endogenously accumulated phenolics in symbiotic seedlings was greater than in non-symbiotic seedlings. The impact of As on the exogenous phenolic content of both *A. bouvetii* associated and free seedlings were less and not significantly affected due to arsenic induce stress conditions (Fig. 4d).

3.6.3. Proline

Proline, a type of free amino acid, was also identified in both the seedlings and the culture media as root exudates (Fig. 4e). The levels of endogenous proline in sunflower seedlings and the amount of proline released by the roots displayed a marked increase ($P = 0.05$) upon the introduction of higher concentrations of As in to the growth media. In non-symbiotic seedlings, the accumulation of endogenous proline and its release from the roots increased by 196.89 % and 118.39 %, respectively, when exposed to 100 ppm of As. In symbiotic seedlings, the accumulation of this amino acid increased by 271.35 % compared to the non-symbiotic control under 100 ppm As stress. However, in symbiotic seedlings subjected to 25 ppm of As, the quantity of endogenous proline decreased, while significantly higher amounts of proline were released. Markedly, the amounts of proline released by *A. bouvetii* associated seedlings under 100 ppm As stress were significantly ($P = 0.05$) reduced, compared to when these same seedlings were subjected to 25 and 50 ppm of As (Fig. 4e).

3.6.4. Glutathione

Regarding the production of glutathione, a notable decrease was perceived in *A. bouvetii* associated sunflower seedlings subjected

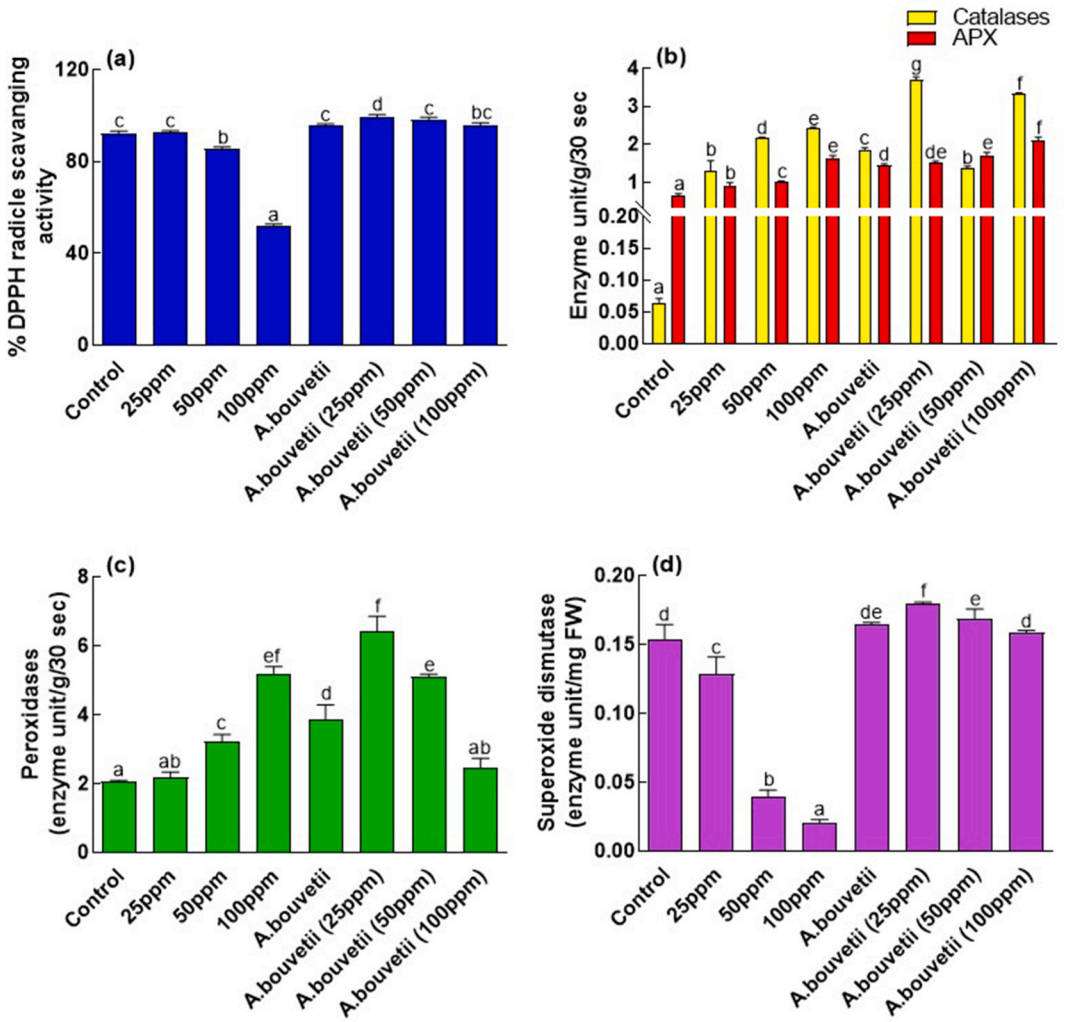


Fig. 5. Effect of different levels of arsenic and *A. bouvetii* on a) DPPH free radical scavenging, b) catalase (CAT)/ascorbic acid peroxidase (APX), d) peroxidases and d) superoxide dismutase activities of sunflower seedlings. The data presented here represent the mean ± standard error of 12 replicates, and the letters indicate significance levels among different treatments ($p < 0.05$).

to elevated As stress, and this decline was significant (P = 0.05) (Fig. 4f). However, in contrast, the symbiotic association of *A. bouvetii* with the seedlings led to accumulate significantly (P = 0.05) higher levels of glutathione, and this effect was further magnified upon exposure to higher levels of As stress (Fig. 4f).

3.7. Modulation in enzymatic response alleviating oxidative stress

3.7.1. 1, 1-diphenyl-2-picrylhydrazyl (DPPH)

A notable decrease in DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging activity was noted in *A. bouvetii* free sunflower seedlings when exposed to 50 and 100 ppm of As stress, and this decrease was significant (P = 0.05) (Fig. 5a). Counter to this, the symbiotic association of *A. bouvetii* with the seedlings led to an improvement in the seedlings' ability to scavenge a higher proportion of DPPH free radicals. Specifically, the DPPH radical scavenging activity of the seedlings associated with the rhizobacteria was either unaffected in media supplemented with high levels of As (50 and 100 ppm) or even enhanced at 25 ppm of As stress (Fig. 5a).

3.7.2. Catalases and ascorbate peroxidases

The activities of both catalase and ascorbate peroxidase (APX) were heightened in non-symbiotic sunflower seedlings as they were exposed to various concentrations of As in a dose-dependent manner (Fig. 5b). Alternatively, in seedlings that had a symbiotic association with *A. bouvetii,* the enzymes' activities were notably higher under As stress-free conditions, as compared to the non-symbiotic control. Specifically, the catalase activity in the symbiotic seedlings was enhanced by 28 % and 140.83 % of that observed in the control seedlings at 25 and 100 ppm of As stress, respectively. However, APX activity consistently increased with the elevation of As levels in the surroundings (Fig. 5b).

3.7.3. Peroxidases

The peroxidase (POD) activity in *A. bouvetii* free sunflower seedlings exhibited an increase corresponding to the rise in As content in the culture media, ranging from 0 ppm to 100 ppm (Fig. 5c). In contrast, in seedlings that were in a symbiotic relationship with *A. bouvetii*, the POD activity was significantly (P = 0.05) greater than that of the non-symbiotic seedlings when exposed to 0, 25, and 50 ppm of As. However, a notable decline in POD activity was perceived in the symbiotic seedlings when they were subjected to 100 ppm of As in the media. Interestingly, the POD activity in *A. bouvetii*-associated seedlings was remarkably similar to that of the non-symbiotic control seedlings (Fig. 5c).

3.7.4. Superoxide dismutase

In *A. bouvetii* free sunflower seedlings, the superoxide dismutase (SOD) activity decreased as the concentration of As in the media increased (Fig. 5d). On the contrary, the SOD activity in *A. bouvetii*-associated seedlings remained non-significantly different from that of the control seedlings when exposed to both 0 and 100 ppm of As. Notably, there was a slight yet significant (P = 0.05) increase in POD activity spotted in symbiotic seedlings that experienced 25 and 50 ppm of As-induced stress (Fig. 5d).

3.7.5. ROS scavenging capabilities of host associated with A. bouvetii

The oxidative stress in sunflower seedlings cultivated in media supplemented with various concentrations of As was visualized by staining the accumulated reactive oxygen species (ROS) using 3,3′-diaminobenzidine (DAB) stain, as depicted in Fig. 6. The vulnerability of *A. bouvetii*-free sunflower seedlings to As stress significantly influenced the generation and buildup of ROS, indicated by the appearance of brown spots or patches (Fig. 6a–d). In *A. bouvetii*-free sunflower leaves, substantial areas surrounding the veins and leaf lamina displayed evident ROS accumulation, particularly pronounced after exposure to 100 ppm of As. In contrast, seedlings

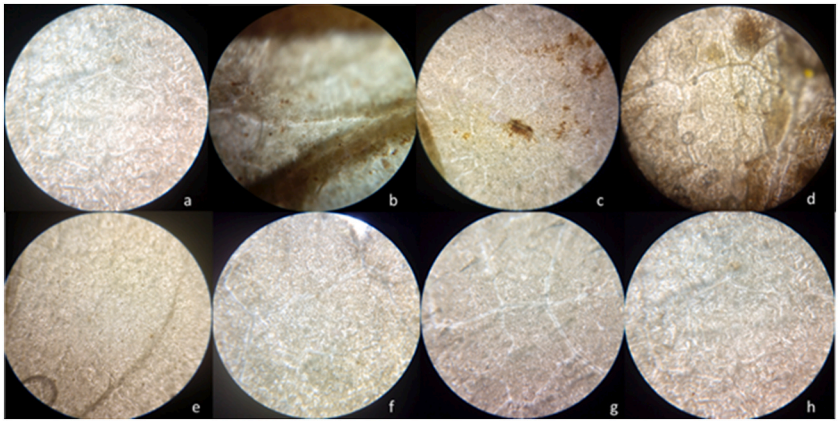


Fig. 6. Accumulation of ROS (total) in the leaves of sunflower seedlings exposed to arsenic (different concentrations) and *A. bouvetii*: a) Control, b) 25 ppm, c) 50 ppm, d) 100 ppm e) *A. bouvetii*, f) *A. bouvetii* +25 ppm, g) *A. bouvetii* +50 ppm and h) *A. bouvetii* +100 ppm. The DAB staining technique was used to visualize ROS accumulation as brown spot under light microscope (400X magnification).

associated with *A. bouvetii* exhibited no visible accumulation of ROS (no presence of brown spots) in response to the various As concentrations (Fig. 6e–h).

3.7.6. Lignification, malonaldehyde contents and electrolyte leakage

The addition of the aforementioned concentrations of As to the growth media led to a noticeable rise in the buildup of lignin in *A. bouvetii*-free sunflower seedlings (Fig. 7a). In contrast, lignin levels remained consistently low in symbiotic seedlings even when exposed to As concentrations of up to 100 ppm. Additionally, the levels of malondialdehyde (MDA) in non-symbiotic seedlings significantly rose with escalating concentrations of As in the media. The introduction of *A. bouvetii* to the seedlings led to a significant reduction in MDA contents (Fig. 7b). Remarkably, under 100 ppm of As stress, *A. bouvetii*-free seedlings exhibited a two-fold increase in electrolyte leakage. Conversely, seedlings associated with *A. bouvetii* effectively mitigated electrolyte leakage in response to As stress (Fig. 7c).

4. Discussion

The presence of heavy metals in water and land resources is a global concern. Among the heavy metals, As is particularly important due to its high level of toxicity. In this current study, we employed a PGPR to enhance sunflower growth and its capacity to accumulate excessive amounts of As in the roots. We observed vigorous growth of the rhizobacterium *A. bouvetii* across varying levels of As, with the maximum growth occurring at 900 ppm. One potential explanation for *A. bouvetii's* ability to tolerate such significant As concentrations could be its biosorption capability. Through biosorption, *A. bouvetii* may bind the heavy metal to its cell surface, thereby safeguarding the living components from the harmful impacts of heavy metal contact [44]. Additionally, it is plausible that *A. bouvetii* utilizes As as a nutrient to facilitate its growth. This notion is supported by previous observations where the Gamma-proteobacterial strain GFAJ-1 exhibited robust growth in media supplemented with As, which could potentially mimic arsenic's role as a substitute for phosphorus [45].

The rhizobacterium *A. bouvetii* established a symbiotic relationship with sunflower, leading to a twofold increase in As accumulation when compared to non-symbiotic sunflower plants. The bioconcentration factor of As in the associated seedlings was approximately 1, indicating that the majority of the added As in the growth medium was taken up by the plants through phytoextraction. Furthermore, the accumulated As primarily existed in the form of arsenite (As^{+3}), as opposed to the originally added arsenate (As^{+5}) present in the growth medium. Markedly, the reduction of arsenate to arsenite represents one potential mechanism for As detoxification. It's worth noting that As^{+3} can be transported outwards to the xylem by the plant and subsequently conveyed to the shoot for vacuolar sequestration [46]. However, in the case of sunflower, only minimal amounts of As were actually translocated into the shoots. An additional As detoxification mechanism involves the potential of phytochelatins to bind with arsenite. This process could facilitate the detoxification of arsenite within the plant [46]. Also, it is conceivable that the rhizobacterium played a role in arsenate detoxification by converting it into arsenite. Subsequently, after sunflower plants absorbed As primarily in the form of arsenite, it's feasible that arsenite efflux pumps were employed to expel As out of the cells. This concerted mechanism involving both the rhizobacterium and the plant's cellular processes could contribute to effective As detoxification [47]. It's important to highlight those growth indicators such as NAR and RGR exhibited higher values in the symbiotic seedlings when compared to the non-symbiotic seedlings under As stress conditions. This unexpected finding might be explained by several factors: 1) *A. bouvetii* bacteria that established residence in the sunflower roots engage in the process of biosorption, which entails binding to heavy metal ions. This activity serves to protect the host tissue from the detrimental effects of As toxicity, 2) the bacteria may employ As for energy production and growth, and 3) retention of As within plant vacuoles or microorganisms and binding through chelation by phytochelatins (PCs) and flavonoids [48]. In the present research, subjecting non-symbiotic sunflower plant to stress led to diminished levels of reduced glutathione. This reduction is implicated in the synthesis of phytochelatins (PCs) through a transpeptidation reaction catalyzed by phytochelatin synthase [49]. In sunflower seedlings associated with *A. bouvetii*, the concentration of this crucial precursor for

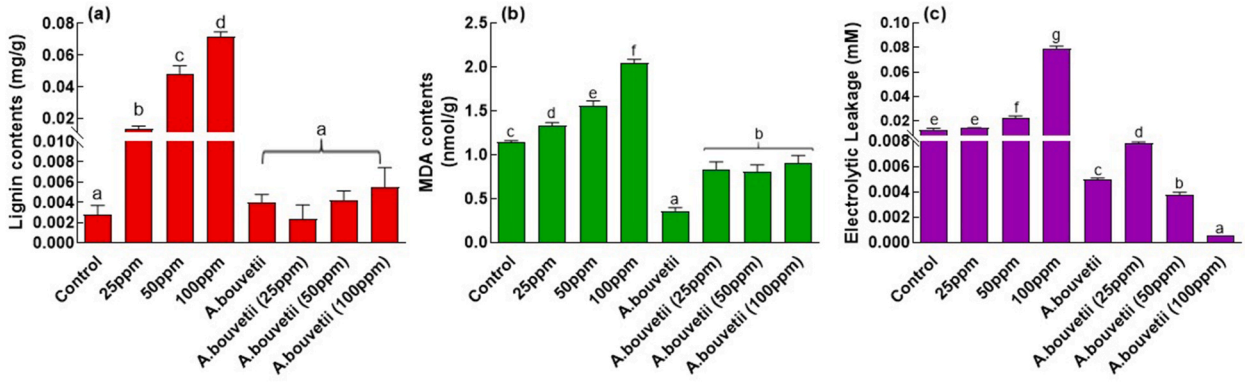


Fig. 7. Effect of arsenic and *A. bouvetii* on: a) root lignification, b) malonaldehyde and c) electrolyte leakage of sunflower roots. The data presented here represent the mean ± standard error of 12 replicates, and the letters indicate significance levels among different treatments ($p < 0.05$).

phytochelatins (PCs) remained consistently elevated. The evident role of rhizobacteria in enhancing sunflower's tolerance to excessive levels of As becomes apparent, as evidenced by the growth parameters when compared to non-symbiotic seedlings. The interplay of biotransformation and biosorption in the current context serves as an effective strategy employed by *A. bouvetii*. This strategy helps to prevent the intrusion of As into cells, subsequently aiding in the mitigation of As toxicity [50–53].

Apart from accumulating and tolerating twofold higher levels of toxic As, symbiotic seedlings exhibited no signs of As toxicity and maintained normal growth. To comprehend the potential mechanism, various essential metabolites that could contribute to stress tolerance and growth enhancement were examined. In sunflower seedlings subjected to As stress, the synthesis of IAA was notably impaired, which could be one among several explanations for their stunted growth. Undoubtedly, reduced levels of IAA can lead to the underdevelopment of the root system, resulting in diminished assimilation of necessary nutrients by the plant [54]. Deprivation of nutrients can induce changes in plant metabolism, which in turn might render the plant more susceptible to adverse environmental conditions [55]. In contrast, symbiotic seedlings exposed to arsenate stress exhibited not only a considerable increase in the accumulation of IAA but also released a substantial proportion of it into the root exudates. Earlier studies of He et al. [56] have reported that the external application of IAA not only reinstated the growth of As-stressed rice plants but also decreased the movement of this metal within the plant. It is plausible that the elevated levels of IAA prompted the synthesis of hemicellulose 1, which in turn enhanced cell division and root growth – both critical factors in augmenting the adsorption of As by the cell wall and obstructing its translocation [56]. In non-symbiotic seedlings, lipid content exhibited an increase under low levels of As, but this elevation sharply declined with higher As concentrations. Conversely, symbiotic sunflower plants showed no significant difference in the total lipid production. In relation to this, endogenous malonaldehyde levels were elevated in non-symbiotic seedlings upon exposure to As. This demonstrates that As triggered lipid peroxidation, consequently leading to membrane degradation, as evidenced by a higher release of electrolytes in As-stressed sunflower plants [57].

Proline serves a dual role, acting as an osmolyte while also playing a crucial part in enhancing plant tolerance to heavy metal stress. This is achieved through a range of mechanisms like metal chelation, enhancement of the antioxidative defense system, and facilitation of signaling processes [58]. Proline induces the formation of phytochelatins which chelate with heavy metals thereby decreasing their toxicity [59]. In a study performed by Sharma et al. [60] exogenous proline protected the activity of glucose-6-phosphate dehydrogenase and nitrate reductase in vitro against inhibition by Cd and Zn. This protection was due to the formation of a proline-metal complex. Similar complex-forming properties of proline were observed by Farago and Mullen [61], where proline formed a complex with Cu in metal-tolerant Armaria. In this current research, sunflower plants under the influence of heavy metal stress, particularly As, exhibited an attempt to counteract the detrimental effects by increasing the production and release of proline at higher concentrations. This reaction was observed consistently in both symbiotic and non-symbiotic sunflower plants, indicating that proline alone could play a major role to mitigate the harmful impact of As. Nonetheless, the elevated proline levels in symbiotic sunflower plants suggest that its presence was a crucial component, working in conjunction with various other factors previously discussed, leading to a significant success in overcoming the stress.

When subjected to metal stress, noticeable hyper-lignification was observed in the sunflower tissues devoid of *A. bouvetii*. This occurrence could be linked to the activation of the plant's defense mechanisms. However, this hyper-lignification might lead to a hindered growth of the plants, subsequently resulting in reduced NAR and RGR [62,63]. The partnership between the chosen *A. bouvetii* and sunflower appeared to normalize tissue lignification, ensuring the appropriate elongation of cells and, consequently, facilitating normal growth.

During our observations, we detected the accumulation of ROS as evidenced by the presence of brown spots in leaves stained with DAB among sunflower seedlings without *A. bouvetii* when exposed to As stress. However, in seedlings associated with *A. bouvetii*, ROS accumulation remained within normal levels even under 100 ppm of As stress. This outcome might be attributed to the robust antioxidative system of the seedlings, as evidenced by the stronger DPPH free radical scavenging activity exhibited by the symbiotic seedlings compared to their non-symbiotic counterparts. The DPPH scavenging activity serves as an effective indicator of the plant's antioxidative defense system [64]. It is important to highlight that sunflower seedling in the presence of *A. bouvetii*, subjected to As stress, exhibited elevated activities of catalase, peroxidase, and APX, which are integral components of the enzymatic antioxidative defense system. In addition, non-enzymatic antioxidants like flavonoids and proline were also increased across various levels of As exposure. These findings emphasize that the overall antioxidative defense mechanism in symbiotic sunflower plants outperformed their non-symbiotic counterparts. However, it's noteworthy that although there was an improvement in the production of several antioxidants, including catalase, APX, peroxidase, flavonoids, and proline, this might not be sufficient to fully combat As stress. Effective mitigation of As stress and thorough scavenging of the resulting ROS necessitates a comprehensive enhancement of all antioxidant components. This is due to the fact that catalase, APX, and peroxidases are particularly effective in managing H_2O_2. Conversely, SOD is crucial for the detoxification of superoxide radicals. Therefore, achieving a balance among these various antioxidant elements is vital to effectively counteract As-induced oxidative stress and maintain the plant's overall health [65]. Research has demonstrated that maintaining a harmonious equilibrium among the activities of distinct components within the antioxidant system (such as catalase, SOD, and APX) is of paramount importance for regulating the appropriate concentrations of H_2O_2 and superoxide radicals. This balance has a pivotal role in preventing oxidative stress. Moreover, in conjunction with this delicate equilibrium, the sequestration of metal ions is recognized as a pivotal factor in curtailing the generation of exceedingly hazardous hydroxyl radicals [65]. The interplay among catalase, SOD, and APX is critical for the regeneration of redox glutathione and ascorbate [66]. The heightened levels of glutathione in symbiotic sunflower plants hold significant implications in this context. This metabolite complex appears to interact with As prior to its ultimate sequestration by PCs, thereby restricting the heavy metal's access to living tissues [66].

A fundamental strategy in rehabilitating soils impacted by heavy metals is to make these metals available for uptake by the selected plants used for remediation. This method seeks to increase the successful removal of heavy metals from the soil [67]. This study

documented that *A. bouvetii* facilitated the bio-transformation of the metal, rendering it bioavailable for uptake by the associated sunflower plants from the soil. Furthermore, As was concentrated within the plant roots and their subsequent transport to the edible portions of the plants was restricted. Plants subjected solely to As treatment exhibited an active bioaccumulation of metals, but this was accompanied by stunted growth, reduced NAR and RGR. In contrast, plants that were inoculated with *A. bouvetii* displayed elevated uptake and accumulation of metals, coupled with improved NAR and RGR when compared to control plants. This observation suggests that *A. bouvetii* positively influenced the host plants' biomass, thereby creating more compartments for As accumulation and translocation. Simultaneously, the presence of *A. bouvetii* facilitated detoxification and compartmentalization, preventing the toxic effects of As on the plants.

5. Conclusion

This study sheds light on the extraordinary proficiencies of *A. bouvetii* in alleviating arsenic-induced stress in sunflower seedlings. The strain's remarkable tolerance to high arsenate concentrations, as well as its capacity to adsorb and biotransform arsenic, underline its aptitude for phytoremediation applications. Besides, the beneficial interactions between *A. bouvetii* and sunflower seedlings were obvious in several aspects. The symbiotic association led to amended host growth attributes, involving improved NAR and RGR, as well as enhanced production of IAA and modulation of lipid content. These effects were chiefly marked under arsenic stress conditions. *A. bouvetii* also performed a critical part in augmenting the antioxidant defense mechanisms of the host, as demonstrated by improved scavenging of ROS, higher activities of catalase, ascorbate peroxidase, peroxidases, and superoxide dismutase, along with elevated levels of flavonoids, phenolics, proline, and glutathione. Furthermore, the symbiotic association diminished lignin accumulation, MDA levels, and electrolyte leakage in the sunflower seedlings subjected to arsenic, signifying the strain's ability to ameliorate oxidative stress and uphold cellular integrity. In short, these findings emphasize the capability of *A. bouvetii* as a helpful collaborator in boosting the resilience and arsenic tolerance of sunflower plants, making it a capable candidate for phytoremediation strategies in arsenic-contaminated environments. Further study is required to discover the practical uses and ecological consequences of this mutually beneficial partnership.

Availability of data and material

All the data is included in the paper.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not Applicable.

Funding

This work is supported by Researchers Supporting Project (#RSP2023R186), King Saud University, Riyadh, Saudi Arabia.

CRediT authorship contribution statement

Muhammad Qadir: Writing – original draft, Methodology, Formal analysis. **Anwar Hussain:** Writing – original draft, Supervision, Project administration, Methodology, Data curation, Conceptualization. **Mohib Shah:** Validation, Supervision, Methodology. **Muhammad Hamayun:** Resources, Project administration, Conceptualization. **Asma A. Al-Huqail:** Writing – review & editing, Resources, Funding acquisition. **Amjad Iqbal:** Writing – review & editing, Writing – original draft, Data curation, Conceptualization. **Sajid Ali:** Writing – original draft, Formal analysis.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgment

We extend our sincere gratitude to Abdul Wali Khan University Mardan for generously providing the necessary laboratory facilities that enabled us to conduct this extensive research.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e33078.

References

[1] V.C. Pandey, et al., Arsenic hazards in coal fly ash and its fate in Indian scenario, Resour. Conserv. Recycl. 55 (9–10) (2011) 819–835.
[2] Z. Rahman, V.P. Singh, The relative impact of toxic heavy metals (THMs)(arsenic (As), cadmium (Cd), chromium (Cr)(VI), mercury (Hg), and lead (Pb)) on the total environment: an overview, Environ. Monit. Assess. 191 (2019) 1–21.
[3] A. Rashid, et al., Heavy metal contamination in agricultural soil: environmental pollutants affecting crop health, Agronomy 13 (6) (2023) 1521.
[4] A. Alengebawy, et al., Heavy metals and pesticides toxicity in agricultural soil and plants: ecological risks and human health implications, Toxics 9 (3) (2021) 42.
[5] WHO, Guidelines for Drinking-Water Quality, vol. 1, World Health Organization, China, 2004.
[6] M.S. Gorby, Arsenic poisoning, West. J. Med. 149 (3) (1988) 308–315.
[7] M.A. Islam, et al., Trace metals accumulation in soil irrigated with polluted water and assessment of human health risk from vegetable consumption in Bangladesh, Environ. Geochem. Health 40 (2018) 59–85.
[8] S. Soltani-Gerdefaramarzi, M. Ghasemi, B. Ghanbarian, Geogenic and anthropogenic sources identification and ecological risk assessment of heavy metals in the urban soil of Yazd, central Iran, PLoS One 16 (11) (2021) e0260418.
[9] D. Hou, et al., Sustainable soil use and management: an interdisciplinary and systematic approach, Sci. Total Environ. 729 (2020) 138961.
[10] D. Padhan, et al., Bioremediation of heavy metals and other toxic substances by microorganisms, in: A.P. Javid, H.A.E.M. Abeer, S. Riyaz (Eds.), Soil Bioremediation: an Approach towards Sustainable Technology, Wiley-Blackwell, USA, 2021, pp. 285–329.
[11] M. Qadir, et al., Phytohormones producing acinetobacter bouvetii P1 mitigates chromate stress in sunflower by provoking host antioxidant response, Antioxidants 10 (12) (2021) 1868.
[12] I. Mallick, et al., Effective rhizoinoculation and biofilm formation by arsenic immobilizing halophilic plant growth promoting bacteria (PGPB) isolated from mangrove rhizosphere: a step towards arsenic rhizoremediation, Sci. Total Environ. 610 (2018) 1239–1250.
[13] M. Nanda, V. Kumar, D. Sharma, Multimetal tolerance mechanisms in bacteria: the resistance strategies acquired by bacteria that can be exploited to 'clean-up'heavy metal contaminants from water, Aquat. Toxicol. 212 (2019) 1–10.
[14] S. Sher, A. Rehman, Use of heavy metals resistant bacteria—a strategy for arsenic bioremediation, Appl. Microbiol. Biotechnol. 103 (2019) 6007–6021.
[15] U. Dahal, K. Paul, S. Gupta, The multifaceted genus Acinetobacter: from infection to bioremediation, J. Appl. Microbiol. 134 (8) (2023) lxad145.
[16] M. Qadir, et al., Pantoea conspicua promoted sunflower growth and engulfed rhizospheric arsenate by secreting exopolysaccharide, Plant Physiol. Biochem. 201 (2023) 107826.
[17] S.S. Dhiman, et al., Metal accumulation by sunflower (Helianthus annuus L.) and the efficacy of its biomass in enzymatic saccharification, PLoS One 12 (4) (2017) e0175845.
[18] V.S. Kanwar, et al., Phytoremediation of toxic metals present in soil and water environment: a critical review, Environ. Sci. Pollut. Control Ser. 27 (2020) 44835–44860.
[19] O. Sytar, et al., Physiological and molecular mechanisms of metal accumulation in hyperaccumulator plants, Physiol. Plantarum 173 (1) (2021) 148–166.
[20] Husna, et al., Pseudocitrobacter anthropi reduces heavy metal uptake and improves phytohormones and antioxidant system in Glycine max L, World J. Microbiol. Biotechnol. 37 (2021) 1–19.
[21] K.S. Prasad, et al., Biosorption of arsenite (As^{+3}) and arsenate (As^{+5}) from aqueous solution by Arthrobacter sp. biomass, Environ. Technol. 34 (19) (2013) 2701–2708.
[22] T. Kazi, et al., Evaluating the mobility of toxic metals in untreated industrial wastewater sludge using a BCR sequential extraction procedure and a leaching test, Anal. Bioanal. Chem. 383 (2005) 297–304.
[23] D.R. Hoagland, D.I. Arnon, The water-culture method for growing plants without soil, Circular. California agricultural experiment station 347 (2nd edit) (1950) 32.
[24] W.A. Hoffmann, H. Poorter, Avoiding bias in calculations of relative growth rate, Ann. Bot. 90 (1) (2002) 37–42.
[25] A. Vernon, J. Allison, A method of calculating net assimilation rate, Nature 200 (4908) (1963) 814, 814.
[26] W.-y. Hao, et al., Allelopathic effects of root exudates from watermelon and rice plants on Fusarium oxysporum f. sp. niveum, Plant Soil 336 (2010) 485–497.
[27] A. Hussain, S. Hasnain, Interactions of bacterial cytokinins and IAA in the rhizosphere may alter phytostimulatory efficiency of rhizobacteria, World J. Microbiol. Biotechnol. 27 (2011) 2645–2654.
[28] M. DuBois, et al., Colorimetric method for determination of sugars and related substances, Anal. Chem. 28 (3) (1956) 350–356.
[29] A.R. Byreddy, et al., A quick colorimetric method for total lipid quantification in microalgae, J. Microbiol. Methods 125 (2016) 28–32.
[30] A.J.D. Fernandes, et al., Total flavonoids content in the raw material and aqueous extractives from Bauhinia monandra Kurz (Caesalpiniaceae), Sci. World J. (2012) 2012.
[31] N. Siddiqui, et al., Spectrophotometric determination of the total phenolic content, spectral and fluorescence study of the herbal Unani drug Gul-e-Zoofa (Nepeta bracteata Benth), Journal of Taibah University Medical Sciences 12 (4) (2017) 360–363.
[32] C.P. Malik, M. Singh, Plant Enzymology and Histo-Enzymology: A Text Manual, Kalyani Publishers, India, 1980, p. 434.
[33] C. Bates, Proline and hydroxyproline excretion and vitamin C status in elderly human subjects, Clin. Sci. Mol. Med. 52 (5) (1977) 535–543.
[34] M. Ikram, et al., IAA producing fungal endophyte Penicillium roqueforti Thom., enhances stress tolerance and nutrients uptake in wheat plants grown on heavy metal contaminated soils, PLoS One 13 (11) (2018) e0208150.
[35] L. Flohe, F. Otting, [10] Superoxide dismutase assays, in: L. Packer (Ed.), Methods in Enzymology, Elsevier, 1984, pp. 93–104.
[36] L.H. Johansson, L.H. Borg, A spectrophotometric method for determination of catalase activity in small tissue samples, Anal. Biochem. 174 (1) (1988) 331–336.
[37] S. Elavarthi, B. Martin, Spectrophotometric assays for antioxidant enzymes in plants, in: R.e. Sunkar (Ed.), Plant Stress Tolerance, Methods in Molecular Biology, Humana Press, India, 2010, pp. 273–280.
[38] J. Pütter, Peroxidases, in: H. Bergmeyer (Ed.), Methods of Enzymatic Analysis, Elsevier, Germany, 1974, pp. 685–690.
[39] M. Szabo, et al., Improved DPPH determination for antioxidant activity spectrophotometric assay, Chem. Pap. 61 (3) (2007) 214–216.
[40] F.C. Moreira-Vilar, et al., The acetyl bromide method is faster, simpler and presents best recovery of lignin in different herbaceous tissues than Klason and thioglycolic acid methods, PLoS One 9 (10) (2014) e110000.
[41] A. Reitznerová, et al., Lipid peroxidation process in meat and meat products: a comparison study of malondialdehyde determination between modified 2-thiobarbituric acid spectrophotometric method and reverse-phase high-performance liquid chromatography, Molecules 22 (11) (2017) 1988.
[42] S. Lutts, J. Kinet, J. Bouharmont, NaCl-induced senescence in leaves of rice (Oryza sativaL.) cultivars differing in salinity resistance, Ann. Bot. 78 (3) (1996) 389–398.
[43] A. Daudi, J.A. O'Brien, Detection of hydrogen peroxide by DAB staining in Arabidopsis leaves, Bio-protocol 2 (18) (2012) e263, e263.
[44] M. Aryal, A comprehensive study on the bacterial biosorption of heavy metals: materials, performances, mechanisms, and mathematical modellings, Rev. Chem. Eng. 37 (6) (2021) 715–754.
[45] F. Wolfe-Simon, et al., A bacterium that can grow by using arsenic instead of phosphorus, Science 332 (6034) (2011) 1163–1166.

[46] F.J. Zhao, et al., Arsenic uptake and metabolism in plants, New Phytol. 181 (4) (2009) 777–794.
[47] G. Yan, et al., Genetic mechanisms of arsenic detoxification and metabolism in bacteria, Curr. Genet. 65 (2019) 329–338.
[48] A. Jain, et al., Therapeutic efficacy of silymarin and naringenin in reducing arsenic-induced hepatic damage in young rats, Ecotoxicol. Environ. Saf. 74 (4) (2011) 607–614.
[49] S. Yadav, Heavy metals toxicity in plants: an overview on the role of glutathione and phytochelatins in heavy metal stress tolerance of plants, South Afr. J. Bot. 76 (2) (2010) 167–179.
[50] B.E. Igiri, et al., Toxicity and bioremediation of heavy metals contaminated ecosystem from tannery wastewater: a review, J. Toxicol. 2018 (2018).
[51] M. Qadir, et al., Comparative assessment of chromate bioremediation potential of Pantoea conspicua and Aspergillus Niger, J. Hazard Mater. 424 (2022) 127314.
[52] T.M. Roane, I.L. Pepper, T.J. Gentry, Microorganisms and metal pollutants, in: L.P. Ian, P.G. Charles, J.G. Terry (Eds.), Environmental Microbiology, Elsevier, USA, 2015, pp. 415–439.
[53] M. Thakare, et al., Understanding the holistic approach to plant-microbe remediation technologies for removing heavy metals and radionuclides from soil, Current Research in Biotechnology 3 (2021) 84–98.
[54] K.K. Meena, et al., Mitigation of salinity stress in wheat seedlings due to the application of phytohormone-rich culture filtrate extract of methylotrophic actinobacterium Nocardioides sp. NIMMe6, Front. Microbiol. 11 (2020) 2091.
[55] W. Xu, et al., Metabolome and RNA-seq analysis of responses to nitrogen deprivation and resupply in tea plant (Camellia sinensis) roots, Front. Plant Sci. 13 (2022) 932720.
[56] Y. He, et al., Exogenous IAA alleviates arsenic toxicity to rice and reduces arsenic accumulation in rice grains, J. Plant Growth Regul. 41 (2) (2022) 734–741.
[57] S. Panda, I. Chaudhury, M. Khan, Heavy metals induce lipid peroxidation and affect antioxidants in wheat leaves, Biol. Plantarum 46 (2) (2003) 289–294.
[58] S. Hayat, et al., Role of proline under changing environments: a review, Plant Signal. Behav. 7 (11) (2012) 1456–1466.
[59] J.A. de Knecht, et al., Phytochelatins in cadmium-sensitive and cadmium-tolerant Silene vulgaris (chain length distribution and sulfide incorporation), Plant Physiol. 104 (1) (1994) 255–261.
[60] S.S. Sharma, H. Schat, R. Vooijs, In vitro alleviation of heavy metal-induced enzyme inhibition by proline, Phytochemistry 49 (6) (1998) 1531–1535.
[61] M. Farago, W. Mullen, Plants which accumulate metals. Part IV. A possible copper-proline complex from the roots of Armeria maritima, Inorg. Chim. Acta. (1979).
[62] J. Díaz, et al., Induction of shikimate dehydrogenase and peroxidase in pepper (Capsicum annuum L.) seedlings in response to copper stress and its relation to lignification, Plant Sci. 161 (1) (2001) 179–188.
[63] Y. Jha, Cell water content and lignification in maize regulated by rhizobacteria under salinity, Brazilian Journal of Biological Sciences 4 (7) (2017) 9–18.
[64] M.M. Rahman, et al., In vitro antioxidant and free radical scavenging activity of different parts of Tabebuia pallida growing in Bangladesh, BMC Res. Notes 8 (1) (2015) 1–9.
[65] R. Mittler, Oxidative stress, antioxidants and stress tolerance, Trends Plant Sci. 7 (9) (2002) 405–410.
[66] C.H. Foyer, G. Noctor, Tansley Review No. 112 Oxygen processing in photosynthesis: regulation and signalling, New Phytol. 146 (3) (2000) 359–388.
[67] X. Li, et al., Health risks of heavy metal exposure through vegetable consumption near a large-scale Pb/Zn smelter in central China, Ecotoxicol. Environ. Saf. 161 (2018) 99–110.