

Article

Enhancing Peanut Crop Quality Under Arsenic Stress Through Agronomic Amendments

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Abstract

Arsenic (As) contamination poses a major challenge to sustainable crop production, particularly in legumes such as peanut (*Arachis hypogaea* L.), where it disrupts growth, nodulation, and redox homeostasis. This study evaluated the potential of circular-economy-based amendments derived from spent mushroom substrate (SMS) of *Pleurotus djamor* and plant growth-promoting bacteria (PGPB) to mitigate As stress in peanut plants. Six growth conditions were tested under 20 μ M arsenate, including single and combined inoculations with *P. djamor* and *Pseudomonas fluorescens*, as well as a residue-only benchmark (E). Results showed that the unamended control (AP) exhibited the highest As accumulation, oxidative stress (H_2O_2 , TBARs), and biomass loss, whereas SMS-based amendments attenuated these effects. Treatments HB (SMS + *P. djamor* + PGPB) and B (SMS + PGPB) combined low As translocation with enhanced antioxidant performance (SOD, CAT), maintaining growth and pigment stability. Amendment H (SMS + *P. djamor*) preferentially activated phytochelatin-related genes (*PCS2*, *CAD1*), while E minimized As uptake but lacked circular applicability. Overall, SMS-PGPB interactions promoted As retention in roots and strengthened ROS-scavenging defenses. These findings highlight SMS-based amendments as viable, sustainable strategies to enhance peanut quality and resilience under As stress, supporting their integration into circular agronomic systems.

Keywords: *Arachis hypogaea*; arsenic; circular economy; plant growth promoting bacteria; *Pleurotus djamor*; spent mushroom substrate



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1. Introduction

In their natural environment, plants are constantly exposed to a variety of stress factors. Among the most concerning, especially in agricultural systems, is the presence of heavy metals and metalloids. These contaminants can interfere with normal plant development, significantly disrupt the soil's microbial community, and ultimately lead to contamination of the food chain [1–3]. Among environmental contaminants, arsenic (As) is of particular concern because it originates from both natural geochemical processes and anthropogenic activities. Millions of people around the world are affected by As toxicity, with a significant portion of agricultural land and drinking water contaminated by this metalloid [4,5]. In agricultural regions worldwide, As is frequently present in groundwater, which under

climate-change-driven rises in the water table increasingly reaches the rooting zone. This enhances the risk of As uptake by crops, thereby compromising plant growth and raising concerns for food safety and human health. In South America, and particularly in Argentina, natural groundwater As concentrations often exceed internationally recommended limits [6,7].

The most common effects of arsenic toxicity in plants include inhibited nutrient uptake, disruptions in photosynthesis, imbalances in water status, interference with enzyme functional groups, and the replacement of essential ions [4,8–12]. The inorganic As(V) form is a chemical analog of phosphate and competes with it for uptake via phosphate transporters [10]. The presence of As in cells leads to oxidative stress, marked by an increase in reactive oxygen species (ROS). In plants, ROS such as superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), and hydroxyl radicals ($\cdot OH$) are naturally produced and tightly regulated to prevent cellular damage caused by their accumulation. Under stress conditions like arsenic exposure, this balance between ROS generation and detoxification mechanisms can be disrupted [12–14]. Several studies have shown that arsenic treatment leads to ROS buildup in various plant species, including peanut [12,15–19]. The overload of cellular antioxidant systems promotes an oxidative environment characterized by lipid peroxidation, protein oxidation, and DNA damage. To counteract the effects of oxidative stress, plants activate a complex antioxidant defense network. In general, tolerance to heavy metals and metalloids has been linked to increased activity of the antioxidant system, which not only neutralizes ROS but also contributes to the chelation and removal of toxic elements [20,21]. This system is divided into two major groups that work in coordination to maintain redox balance under stress conditions: enzymatic and non-enzymatic antioxidants. The enzymatic group, considered the first line of defense, includes superoxide dismutase (SOD) [22,23], and catalase (CAT) [24]. It also encompasses enzymes involved in glutathione (GSH) metabolism, such as glutathione S-transferase (GST), glutathione peroxidase or peroxiredoxin (GPX/PRX), and glutathione reductase (GR), as well as those participating in the ascorbate-glutathione cycle (AsA-GSH), including ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), and the aforementioned GR. On the other hand, non-enzymatic antioxidants also play a crucial role, with GSH standing out as one of the most important components [25,26].

The widespread use of agrochemicals is the conventional method for maintaining crop productivity under various stress conditions. However, research indicates that employing plant growth-promoting bacteria (PGPB) offers a far more sustainable strategy to reduce damage caused by abiotic and biotic stressors [27–30]. Additionally, numerous studies have identified soil microbiome bacteria with the potential to partially or even completely replace conventional nitrogen fertilizers [31,32]. Among rhizosphere bacteria, those capable of biological nitrogen fixation (BNF) offer a cost-effective and environmentally attractive alternative to synthetic nitrogen fertilizers [33]. In addition to fixing atmospheric nitrogen (N_2) in the soil, many of these bacteria can solubilize essential nutrients such as phosphorus through the production of organic acids or other chelating compounds, enhancing root absorption and promoting plant growth [34]. In the specific case of the legume peanut (*Arachis hypogaea* L.), symbiotic bacteria have been primarily classified within the genus *Bradyrhizobium* [35]. This legume crop is an annual species native to warm regions, originating from northwestern Argentina and adjacent regions of South America. Global peanut production exceeds 42 million tons, with Asian countries leading cultivation worldwide [36]. This legume produces geocarpic fruits, which develop underground and interact directly with the soil and rhizosphere microbiome [37]. These characteristics make peanut an interesting model for studying plant–microbe interactions and the uptake of metals from the soil environment.

Given the widespread impact of arsenic toxicity on human health and the contamination of agricultural land and water sources, there is growing interest in biotechnological strategies that use PGPB inoculants and organic amendments to reduce arsenic uptake by plants. This interest stems from the ability of soil microbial activity to influence arsenic adsorption/desorption, solubility, bioavailability, mobility, and its transfer from soil to plants by altering its chemical speciation [38,39]. Notably, in agriculturally important crops such as peanut, studies on arsenic impact remain limited, especially under conditions that include interactions with PGPB [17,18,40]. Certain *Bradyrhizobium* strains have shown effectiveness in reducing arsenic translocation to the aerial parts of the plant [17,41]. Due to their broad spectrum of action, arsenic-tolerant PGPB offer promising potential as bioinoculants to support plant growth in metal-stressed soils. Macrofungi are also recognized for their tolerance to pollutants and their ability to accumulate high concentrations of heavy metals and metalloids, making them a promising tool for bioremediation and soil and water decontamination strategies [42,43]. However, using edible mushrooms for pollutant removal is not advisable due to their valuable nutritional and medicinal properties. Instead, spent mushroom substrates (SMS) are recommended as a sustainable alternative, as they are produced in large quantities after mushroom harvesting and retain a notable capacity for pollutant sorption or degradation [44,45]. Additionally, reusing organic waste as soil amendments offers a sustainable and practical alternative, promoting a shift from a linear production model to a circular economy, where by-products from one activity are reintegrated into another productive system [46–48]. This is particularly relevant for SMS, which has traditionally been disposed of through environmentally harmful methods such as land-filling and open burning [49]. Moreover, spent mushroom substrate (SMS), a byproduct of mushroom cultivation, stands out for its high organic matter content, neutral pH, and good porosity, all of which contribute to improved soil structure and fertility [50–52]. Its application positively influences soil microbial populations and biological pest control, while also enhancing crop yield and fruit quality. Beyond its value as an agricultural amendment, SMS contains lignin and enzymes capable of degrading both organic and inorganic pollutants, making it a promising tool for bioremediation and soil and water decontamination strategies. In particular, SMS derived from *Pleurotus* spp., a group of edible mushrooms with high agronomic and nutritional value [53], has demonstrated remarkable heavy metal bioadsorption capacity [54,55]. While PGPB and SMS-based amendments have been studied independently in numerous works, combining arsenic-tolerant PGPB with *Pleurotus* SMS as a soil amendment represents an innovative biological strategy. This synergy could help reduce metal concentrations in the rhizosphere, limiting their uptake by plants.

Given the growing concern over high arsenic concentrations in groundwater, the ability of plants to absorb it through this water in agricultural areas, and its potential impact on high-value crops such as peanut, it is essential to develop strategies that minimize the uptake and translocation of this metalloid into edible plant tissues. Therefore, this study uses peanut as a model crop to evaluate the effectiveness of biological tools for arsenic mitigation while maintaining plant performance. We hypothesize that the use of sustainable amendments based on spent mushroom substrate (SMS), *Pleurotus djamor*, and plant growth-promoting bacteria (PGPB) improves plant growth and tolerance to arsenic stress by limiting As translocation and effectively modulating the oxidative response. This study aimed to evaluate the potential of sustainable amendments derived from *Pleurotus djamor* spent substrate and plant growth-promoting bacteria (PGPB) to improve peanut tolerance to arsenic stress. The effects of these amendments were assessed on (i) arsenic mobility and translocation, (ii) plant growth and pigment retention, (iii) oxidative stress

indicators and antioxidant enzyme activities, and (iv) the expression of key genes involved in arsenic detoxification and homeostasis.

2. Materials and Methods

2.1. Preparation and Evaluation of Fungal and Bacterial Strains

The strain PQ of *Pleurotus djamor* is a wild isolate collected (2019) by A. Riofrío from Quevedo, Los Ríos Province, Ecuador (coordinates: 01°00'05" S; 79°26'59" W). The isolate was morphologically identified as *Pleurotus* and molecularly confirmed as *P. djamor* using three genetic markers (ITS, EF1 α , and RPB2), showing over 93% sequence identity through the National Center for Biotechnology Information (NCBI) primer web blast tool (Biolegio, Nijmegen, The Netherlands) platform. Later, *P. djamor* PQ was domesticated for research by our group in the Plant Physiology Laboratory of the UAB. Mycelial plugs (0.5 cm in diameter) from a previous culture were inoculated onto Petri dishes containing Potato Dextrose Agar (PDA) medium (Neogen® Lansing, Lansing, MI, USA.) and cultured in static controlled conditions of temperature at 28 °C for 21 days in darkness. In the case of prokaryotic microorganisms, two strains of PGPB were used: 1.2 (*Pseudomonas fluorescens*, MT218315) which was isolated from soils of former heavy metal mines [56], and J49 (*Enterobacter* sp.) which has been studied as PGPB in peanut crops [57,58], with plant growth-promoting traits experimentally verified in both strains. The bacteria were cultured in tryptone-yeast extract (TY) medium (Fisher Scientific®, Waltham, MA, USA) at 25 °C in a thermostatic bath with rotary agitation (150 rpm).

2.2. Fungal-Bacterial Compatibility Tests

The compatibility of *P. djamor* PQ and PGPB when grown together in vitro was evaluated in Petri dishes. The fungus was cultured on PDA medium for 4 days, and then two sterile filter paper disks (6 mm in diameter) were placed equidistant from each other and one centimeter from the edge of the fungal mycelium growth. Each disk was inoculated with 10 μ L of bacterial suspension (1×10^8 CFU mL⁻¹) of each of the bacterial strains studied (J49, 1.2), and the plates were incubated again under the same conditions (25 °C, darkness). During the 24 and 48 h following bacterial inoculation, macro and microscopic observations were made of the presence or absence of bacterial growth, or of possible interactions, compatibility, or inhibition between fungi and bacteria. Photographic records were made to document these observations. For each co-culture of the fungus and each bacterial strain, three replicates were made.

2.3. Arsenic Tolerance Assay

Based on previous results, *P. djamor* PQ and the bacterial strain *P. fluorescens* 1.2 were selected to evaluate their in vitro tolerance to As. Increasing concentrations of the metalloid (supplied as sodium arsenate, Na₂HAsO₄ 7H₂O) were incorporated to PDA or TY culture media. In the case of bacteria, eight concentrations of As (0, 0.2, 0.5, 1, 2, 4, 6, and 8 mM) were evaluated, and four concentrations in the case of the fungus (0, 5, 10, and 20 mM). Metalloid tolerance was determined in bacteria by counting viable microorganisms using the microdroplet technique described by Somasegaran and Hoben [59]. This counting method consists of placing three drops (10 μ L) per dilution in a quarter of a Petri dish containing solid TY medium, then incubating them for 24 h at 26 °C until between 3 and 30 colonies are obtained and the colony-forming units per mL were counted (CFU mL⁻¹). To evaluate fungal viability, mycelial growth was recorded in millimeters starting on the fourth day of growth, at one-day intervals. Growth averages based on the mycelial diameter were calculated for days 6, 8 and 11. Three replicates were performed for each concentration and for each organism.

2.4. Setup of Peanut Growth Assays and Physiological Measurements Under As Exposure

2.4.1. Preparation of Substrates

Six different substrates were used in this study: five were amended with crushed maize stover, and in four of them, the stover had previously been used to cultivate *P. djamor* PQ mushrooms, thus qualifying as spent mushroom substrate (SMS). The material was mechanically chopped using a manual lawnmower, then soaked in water for full hydration and subsequently drained to remove excess moisture. After draining, the required portions were weighed for each experimental unit: 500 g of substrate packed in 1 L heat-resistant polypropylene bags. The substrate formulation was as follows: 53% maize stover, 6% wheat bran, 3% calcium carbonate, and 38% water [60]. Pasteurization was carried out with steam at 80 °C for 8 h and cooled at room temperature. To prepare the amendments from the SMS of PQ, substrates and propagules were prepared following standard protocols [60]. Briefly, grain-based maize substrate was sterilized, inoculated with standardized mycelial disks of *P. djamor* PQ, and incubated until colonization. Three consecutive fruiting trials were conducted at the UAB facilities, and the remaining material was subsequently used to prepare the SMS amendments. Full methodological details are provided in the Supplementary Materials (File S1). Pots used to grow peanut plants for the experiment were filled with 200 g of sand, 10 g of perlite, and 15 g of SMS, except for the control group and one of the amendments. The six substrates were prepared as follows: AP (control): sand + perlite only (no amendment); E: mechanically crushed maize stover (not SMS-derived and without microorganisms); H: SMS supplemented with live mycelium of *P. djamor* PQ obtained from a 3-week Petri culture (inoculum details in Supplementary Materials File S1); B: SMS plus 10 mL of *P. fluorescens* 1.2 inoculum (1×10^8 CFU mL⁻¹) in physiological saline solution; HB: SMS plus a mixed inoculum of *P. djamor* PQ and *P. fluorescens* 1.2 (inoculum ratios in Supplementary Materials File S1); HA: SMS sterilized twice in an autoclave at 121 °C for 20 min (SMS without alive fungus). To contextualize performance, we included a residue-only benchmark (E). However, because E does not contain SMS (the practical target in a circular-economy framework) it serves strictly as a reference and not as a deployable strategy.

2.4.2. Plant Bioassays and Treatment with As

An experiment was conducted to evaluate the effect of six substrates (five amendments and a control) on peanut plants. Runner peanut seeds of the Granoleico variety (from the 'El Carmen' Genetic Improvement Farm, General Cabrera, Córdoba, Argentina) were used, which are widely grown in peanut-producing regions due to their high oleic acid content. The seeds were superficially disinfected according to the adapted protocol of Vincent [61]. Then, they were then placed in Petri dishes on a base of sterile cotton wool and filter paper moistened with 10 mL of distilled water. The dishes were incubated at 28 °C in the dark for 3 days until the radicles reached a length of between 2 and 3 cm. The pre-germinated seeds were transferred to pots with the previously defined substrates and placed in trays. These pots had holes in the bottom through which they absorbed the Hoagland nutrient solution [62]. For the treatments, the plants were watered with a Hoagland solution prepared without nitrogen and, with or without the addition of As, being the only source of N that provided by their microsymbiont *Bradyrhizobium* sp. SEMIA6144 through the biological nitrogen fixation process. Peanut plants were inoculated 3 days after germination with 3 mL of a suspension (1×10^8 CFU mL⁻¹) placed on the root crown to prevent inoculant dispersion. Five days after inoculation with SEMIA6144, half of the plants were treated with 20 µM As arsenate (as Na₂HAsO₄·7H₂O). Throughout the trial, the level of nutrient solution in the trays was maintained constant to ensure a continuous supply. Five replicates were performed per treatment, and all plants were grown in a

growth chamber controlled at 28 °C with a photoperiod of 16 h light/8 h dark. The chosen As concentration is in correspondence to the natural concentration of metalloids found in groundwater in certain regions of Latin America [7].

The plants were collected 30 days after inoculation, at the flowering phenological stage (R1), according to the scale proposed by Boote [63], and subsequently, leaves, roots and nodules were separated. Part of the plant material was oven-dried at 70 °C, while another portion was frozen in liquid nitrogen and stored at −80 °C until processing. In addition, fresh samples were reserved for immediate histochemical analysis after harvest (see timeline in Supplementary Materials Figure S1).

2.4.3. Growth and Photosynthesis

Growth parameters, including shoot and root length, dry biomass, and the number and dry weight of nodules, were determined for peanut plants. All harvested materials were immediately frozen at −80 °C until further analyses. For the photosynthetic variables, the maximum quantum yield of photosystem II ($F_v F_m^{-1}$) was measured in fully expanded second-nodal leaves that had been dark-adapted for at least 30 min, using a Mini-PAM II fluorometer (Heinz Walz GmbH, Effeltrich, Germany). In parallel, chlorophyll a, chlorophyll b, and carotenoid contents were quantified following the method of Vernon [64] with slight modifications. Briefly, 0.05 g of fresh leaf tissue was extracted in 15 mL of 80% (v/v) ethanol and heated at 100 °C for 15 min in a thermostatic water bath. After extraction, absorbance was recorded at 665, 650, and 450 nm, corresponding to the maximum absorption peaks of chlorophyll a, chlorophyll b, and carotenoids, respectively. Pigment concentrations were calculated according to Vernon [64], using the specific absorption coefficients reported by MacKinney [65], and expressed as mg pigment g^{−1} dry weight.

2.4.4. Osmotic Potential

From each substrate and treatment, 0.2 g of fresh leaves were collected and placed in 0.5 mL Eppendorf tubes with perforated sides. Next, 1.5 mL of extraction solution was added to each tube and heated in a water bath at 100 °C for 20 min, then rapidly cooled on ice. The samples were centrifuged at 12,000 × g for 15 min, and 56 µL of the supernatant was transferred to new 0.5 mL tubes to measure the osmotic potential using a OSMOMAT 3000 device (Osmomat 3000, Gonotec®, Logan, UT, USA).

2.4.5. Peanut As Concentration and Translocation

Metalloid concentration was determined in peanut shoots, roots and nodules, by using an inductively coupled plasma mass spectrometry (ICP-MS). For analysis, dried plant tissues (0.1 g for leaves and roots, and 0.05 g for nodules) were subjected to acid digestion using a mixture composed of 0.3 mL HNO₃, 0.5 mL H₂O, and 0.2 mL H₂O₂, following the general procedure of Ortega-Villasante [66] and Sobrino-Plata [67]. Digestions were carried out in sealed vessels under high pressure in an autoclave at 121 °C for 20 min. The resulting solutions were filtered through PVDF membranes and diluted to a final volume of 5 mL with Milli-Q water. Arsenic concentrations were then determined using a NexION 300 (PerkinElmer, Inc., Waltham, MA, USA) and the translocation factor (TF) was calculated according to Singh & Agrawal [68].

2.4.6. Determination of ROS Production

Nicotinamide Adenine Dinucleotide Phosphate (NADPH) oxidase activity was determined spectrophotometrically in peanut roots by Nitroblue tetrazolium (NBT) reduction at 560 nm [69]. The enzyme activity was measured in 1 mL reaction buffer containing: 0.02 mg protein extract, 0.5 mg mL^{−1} NBT, 0.2 mM NADPH, 4 mM CaCl₂ and 0.2 mM MgCl₂. One unit of NADPH oxidase was defined as the quantity of enzyme needed to

reduce 1 mmol NADPH per minute. Protein concentration in plant extracts was determined by Bradford procedure, using bovine serum albumin as standard [70]. For superoxide anion ($O_2^{\cdot -}$) visualization, leaf samples were immersed in a 1 mM nitro blue tetrazolium (NBT) solution prepared in 10 mM sodium citrate buffer (pH 6) and incubated under the same conditions, as described by Frahy and Schopfer [71]. The photographs were used to measure the blue stained leaf areas and pixel quantification using Image-Pro Plus software (version 6.0; Media Cybernetics, Rockville, MD, USA). After staining, tissues were bleached in boiling 96% ethanol to remove chlorophyll. Hydrogen peroxide (H_2O_2) was visually detected incubating freshly leaves segments in 1 mg mL^{-1} 13,3-diaminobenzidine (DAB) for 8 h following Alexieva et al. procedure [72]. Leaves were photographed under a stereoscopic microscope equipped with a digital camera (Stemi SV6, Carl Zeiss, Oberkochen, Baden-Württemberg, Germany). The amount of H_2O_2 was also quantified spectrophotometrically after reaction of roots extracts with potassium iodide (KI) [72]. In brief, fresh leaf tissue was homogenized in 0.5% (*w/v*) trichloroacetic acid (TCA) and centrifuged at $12,000\times g$ for 15 min. For H_2O_2 determination, an aliquot of the supernatant was mixed with 100 mM phosphate buffer (pH 7.0) and 1 M KI. The reaction mixture was incubated in the dark for 1 h, and the absorbance was measured at 390 nm. A standard curve of known H_2O_2 concentrations was used for quantification, and results are expressed as mmol $H_2O_2\text{ g}^{-1}\text{ FW}$.

2.4.7. Oxidative Stress Marker

The level of lipid peroxidation was estimated by determining the concentration of thiobarbituric-reactive substances (TBARs) following the method of Heath and Packer [73]. Approximately 0.1 g of fresh leaf tissue was homogenized in 0.1% (*w/v*) trichloroacetic acid (TCA) and centrifuged at $12,000\times g$ for 15 min. For TBARs determination, 0.5 mL of the resulting supernatant was mixed with 0.5 mL of 0.5% (*w/v*) thiobarbituric acid (TBA) in 20% (*w/v*) TCA. The mixture was incubated at $95\text{ }^\circ\text{C}$ for 25 min and then rapidly cooled in an ice bath. After cooling, the mixture was centrifuged at $8000\times g$ for 6 min. TBARs were quantified by measuring absorbance at 535 nm and correcting for non-specific turbidity by subtracting the absorbance at 600 nm, using a UV-visible spectrophotometer (Spectronic® Genesys 2, Waltham, MA, USA).

2.4.8. Antioxidants Enzymatic Assay

The preparation of extracts for determining superoxide dismutase (SOD, EC 1.15.1.1) and catalase (CAT, EC 1.11.1.6) activities followed the method of Beauchamp and Fridovich [74] and Aebi [75], respectively. Frozen root plant tissue (0.1 g) was homogenized in 50 mM potassium phosphate buffer (pH 7.4) containing 0.5% (*v/v*) Triton X-100, 1 mM Ethylenediaminetetraacetic acid (EDTA), and polyvinylpyrrolidone (PVP) in a pre-cooled mortar. The homogenate was centrifuged at $10,000\times g$ for 15 min at $4\text{ }^\circ\text{C}$, and the supernatant was used for enzymatic activity assays. Protein concentration in the extracts was quantified using Bradford reagent [70]. For SOD assay superoxide anions generated by slightly excited riboflavin reduce nitroblue tetrazolium (NBT), forming a blue formazan product measured at 560 nm. The reaction mixture contained 0.02 mg mL^{-1} of protein extract, $75\text{ }\mu\text{M}$ NBT, $777\text{ }\mu\text{M}$ methionine, $0.54\text{ }\mu\text{M}$ EDTA, and $8\text{ }\mu\text{M}$ riboflavin in final volume of 1 mL. Absorbance was measured at 560 nm, first in the dark and then after 7 min of UV light exposure. One unit of SOD activity was defined as the amount of enzyme required to inhibit in a 50% reduction in NBT. For CAT activity the assay mixture was prepared with 50 mM phosphate buffer (pH 7.4), 12.5 mM H_2O_2 , and $100\text{ }\mu\text{g}$ of protein extract. CAT activity was defined as the amount of enzyme to degrade 1 mmol of H_2O_2 per minute, measured by a decrease in absorbance at 240 nm.

2.4.9. Gene Expression Analysis Using qRT-PCR

Total RNA of about 100 mg of plant root material was extracted using the MaxwellRSC plant RNA kit following the manufacturer's instructions (Promega Corporation, Madison, WI, USA). Two micrograms of total RNA were used as a template to synthesize first-strand cDNA with an iScriptTM cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA). This cDNA was then used as a template for Reverse-Transcriptase quantitative real-time PCR (RT-qPCR) using iTaqTM Universal SYBR Green Supermix (Bio-Rad, Hercules, CA, USA). Real-time detection of fluorescence emission was performed on a CFX384 Real-Time System (Bio-Rad, Hercules, CA, USA), and plates were edited using the CFX manager version 3.1 software. Primers used for peanuts genes transcript quantification are detailed in Supplementary Materials (File S2), and relative quantifications were performed using the housekeeping Ah-ACT2 gene as internal reference. The relative gene expression data was calculated by the $2^{-\Delta\Delta C_t}$ method of Livak and Schmittgen [76].

2.4.10. Statistical Analysis

Experiments were carried out in a completely randomized design in independent experiments. The data were analyzed using the InfoStat software 2020 [77]. Differences among treatments were analyzed using ANOVA, taking $p < 0.05$ as significant according to Duncan test. Before the test of significance, the normality and homogeneity of variance were corroborated using the modified Shapiro–Wilk and Levene tests, respectively.

3. Results

3.1. Bacterial Coexistence and Arsenic Tolerance on Selected Microorganisms

In vitro co-culture assays revealed no visible inhibition zones among strains J49, 1.2, and the fungus *Pleurotus djamor* PQ when grown on the same plate (Supplementary Materials, Figure S2). The mycelial growth of PQ was significantly affected by increasing arsenic concentrations and exposure time (Supplementary Materials, Figure S3). At each time point (6, 8, and 11 days), higher metalloid concentrations resulted in reduced mycelial expansion. Growth under 5 and 10 mM As remained statistically similar on days 6 and 8 but decreased markedly at 20 mM As across all time points. In vitro arsenic tolerance assays with *Pseudomonas fluorescens* strain 1.2 revealed a significant reduction in bacterial growth as the arsenic concentration in the medium increased. Growth inhibition became noticeable at 250 μ M, but the strain was able to tolerate up to 8 mM As (Supplementary Materials, Figure S4). Although compatibility between the fungus and the PGPB strains was confirmed, and previous studies [78] showed that strain J49 tolerated arsenic only up to 4 mM, further experiments were conducted using strain 1.2, which exhibited greater As tolerance and was originally isolated from metal(loid)s contaminated mining soils.

3.2. Response of Peanut Growing on Different Amendments and Exposed to As

3.2.1. Growth and Nodulation

At the R1 developmental stage (40 days), morphological observations revealed reduced shoot development in As-treated plants, particularly under the AP condition (no amendment) (Figure 1). Shoot dry weight (SDW) significantly decreased under As exposure in the AP, H (fungus), and HB (fungus + bacterium) treatments compared to their respective controls (Table 1). Among the As-treated plants, HA (autoclaved substrate) and AP exhibited the lowest SDW values. In contrast, the E (no microbes) and B (bacterium only) treatments showed significantly higher SDW than HA and AP under the same conditions. Root dry weight (RDW) did not show statistically significant differences between control and As-treated plants across all growth conditions. In the absence of As, the highest root biomass was observed in the AP and HB treatments. Under As treatment, however,

the B treatment resulted in significantly higher RDW compared to HA. Shoot length was significantly reduced by As in all growth conditions compared to the respective controls. Under metalloid exposure, no major differences were observed among growth conditions; however, HA and HB showed significantly higher shoot length than B and AP. Root length was significantly reduced by As only in the H condition compared to its control. In the absence of the metalloid, H and B exhibited significantly longer roots than HA, while no significant differences were observed among treatments under As exposure. As a general indicator, when considering total dry weight (DW), arsenic induced changes of -40% in AP, -19% in H, -29% in HB, -10% in B, $+4\%$ in E, and -1.5% in HA relative to their respective controls. Under As conditions, E and B exhibited $+55\%$ and $+49.6\%$ higher total biomass than AP, respectively.

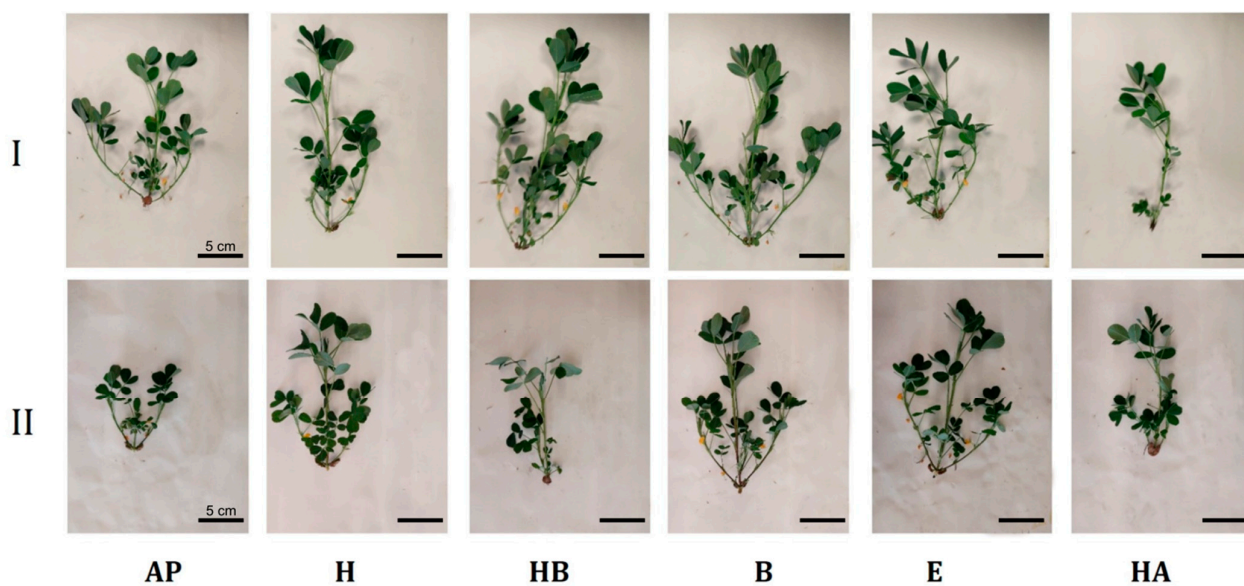


Figure 1. Morphology of peanut shoot under different treatments. (I) Plants grown under control conditions. (II) Plants exposed to $20\ \mu\text{M}$ arsenic. Abbreviations: AP: no amendment (sand:perlite); H: spent growth substrate (SMS) with live *Pleurotus djamor* mycelium; HB: SMS with plant growth promoting bacteria (PGPB) and live mycelium; B: SMS with PGPB only; E: maize stover amendment (no microbial inoculants); HA: SMS with autoclaved mycelium.

Regarding nodulation parameters, arsenic significantly reduced nodule number in all growth conditions, especially in AP (-67%), H (-39%), and B (-42%), compared to their respective controls (Table 2). In the absence of As, AP and E showed significantly higher nodules number than HB, whereas in the presence of As, AP was the most severely affected condition, with significantly fewer nodules than B, E, and HA. Nodule dry weight was also significantly decreased by As, particularly in AP (-69%), H (-41%), and B (-41%), in line with the observed reductions in nodule number. Under As treatment, the E condition exhibited the highest nodule dry weight compared to the other evaluated treatments. Consequently, the percentage of effective nodules (i.e., those showing such coloration per plant) remained consistent ($\sim 80\%$) between control and As treatments across all growth conditions. Nitrogen content was most affected in the AP treatment (without amendment), showing a 16% reduction. In contrast, in the H, HB, B, E, and HA conditions, nitrogen levels remained comparable to their respective controls.

Table 1. Role of different soil amendments on the effect of arsenic (As) on peanut plant growth.

Growth Conditions	Shoot Length (cm)			Root Length (cm)			Shoot Dry Weight (g)			Root Dry Weight (g)			% Change in Total (DW)	
	0 μ M As	20 μ M As	%	0 μ M As	20 μ M As	%	0 μ M As	20 μ M As	%	0 μ M As	20 μ M As	%		
AP	20.90 $\pm$ 0.95 ^{a1}	16.17 $\pm$ 0.73 ^{b2}	↓23	17.60 $\pm$ 1.66 ^{ab1}	14.50 $\pm$ 0.65 ^{a1}	↓18	2.01 $\pm$ 0.18 ^{a1}	1.16 $\pm$ 0.23 ^{c2}	↓42	0.28 $\pm$ 0.03 ^{a1}	0.21 $\pm$ 0.02 ^{ab1}	↓25	↓40	
H	23.90 $\pm$ 1.35 ^{a1}	17.97 $\pm$ 0.60 ^{ab2}	↓24	19.00 $\pm$ 1.15 ^{a1}	15.30 $\pm$ 1.09 ^{a2}	↓20	1.81 $\pm$ 0.13 ^{a1}	1.42 $\pm$ 0.10 ^{bc2}	↓21	0.26 $\pm$ 0.02 ^{ab1}	0.25 $\pm$ 0.03 ^{ab1}	↓4	↓19	
HB	21.33 $\pm$ 1.50 ^{a1}	16.25 $\pm$ 0.75 ^{a2}	↓24	16.00 $\pm$ 0.95 ^{ab1}	14.17 $\pm$ 0.95 ^{a1}	↓11	1.93 $\pm$ 0.25 ^{a1}	1.36 $\pm$ 0.07 ^{bc2}	↓29	0.31 $\pm$ 0.02 ^{a1}	0.22 $\pm$ 0.03 ^{ab1}	↓29	↓29	
B	23.13 $\pm$ 1.23 ^{a1}	16.73 $\pm$ 0.59 ^{b2}	↓28	19.20 $\pm$ 1.28 ^{a1}	16.00 $\pm$ 0.71 ^{a1}	↓17	2.04 $\pm$ 0.12 ^{a1}	1.76 $\pm$ 0.12 ^{ab1}	↓14	0.25 $\pm$ 0.03 ^{ab1}	0.29 $\pm$ 0.05 ^{a1}	↑16	↓10	
E	26.17 $\pm$ 0.44 ^{a1}	17.63 $\pm$ 0.24 ^{ab2}	↓36	16.50 $\pm$ 0.29 ^{ab1}	16.00 $\pm$ 0.98 ^{a1}	↓3	1.86 $\pm$ 0.07 ^{a1}	1.88 $\pm$ 0.13 ^{a1}	↑2	0.19 $\pm$ 0.004 ^{bc1}	0.25 $\pm$ 0.03 ^{ab1}	↓31	↑4	
HA	23.60 $\pm$ 1.29 ^{a1}	19.13 $\pm$ 0.85 ^{a2}	↓19	14.80 $\pm$ 0.75 ^{b1}	14.75 $\pm$ 0.95 ^{a1}	↓0.3	1.27 $\pm$ 0.11 ^{b1}	1.25 $\pm$ 0.13 ^{c1}	↓2	0.16 $\pm$ 0.02 ^{c1}	0.16 $\pm$ 0.01 ^{b1}	Ø	↓1.5	

Data are presented as mean $\pm$ SE ($n = 5$). Different letters indicate statistically significant differences among growth conditions within the same treatment. Different numbers indicate statistically significant differences between treatments within the same growth condition ($p < 0.05$, according to Duncan's test). Symbols: ↓ = reduction; ↑ = increase; Ø = no significant change.

Table 2. Evaluation of nodulation response and nitrogen content in peanut plants.

Growth Conditions	Nodules Numbers			Nodules Dry Weight (g Plant)			N Content (mg g ⁻¹ DW)		
	0 μ M As	20 μ M As	%	0 μ M As	20 μ M As	%	0 μ M As	20 μ M As	%
AP	55.50 $\pm$ 2.99 ^{a1}	18.50 $\pm$ 2.78 ^{c2}	↓67	0.081 $\pm$ 0.003 ^{a1}	0.025 $\pm$ 0.003 ^{b2}	↓69	13.56 $\pm$ 0.21 ^{b1}	11.38 $\pm$ 0.003 ^{b2}	↓16
H	38.40 $\pm$ 3.11 ^{bc1}	23.50 $\pm$ 2.51 ^{bc2}	↓39	0.064 $\pm$ 0.005 ^{ab1}	0.038 $\pm$ 0.005 ^{b2}	↓41	14.42 $\pm$ 1.56 ^{ab1}	14.19 $\pm$ 1.30 ^{a1}	↓2
HB	26.00 $\pm$ 2.48 ^{c2}	22.50 $\pm$ 3.73 ^{bc1}	↓13	0.046 $\pm$ 0.011 ^{b1}	0.041 $\pm$ 0.004 ^{b1}	↓11	14.40 $\pm$ 0.85 ^{ab1}	13.50 $\pm$ 0.50 ^{a1}	↓6
B	55.60 $\pm$ 5.74 ^{bc1}	32.25 $\pm$ 1.18 ^{ab2}	↓42	0.064 $\pm$ 0.005 ^{ab1}	0.038 $\pm$ 0.006 ^{b2}	↓41	15.94 $\pm$ 1.64 ^{ab1}	14.94 $\pm$ 0.67 ^{a1}	↓6
E	35.00 $\pm$ 3.79 ^{ab1}	32.00 $\pm$ 5.03 ^{ab1}	↓9	0.047 $\pm$ 0.012 ^{b1}	0.061 $\pm$ 0.007 ^{a1}	↑30	17.19 $\pm$ 0.68 ^{a1}	16.50 $\pm$ 0.76 ^{a1}	↓4
HA	40.60 $\pm$ 2.48 ^{b1}	38.67 $\pm$ 5.78 ^{a1}	↓5	0.035 $\pm$ 0.009 ^{b1}	0.042 $\pm$ 0.004 ^{b1}	↑20	15.75 $\pm$ 0.37 ^{ab1}	15.50 $\pm$ 1.06 ^{a1}	↓2

Data are presented as mean $\pm$ SE ($n = 5$). Different letters indicate statistically significant differences among growth conditions within the same treatment. Different numbers indicate statistically significant differences between treatments within the same growth condition ($p < 0.05$, according to Duncan's test). Symbols: ↓ = reduction; ↑ = increase.

3.2.2. Photosynthetic Efficiency and Photosynthetic Pigment Levels

Table 3 shows the photosynthetic efficiency and Table 4 the levels of chlorophyll a, chlorophyll b, total chlorophyll, and carotenoids in peanut plants grown under different amendments and exposed to As. Regarding the maximum efficiency of PSII ($F_v F_m^{-1}$), no significant differences were detected between control and As treatments within each growth condition. Under control conditions, E displayed significantly higher $F_v F_m^{-1}$ values compared to H, HB, and HA. In the presence of As, E maintained the highest values, with no significant difference compared to B. Both chlorophyll a and chlorophyll b exhibited a similar response pattern. A significant decrease in both pigments was observed under H and B conditions compared to their respective controls, whereas AP, HB, E, and HA showed no significant differences between control and As treatments. In the absence of As, H and B exhibited significantly higher chlorophyll a and b levels than AP, HB, E, and HA. In contrast, under As exposure, AP and E reached the highest values of both pigments, surpassing H and HB. For total chlorophyll, the response was dependent on the growth condition. A significant reduction was detected in H and B under As stress compared to their controls. In the control treatment, H and B displayed significantly higher levels than AP, HB, E, and HA. Under As exposure, however, AP and E showed the highest total chlorophyll values, significantly exceeding H and HB. Similarly, carotenoid content exhibited a condition-dependent response. Arsenic exposure caused a significant decrease in H and B compared to their controls. In the absence of As, H and B had significantly higher carotenoid levels than AP, HB, E, and HA. Under As treatment, AP and E displayed the highest carotenoid contents, surpassing H and HB.

Table 3. Photosynthetic efficiency of peanut plants.

Growth Condition	Photosynthetic Efficiency ($F_v F_m^{-1}$)	
	0 μM As	20 μM As
AP	0.779 $\pm$ 0.018 ^{ab1}	0.780 $\pm$ 0.025 ^{abc1}
H	0.755 $\pm$ 0.021 ^{b1}	0.732 $\pm$ 0.019 ^{c1}
HB	0.745 $\pm$ 0.023 ^{b1}	0.756 $\pm$ 0.023 ^{bc1}
B	0.786 $\pm$ 0.011 ^{ab1}	0.795 $\pm$ 0.006 ^{ab1}
E	0.825 $\pm$ 0.011 ^{a1}	0.816 $\pm$ 0.004 ^{a1}
HA	0.767 $\pm$ 0.026 ^{b1}	0.740 $\pm$ 0.016 ^{c1}

Data are presented as mean $\pm$ SE ($n = 5$). Different letters indicate statistically significant differences among growth conditions within the same treatment. Different numbers indicate statistically significant differences between treatments within the same growth condition ($p < 0.05$, according to Duncan's test).

Table 4. Photosynthetic pigment content in peanut plants exposed to arsenic (As) and grown under different amendments.

Growth Condition	Pigment Content (mg g^{-1} DW)											
	Chlorophyll a			Chlorophyll b			Total Chlorophyll			Carotenoids		
	0 μM As	20 μM As	%	0 μM As	20 μM As	%	0 μM As	20 μM As	%	0 μM As	20 μM As	%
AP	3.29 $\pm$ 0.35 ^{b1}	4.51 $\pm$ 0.85 ^{a1}	$\uparrow$ 37	2.12 $\pm$ 0.17 ^{b1}	2.56 $\pm$ 0.37 ^{a1}	$\uparrow$ 20	5.42 $\pm$ 0.52 ^{b1}	7.07 $\pm$ 1.22 ^{a1}	$\uparrow$ 30	0.73 E2 $\pm$ 0.07 ^{b1}	0.97 E2 $\pm$ 0.17 ^{a1}	$\uparrow$ 33
H	5.10 $\pm$ 0.54 ^{a1}	2.88 $\pm$ 0.26 ^{b2}	$\downarrow$ 43	3.30 $\pm$ 0.29 ^{a1}	2.04 $\pm$ 0.16 ^{ab2}	$\downarrow$ 38	8.39 $\pm$ 0.81 ^{a1}	4.92 $\pm$ 0.41 ^{b2}	$\downarrow$ 41	1.06 E2 $\pm$ 0.12 ^{a1}	0.61 E2 $\pm$ 0.05 ^{c2}	$\downarrow$ 42
HB	2.95 $\pm$ 0.40 ^{b1}	2.55 $\pm$ 0.33 ^{b1}	$\downarrow$ 13	2.11 $\pm$ 0.24 ^{b1}	1.78 $\pm$ 0.19 ^{b1}	$\downarrow$ 15	5.06 $\pm$ 0.63 ^{b1}	4.33 $\pm$ 0.52 ^{b1}	$\downarrow$ 14	0.65 E2 $\pm$ 0.09 ^{b1}	0.54 E2 $\pm$ 0.07 ^{c1}	$\downarrow$ 17
B	5.14 $\pm$ 0.32 ^{a1}	3.17 $\pm$ 0.36 ^{ab2}	$\downarrow$ 38	3.12 $\pm$ 0.18 ^{a1}	2.07 $\pm$ 0.23 ^{ab2}	$\downarrow$ 33	8.26 $\pm$ 0.43 ^{a1}	5.24 $\pm$ 0.58 ^{ab2}	$\downarrow$ 36	1.05 E2 $\pm$ 0.06 ^{a1}	0.69 E2 $\pm$ 0.69 ^{bc2}	$\downarrow$ 34
E	3.50 $\pm$ 0.19 ^{b1}	4.54 $\pm$ 0.23 ^{a1}	$\uparrow$ 29	2.35 $\pm$ 0.08 ^{b1}	2.42 $\pm$ 0.28 ^{a1}	$\uparrow$ 2	5.84 $\pm$ 0.84 ^{b1}	7.13 $\pm$ 0.50 ^{a1}	$\uparrow$ 22	0.72 E2 $\pm$ 0.11 ^{b1}	0.93 E2 $\pm$ 0.07 ^{ab1}	$\uparrow$ 29
HA	3.18 $\pm$ 0.90 ^{b1}	3.48 $\pm$ 0.90 ^{ab1}	$\uparrow$ 9	2.18 $\pm$ 0.95 ^{b1}	3.18 $\pm$ 0.95 ^{ab1}	$\uparrow$ 45	5.36 $\pm$ 0.27 ^{b1}	5.89 $\pm$ 0.51 ^{ab1}	$\uparrow$ 9	0.68 E2 $\pm$ 0.04 ^{b1}	0.72 E2 $\pm$ 0.05 ^{abc1}	$\uparrow$ 6

Data are presented as mean $\pm$ SE ($n = 5$). Different letters indicate statistically significant differences among growth conditions within the same treatment. Different numbers indicate statistically significant differences between treatments within the same growth condition ($p < 0.05$, according to Duncan's test). Symbols: $\downarrow$ = reduction; $\uparrow$ = increase.

3.2.3. Impact of Osmotic Potential on Peanut Exposed to As Growing Under Different Amendments

The osmotic potential (Ψ s) showed differences associated with the growth condition (Table 5). Under control conditions, AP exhibited the most negative value compared to all other amendments, while in the presence of As no significant differences were detected among growth conditions. However, when compared to their respective controls, As exposure caused a significant reduction only in AP.

Table 5. Osmotic potential in peanut plants exposed to arsenic (As).

Growth Condition	Osmotic Potential (Ψ s)		
	0 μ M As	20 μ M As	%
AP	-0.800 ± 0.022 ^{c1}	-0.698 ± 0.017 ^{a2}	↓12.8
H	-0.665 ± 0.041 ^{bc1}	-0.533 ± 0.053 ^{a1}	↓19.8
HB	-0.574 ± 0.047 ^{ab1}	-0.647 ± 0.051 ^{a1}	↑12.7
B	-0.545 ± 0.061 ^{ab1}	-0.639 ± 0.049 ^{a1}	↑17.2
E	-0.516 ± 0.067 ^{ab1}	-0.627 ± 0.046 ^{a1}	↑21.5
HA	-0.477 ± 0.060 ^{a1}	-0.621 ± 0.055 ^{a1}	↑30.1

Data are presented as mean $\pm$ SE ($n = 5$). Different letters indicate statistically significant differences among growth conditions within the same treatment. Different numbers indicate statistically significant differences between treatments within the same growth condition ($p < 0.05$, according to Duncan's test). Symbols: ↓ = reduction; ↑ = increase.

3.2.4. Arsenic and Translocation Factor of Peanut Plants Growing Under Different Amendments

Table 6 summarizes metalloid accumulation in each plant organ and its translocation. Overall, the distribution followed a similar pattern across treatments, with roots $\geq$ nodules $\gg$ leaves; the only exceptions were E and HA, where nodule concentrations were comparable to or slightly higher than those in roots. Leaves and roots of plants grown under AP contained significantly more As than all other conditions. For nodules, AP and HA formed the highest statistical group. As was not detected in leaves of plants grown with the E and HA amendments. The translocation factor (TF) was highest in AP, H, and B, was significantly reduced in HB, and was zero in E and HA. Because a lower TF limits the movement of As from roots to shoots, and by extension to the grain, E and HA exhibited the strongest restriction capacity, followed by HB; in contrast, AP, H, and B allowed greater mobilization of the metalloid to aerial tissues.

Table 6. Arsenic content in peanut plants.

Growth Condition	Shoot	Root	Nodules	Translocation Factor
		$\mu\text{g g}^{-1}$ DW		
AP	13.41 ± 1.21 ^a	143.12 ± 1.92 ^a	127.42 ± 3.01 ^a	0.09 ± 0.01 ^a
H	7.24 ± 1.81 ^{bc}	103.14 ± 9.99 ^{cd}	44.11 ± 2.62 ^c	0.07 ± 0.02 ^a
HB	4.65 ± 0.55 ^c	130.62 ± 2.95 ^{ab}	60.29 ± 0.62 ^b	0.04 ± 0.01 ^b
B	8.73 ± 1.59 ^b	121.81 ± 2.57 ^{bc}	56.65 ± 0.95 ^b	0.07 ± 0.01 ^a
E	ND	55.69 ± 9.45 ^e	59.62 ± 6.24 ^b	0 ^c
HA	ND	98.3 ± 4.32 ^d	127.53 ± 2.64 ^a	0 ^c

Data are presented as mean $\pm$ EE ($n = 3$). Different letters indicate statistically significant differences among growth conditions within the same organ ($p < 0.05$, according to Duncan's test). Abbreviation: ND: not detected. Growth conditions: AP, no amendment; H, fungus; HB, fungus + bacterium; B, bacterium; E, no microbes; HA, autoclaved substrate.

3.2.5. Markers of ROS Production and Oxidative Damage

In the previous sections, the effects of As on morphophysiological variables of plants grown under control and treated conditions were addressed using absolute values to describe overall responses. From this point onward, the analysis focuses on the root tissue, the

first barrier in contact with the metalloid and the site where early oxidative-stress responses are triggered. Nevertheless, complementary histochemical observations in leaves are included: NBT for $O_2^{\cdot-}$ and DAB for H_2O_2 , to contextualize organ-specific ROS distribution and to contrast the systemic redox signal with the changes detected in roots. Methodologically, results are presented relatively, expressing each value measured under metalloid as the ratio to the corresponding control mean. This approach (i) normalizes biochemical responses with different scales, (ii) reduces between-experiment variability (particularly for antioxidant enzymes and redox metabolites) and (iii) highlights the magnitude of the stress effect, facilitating the interpretation of response/tolerance mechanisms.

Activity of NAPH Oxidase in Peanut Roots

Root NADPH oxidase activity showed statistically significant differences among growth conditions (Figure 2). AP exhibited the highest activity, significantly greater than HB, E, and HA. In these three conditions, the As-induced activation of NADPH oxidase was markedly attenuated compared with AP, suggesting a modulatory effect on the generation of superoxide anion, the principal ROS produced by this enzyme. H and B displayed intermediate values that did not differ statistically from either AP or the lower group, indicating a less pronounced, though not fully suppressed, response.

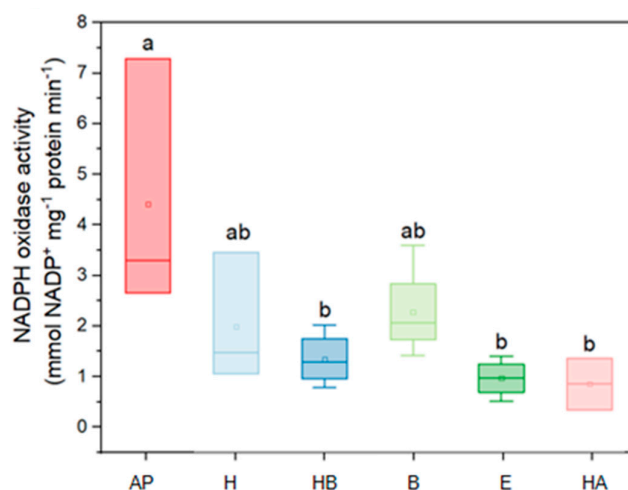


Figure 2. Relative values of peanut root NADPH oxidase activity, calculated as the ratio of enzyme activity under arsenic (As, 20 μ M) to the corresponding control. Different letters indicate statistically significant differences among treatments ($p < 0.05$), according to Duncan's multiple range test ($n = 3$). Growth conditions: AP, no amendment; H, fungus; HB, fungus + bacterium; B, bacterium; E, no microbes; HA, autoclaved substrate.

In Situ Histochemical Detection of Superoxide Anion ($O_2^{\cdot-}$) in Peanut Leaves

The histochemical detection of $O_2^{\cdot-}$ revealed that As exposure increased leaf staining relative to the control, evidenced by the appearance of blue puncta due to the accumulation of formazan (blue) following NBT reduction (Figure 3). This increase was most pronounced under the AP condition, where intense dark staining was distributed across the entire leaf blade. In the other conditions, staining was present but less intense, suggesting that superoxide production in response to As is differentially modulated depending on the growth context (see Supplementary Materials Figure S5 for Visualization and quantification of blue-stained leaf areas under arsenic stress).

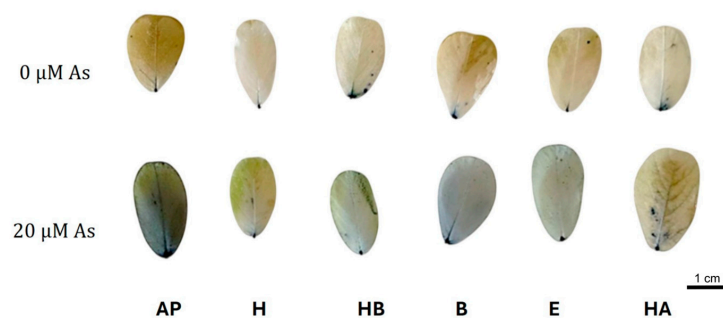


Figure 3. In situ histochemical detection of superoxide anion ($O_2^{\bullet-}$) in peanut leaves by NBT staining, in the absence and presence of arsenic (As, 20 μ M). The intensity of blue formazan staining indicates relative $O_2^{\bullet-}$ accumulation resulting from NBT reduction. Growth conditions: AP, no amendment; H, fungus; HB, fungus + bacterium; B, bacterium; E, no microbes; HA, autoclaved substrate.

In Situ Histochemical Detection (Leaves) and Quantification (Roots) of Hydrogen Peroxide (H_2O_2) in Peanut

DAB staining revealed that, in the absence of As, leaves from all conditions showed low H_2O_2 with faint, localized staining (Figure 4I). In the presence of 20 μ M As, H_2O_2 brown spot accumulation increased, particularly in AP, which displayed intense, widespread brown deposits. In amended plants (H, HB, B, E, HA), staining was less marked; HB and HA showed the faintest, localized signal, H and E exhibited a visible increase but lower than AP, and B was intermediate. These observations indicate that amendments generally modulate As-induced H_2O_2 accumulation in leaves, with the fungus + bacterium combination (HB) showing the strongest attenuation. Root H_2O_2 content (Figure 4II) differed significantly among growth conditions. AP exhibited the lowest values, whereas H and E showed the highest concentrations. HB also displayed low values, not differing from AP. B and HA were intermediate and did not differ significantly from either the highest or the lowest group. Overall, H_2O_2 accumulation in roots was condition-dependent, with marked attenuation in AP and HB, and stronger induction in H and E under As exposure.

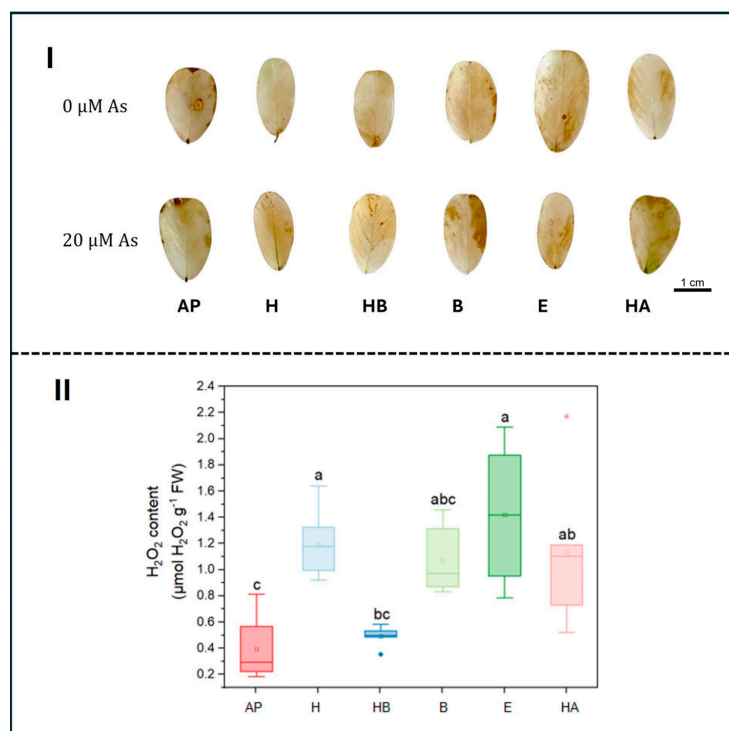


Figure 4. In situ histochemical detection and quantitative determination of hydrogen peroxide (H_2O_2) in peanut. Panel (I). Leaves stained with DAB under control (0 μ M As) and arsenic (As, 20 μ M) conditions;

the brown polymerized DAB precipitate indicates relative H_2O_2 accumulation. Panel (II). Root H_2O_2 levels expressed as relative values. Different letters indicate statistically significant differences among treatments ($p < 0.05$), according to Duncan's multiple range test ($n = 5$). Growth conditions: AP, no amendment; H, fungus; HB, fungus + bacterium; B, bacterium; E, no microbes; HA, autoclaved substrate.

Oxidative Damage of Peanut Roots

Figure 5 shows the relative membrane lipid peroxidation (measured as thiobarbituric acid–reactive substances, TBARs) in peanut roots grown under different amendments and exposed to As. AP displayed the highest values. Amendment E showed elevated levels that were not statistically different from AP but were significantly higher than H and HB. The latter two conditions exhibited the lowest TBARs, indicating a marked attenuation of oxidative damage relative to AP. B and HA were intermediate, lower than AP and not significantly different from either E or the low group (H/HB).

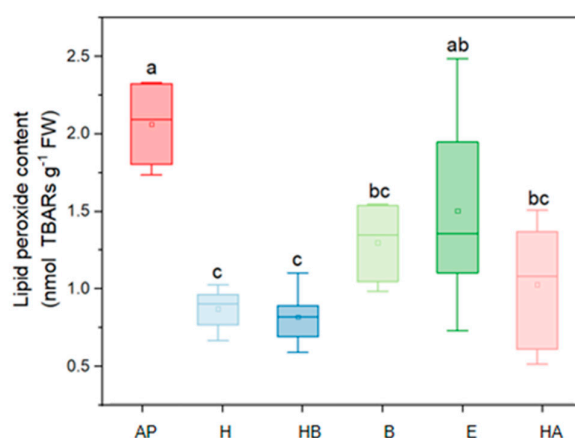


Figure 5. Relative membrane lipid peroxidation in peanut roots under arsenic (As, 20 μ M) across growth conditions. Lipid peroxide content was measured as TBARs and expressed as nmol TBARs g^{-1} fresh weight (FW). Different letters indicate statistically significant differences among treatments ($p < 0.05$), according to Duncan's test ($n = 5$). Growth conditions: AP, no amendment; H, fungus; HB, fungus + bacterium; B, bacterium; E, no microbes; HA, autoclaved substrate.

3.2.6. Specific Activity of Antioxidant Enzymes

Both SOD and CAT activities differed significantly among growth conditions (Figure 6). For SOD, B exhibited the highest activity, significantly exceeding all other treatments. AP, H, and HB showed intermediate values with no differences among them, HA was significantly lower, and E displayed the lowest SOD activity. For CAT, E and H showed significantly higher activities than the AP/HB group, whereas B and HA were intermediate and did not differ clearly from either extreme. Thus, growth conditions elicited opposite trends for these two antioxidant enzymes: SOD was primarily enhanced in B and depressed in E, whereas CAT was maximized in E and H and minimized in AP and HB.

3.2.7. Differential Expressions of Genes Involved in Arsenic Homeostasis Across Growth Conditions

Figure 7 shows the relative gene expression in peanut roots exposed to As, revealing distinct patterns among the three conditions analyzed (AP, H, and E). *ABCC1* expression was highest in AP and significantly lower in H and E, which did not differ from each other. *ACR3* showed no detectable changes among treatments. For *CAD1*, H exhibited the highest expression, E was intermediate, and AP the lowest. *GR* expression remained stable, with no differences among conditions. Finally, *PCS2* reached its maximum expression in H, the

lowest in AP, and an intermediate level in E. Altogether, these data suggest preferential use of the vacuolar sequestration route in AP (high *ABCC1*) but with limited phytochelatin synthesis capacity (low *PCS2*), whereas H strongly activates the phytochelatin-based chelation pathway (*PCS2*↑, *CAD1*↑). E shows an intermediate response, consistent with the previously observed physiological phenotypes.

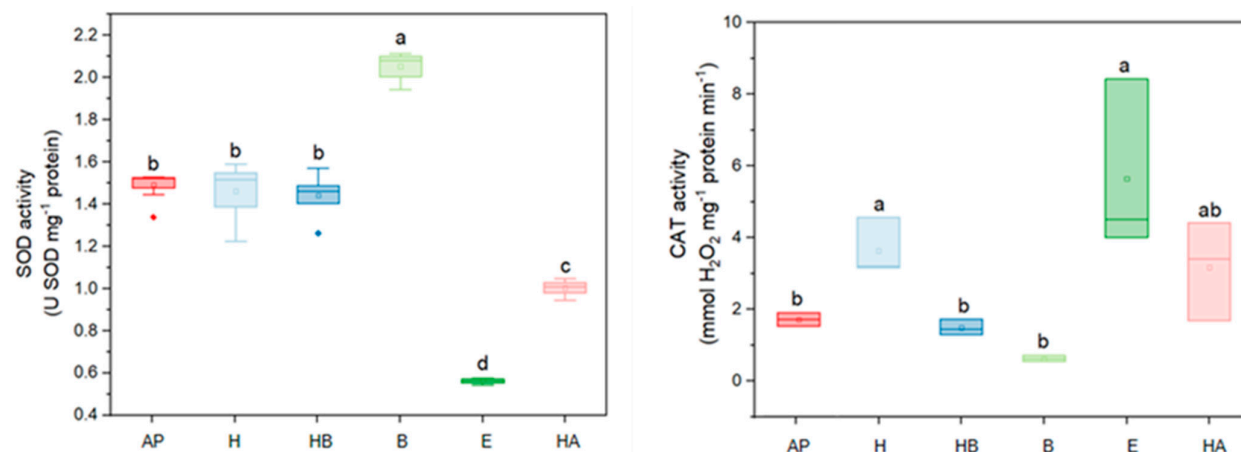


Figure 6. Relative activities of superoxide dismutase (SOD) and catalase (CAT) on peanut roots under arsenic (As, 20 μ M). Activities were measured in peanut roots. Different letters indicate statistically significant differences among treatments ($p < 0.05$), according to Duncan's test ($n = 5$). Growth conditions: AP, no amendment; H, fungus; HB, fungus + bacterium; B, bacterium; E, no microbes; HA, autoclaved substrate.

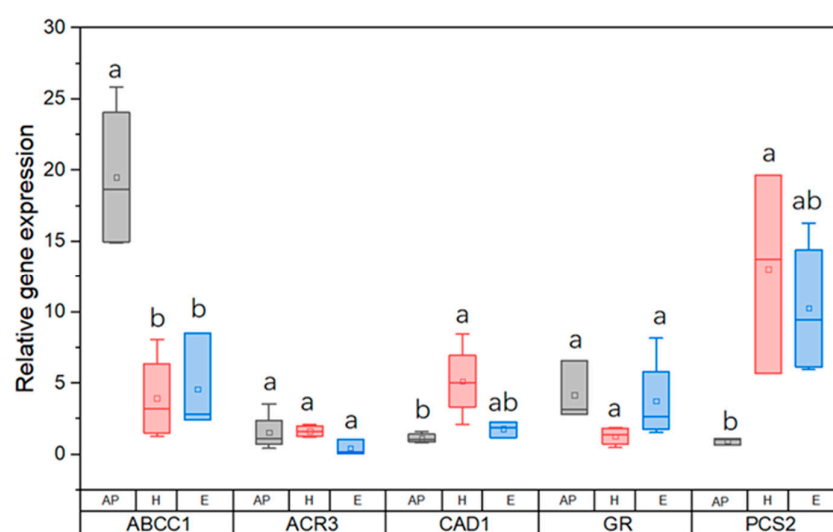


Figure 7. Relative expression of arsenic homeostasis-related genes on peanut. Values are normalized to the ACT reference gene and expressed as fold change (As, 20 μ M/Control) for each gene and growth condition. Gene expression was measured in roots. Genes analyzed: *ABCC1* (vacuolar ABC transporter), *ACR3* (arsenite efflux transporter), *CAD1* (phytochelatin pathway), *GR* (glutathione reductase), and *PCS2* (phytochelatin synthase 2). Growth conditions: AP (no amendment; control), H, and E. Different letters indicate statistically significant differences among treatments ($p < 0.05$), according to Duncan's test ($n = 7$).

4. Discussion

4.1. Effect of As on the Growth of *Pleurotus djamor* and *Pseudomonas fluorescens* 1.2

The ability of *Pleurotus* spp. to grow in contaminated environments has already been reported for several heavy metals, including Pb(II) [55], and its tolerance has been described

as generally superior to that of bacteria exposed to the same contaminants [79]. In our assays, the isolate *Pleurotus djamor* PQ exhibited high tolerance to As, with growth inhibition only becoming evident at 5 mM and a marked reduction at 20 mM (1500 mg L⁻¹). These thresholds are comparable to those reported for *P. pulmonarius*, where growth was slightly reduced at 80 mg L⁻¹ and severely inhibited at 1280 mg L⁻¹ [80]. To our knowledge, no previous studies have specifically assessed in vitro As tolerance in *P. djamor*, highlighting the novelty of our findings.

For *Pseudomonas fluorescens* 1.2, significant growth inhibition was observed from 250 µM As, with survival maintained up to 8 mM and complete inhibition at 10 mM. This confirms a high but lower tolerance than the fungus, consistent with prior reports showing that arsenic exerts a dose-dependent toxic effect on PGPB growth and plant growth-promoting traits [81]. These authors demonstrated that As exposure progressively impairs both symbiotic and free-living PGPB, reducing adhesion, siderophore production, and IAA synthesis, but with strain-dependent resilience. Importantly, compatibility assays showed no antagonism between *P. djamor* PQ and the PGPB strain, supporting the feasibility of their co-inoculation in agricultural systems. An adequate microbial partnership can even enhance the colonization success of PGPB, as demonstrated for *Pseudomonas striata* in combination with the fungus *Piriformospora indica* in maize [82].

4.2. Role of Amendments in Peanut Growth Under As

The toxic effect of As on plant growth has been widely documented [10,41,83–87]. In legumes, biomass and nodulation responses to As are strongly strain-dependent, as demonstrated in peanut (*Bradyrhizobium* sp. SEMIA6144 vs. C-145) and soybean [41,86], as well as in maize-*Azospirillum* interactions [19]. In our study, biomass responses were clearly amendment dependent. The unamended condition (AP) showed the greatest reduction (−40%), confirming high sensitivity without mitigation. Treatments with fungal inoculum (H, HB) showed intermediate reductions, while the bacterial amendment (B) maintained biomass near control levels (without As), and the residue-only amendment (E) even displayed a slight increase relative to control. Autoclaved SMS (HA) produced minimal change, though absolute values remained low.

The performance of B and E is consistent with previous reports where fungal spent substrate or PGPB amendments supported plant growth under metal stress through improved substrate structure, water retention, and beneficial microbiota [88–90]. Likewise, PGPB inoculation has been shown to maintain photosynthesis and biomass in halophytes and legumes exposed to heavy metals [91].

Nodulation was markedly reduced by As in AP, H, and B, while E and HA preserved higher nodule dry weight under stress. This aligns with reports showing that As impairs nodule initiation and infection thread development [92], yet established nodules remain functionally active [93].

4.3. Photosynthetic Pigments and Efficiency

Chlorophylls and carotenoids are early targets of As toxicity due to redox imbalance [10,85,86]. While many studies reported decreases under As exposure [94,95], variable responses also exist [96,97]. In our assays, Fv Fm⁻¹ remained stable across treatments, but pigment responses varied: E and AP retained or increased chlorophyll and carotenoids under As, consistent with either photoprotective compensation (AP) or restricted foliar As accumulation (E). In contrast, H and B showed reductions, though B maintained PSII efficiency, suggesting activation of non-photochemical quenching. Similar compensation has been observed in PGPB- or mycorrhiza-associated plants under metal stress [88,98].

4.4. Osmotic Status

The osmotic potential (Ψ_s) was most negatively affected in AP, with As exposure exacerbating the reduction, whereas other amendments maintained stability. This indicates activation of osmotic adjustment mechanisms via SMS and microbial interactions, consistent with prior evidence that PGPB and rhizobia enhance compatible solute accumulation and water balance under metal stress [90,91]. A previous study reported negative osmotic effects from SMS applications in certain turfgrass species, effects not observed in our work. Those authors found reduced grass germination when plants were treated with SMS extracts, like controls exposed to polyethylene glycol with equivalent osmotic potentials [99].

4.5. Arsenic Accumulation and Translocation

Across treatments, As was predominantly retained in roots > nodules $\gg$ shoots, as reported for non-hyperaccumulating legumes [86,100–102]. All amendments reduced foliar As compared with AP, with undetectable levels in E and HA (TF = 0). This is agronomically relevant since it minimizes contamination risk in edible tissues. Previous reports with SMS-based biochar also described reduced heavy metal absorption and translocation in oilseed rape and maize [48,103]. Mechanistically, As added as arsenate (As V) can be reduced to arsenite (As III), sequestered by phytochelatins, and compartmentalized in vacuoles, limiting translocation [104,105].

While the SMS matrix likely contributed to lowering As availability through its ability to bind and immobilize arsenic, the differential responses among treatments indicate that this physicochemical effect alone cannot explain the magnitude of As reduction. The presence of living inoculants (*Pleurotus djamor* and PGPB) further modulated As dynamics by altering the rhizosphere microenvironment and activating plant defense mechanisms. Both microorganisms can modify pH, redox conditions, and As speciation through the release of organic acids, siderophores, and extracellular polymers [106,107], thereby reinforcing As immobilization within the substrate. In parallel, their interaction with roots enhanced antioxidant activity and ion homeostasis, reducing As translocation and oxidative injury. Consequently, the combined HB treatment achieved the strongest mitigation effect, reflecting the complementarity between the physicochemical retention provided by the SMS and the biological regulation driven by the fungal–bacterial consortium.

4.6. ROS Production and Oxidative Damage

As exposure strongly activated NADPH oxidase in AP roots and induced $O_2^{\cdot-}$ and H_2O_2 accumulation in leaves, consistent with systemic redox signaling [18,40,86,108,109]. Among the SMS-based treatments, HB clearly attenuated ROS production, while H showed elevated H_2O_2 levels consistent with a controlled redox adjustment rather than oxidative damage. These patterns indicate that the amendments modulated ROS generation and signaling to different extents, rather than uniformly reducing ROS levels. TBARs confirmed oxidative membrane damage was highest in AP and E, and lowest in H and HB, consistent with the protective role of SMS- and microbe-based amendments. The combined HB treatment showed intermediate yet functionally complementary effects (lower As translocation and oxidative damage compared with individual treatments), suggesting biochemical cooperation between the fungal and bacterial inoculants rather than antagonism. Importantly, the compatibility assay (Supplementary Figure S2) confirmed that *P. djamor* and the PGPB strain displayed no inhibitory effects on each other's growth, further supporting their coexistence potential.

4.7. Antioxidant Enzyme Responses

As expected, SOD and CAT exhibited contrasting responses depending on the amendment. AP showed the “typical As profile”: high lipid peroxidation, high NADPH oxidase,

and unbalanced antioxidant system. B showed enhanced SOD activity, whereas E and H maximized CAT activity, consistent with distinct ROS scavenging strategies. Higher SOD and CAT activities in B and E treatments suggest an enhanced detoxification capacity that likely contributes to reduced oxidative damage and limited As translocation to shoots. No previous studies have reported antioxidant enzyme modulation by SMS amendments in planta; most literature has focused on SMS sorption capacity and enzyme activities in the substrate [103,110]. Our results fill this gap, showing that SMS-based amendments can indirectly modulate plant antioxidant responses.

Although the experiment was performed under sterile substrate conditions to isolate plant responses, the differential outcomes among treatments H, B, and HB suggest functional complementarity between *Pleurotus djamor* and the PGPB strain. The compatibility assay (Supplementary Figure S2) confirmed the absence of antagonistic growth effects between both inoculants, supporting their coexistence potential. In natural soils, such associations could extend beyond the plant level, affecting As dynamics through pH modulation, chelator secretion, and competition for adsorption sites. Similar tripartite plant–microbe–fungal systems have been shown to enhance metal immobilization, nutrient exchange, and oxidative stress mitigation via secretion of siderophores, organic acids, and extracellular polymers [106,107,111,112]. These mechanisms, though not directly evaluated here, may underlie cooperative behavior observed in HB and provide a conceptual framework for future soil-based studies assessing the ecological dimension of SMS + PGPB consortia.

4.8. Gene Expression Related to As Homeostasis

The molecular analysis was intentionally restricted to three representative conditions (AP, H, and E) that encompassed the full physiological range observed in growth, pigment, translocation, and oxidative responses. These treatments were selected as contrasting models: AP (unamended control) represented the sensitive condition with high As accumulation and oxidative damage; H (SMS + *Pleurotus djamor*) showed partial tolerance and mitigation; and E (non-microbial benchmark) exhibited minimal As translocation (TF = 0) and maintained growth and pigment stability. This preliminary qRT-PCR screening aimed to identify distinct detoxification routes rather than to provide a comprehensive transcriptional profile for all treatments, focusing instead on the most physiologically contrasting responses. Gene expression data showed amendment-dependent detoxification strategies: AP relied on high *ABCC1* expression (vacuolar sequestration), while H induced *PCS2* and *CAD1* (phytochelatin synthesis and metal binding). E showed intermediate expression patterns, aligning with its physiological performance. Similar regulation of *PCS* and *ABCC1* has been described in legumes and cereals under As and Cd stress [18,40,113,114]. Future transcriptomic work will extend this analysis to the mixed and bacterial treatments (HB and B) to better characterize their combined regulatory mechanisms.

Based on our findings and current literature, the B amendment (SMS + PGPB) appears suitable as a cost-effective option for low-to-moderate As loads in well-buffered soils, where PGPB mechanisms can assist plant tolerance and metal(loid) handling [5,107,115]. In turn, the HB amendment (SMS + *Pleurotus djamor* + PGPB) may be more appropriate for slightly As-contaminated, OM-poor or weakly structured soils, where fungal–bacterial complementarity can foster As immobilization/biotransformation and improve the substrate matrix [116,117]. These differentiated application scenarios are consistent with reports on PGPB-assisted remediation and on the role of spent mushroom substrates in mitigating As effects and deserve field-scale testing to establish practical guidelines.

4.9. Framing the Role of E vs. SMS-Based Amendments

E performed best in limiting As uptake and translocation while sustaining biomass and pigments. However, because E does not contain SMS, it is used here as a benchmark rather than a practical deployment target. This work explicitly prioritizes SMS-based circular-economy strategies. Moreover, E can serve other purposes before being applied as an amendment to mitigate As stress in plants. Notably, maize stover has proven to be an excellent substrate for *Pleurotus* cultivation, offering higher biological efficiency compared to sawdust [60]. Within this framework, HB (SMS + *P. djamor* + PGPB) combines low translocation with oxidative stress attenuation (H_2O_2 /TBARs) and intermediate growth; B (SMS + PGPB) provides a robust compromise by stabilizing biomass with low impact on N; and H (SMS + *P. djamor*) preferentially activates chelation pathways (*PCS2*, *CAD1*), albeit with higher pigment sensitivity. HA (autoclaved SMS), while effective at restricting As ($TF \approx 0$), is considered a non-transferable control. Thus, for circular applications, HB is recommended as the primary option and B as a strong alternative, with H considered conditionally depending on crop and scenario.

5. Conclusions

The E amendment (without SMS) acted as the best benchmark for minimizing As uptake and translocation without compromising peanut crop performance (biomass and pigments). However, because it does not include SMS, E is considered a reference rather than a practical deployment strategy. Although E minimized As uptake, its non-circular origin limits its application as a sustainable amendment; thus, it is considered a benchmark rather than a practical deployment strategy. The objective of this work was to evaluate viable SMS-based approaches within a circular economy model, and under this framework the amendments showed differentiated behaviors. HB (SMS + *Pleurotus djamor* + PGPB) combined low As translocation with attenuation of oxidative stress (reduced H_2O_2 and TBARs) and intermediate effects on growth. B (SMS + PGPB) provided a robust compromise, sustaining biomass with low impact on N and enhanced SOD activity. H (SMS + *P. djamor*) preferentially activated chelation pathways (*PCS2*, *CAD1*), although with higher sensitivity in pigments. HA (autoclaved SMS) was very effective in restricting As ($TF \approx 0$) but is considered a non-transferable control. AP (no amendment) remained the most sensitive condition, showing high As uptake, strong oxidative damage, and growth inhibition.

Mechanistic interpretation suggests that HB and B favor complementary routes of chelation/sequestration and redox modulation in the rhizosphere, consistent with restricted As mobilization, lower membrane damage, and adjusted antioxidant responses. Thus, for SMS-based circular applications, HB is recommended as the primary option and B as a robust alternative, while H may be considered conditionally depending on the crop or scenario. To validate these findings, field trials with realistic As gradients and different crops are required to confirm whether SMS maintains its barrier effect against As translocation and supports yield performance in complex soils.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/agriculture15212300/s1>, Supplementary Materials Figure S1. Timeline of fungal substrate preparation (A) and pot experiment (B). The timing and sequence correspond to the methodological description provided in Sections 2.4.1 and 2.4.2 and Supplementary Materials File S1. Supplementary Materials Figure S2. Compatibility tests between *Pleurotus* strain PQ and plant growth-promoting bacteria (PGPB) on PDA medium. The main plate shows the *Pleurotus* colony (PQ) co-cultured with bacterial isolates J49 and 1.2, and the control (C). Supplementary Materials Figure S3. Effect of arsenic on in vitro mycelial growth of *Pleurotus djamor* PQ. Supplementary Materials Figure S4. Viability of *Pseudomonas fluorescens* strain 1.2 in TY medium supplemented with arsenic. Supplementary Materials Figure S5. Visualization and quantification of blue-stained leaf areas under

arsenic stress. Representative peanut leaves grown under control and arsenate conditions for each treatment. Panel I shows digitally enhanced images highlighting the blue-stained areas (as in Figure 3), while panel II displays only the detected blue regions corresponding to the quantified signal. The table (III) indicates the percentage of total leaf area covered by blue coloration, including all blue pixels regardless of intensity. Supplementary Materials File S1. Methodological details to prepare the SMS amendments. Supplementary Materials File S2. Table S1: Peanut's primers. Supplementary Dataset S1.

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Abbreviations

APX	Ascorbate peroxidase
As	Arsenic
BNF	Biological nitrogen fixation
CAT	Catalase
CFU	Colony-forming units
DHAR	Dehydroascorbate reductase
EDTA	Ethylenediaminetetraacetic acid
GPX/PRX	Glutathione peroxidase or peroxiredoxin
GR	Glutathione reductase
GST	Glutathione S-transferase
KI	Potassium iodide
MDHAR	Monodehydroascorbate reductase
NBT	Nitroblue tetrazolium
PDA	Potato dextrose agar
PGPB	Plant growth-promoting bacteria
PVP	Polyvinylpyrrolidone
ROS	Reactive oxygen species
SMS	Spent mushroom substrate
SOD	Superoxide dismutase
TBARs	Thiobarbituric-reactive substances
TCA	Trichloroacetic acid
TY	Tryptone-yeast extract

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