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Insights into the physiological and biochemical responses of peanut plants under combined arsenic and flooding stress

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ABSTRACT

Plants in nature constantly face multiple simultaneous stresses. In this study, we assessed for the first time the impact of environmentally relevant arsenic (As) concentrations combined with a flooding (F) event on peanut plants. We analyzed growth, nodulation, mineral composition, redox metabolism, oxidative damage, organic acid levels, and phytohormone concentrations. Each treatment, whether applied alone or combined, elicited distinct responses in roots and leaves. Peanut plants exhibited increased reactive oxygen species (ROS) production under all treatments, resulting in oxidative stress, disruptions in organic acid metabolism, and changes in hormone profiles. The responses were more severe under combined As + F stress than under individual stress treatments, indicating synergistic effects. The different stress treatments operated through distinct pathways. Arsenic caused metalloid toxicity, triggering oxidative stress and disrupting vital processes. In contrast, flooding-induced stress was attributed to oxygen deprivation, forcing a metabolic shift from aerobic respiration to fermentation, ultimately leading to oxidative stress. This study highlights the complex interactions between individual and combined stresses, which can lead to either additive or synergistic effects. Despite consistent declines in growth and key physiological processes like photosynthesis, the specific response mechanisms varied depending on the type of stress imposed, with combined stress exerting the most severe effects.

1. Introduction

The combined stress of arsenic (As) toxicity and flooding is a growing concern for agriculture, especially in regions where elevated groundwater As levels intersect with poor drainage or waterlogging events. This issue is well-documented in paddy rice systems of South and Southeast Asia (Mondal et al., 2024). Although rice is tolerant to waterlogging, flooding substantially increases As phytoavailability, leading to serious health risks due to grain contamination (Kong et al., 2024). However, this environmental challenge is not restricted to Asia. The increasing incidence of climate-change-induced rises in the groundwater level reaching the rooting zone is already causing severe problems. A clear example can be found in peanut cultivation in Cordoba, Argentina, one of the world's top five peanut-exporting regions (OEC, 2024). In the 2016/2017 and 2018/2019 campaigns, crop losses due to flooding episodes were alarming, with thousands of hectares of peanuts and other crops lost (BCCBA, 2019, 2024). Moreover, in this region the natural As concentrations in groundwater frequently exceeds internationally recommended maximum values (Bécher Quinodóz et al., 2019). High As concentrations (≥20 μM) have been reported to inhibit growth, alter root architecture, and induce strong oxidative stress in peanut plants (Bianucci et al., 2017). Even low concentrations, such as 3 μM As, the average As concentration in Cordoba's groundwater (Bécher Quinodóz et al., 2019), can enhance the formation of reactive oxygen species (ROS) in nodulated peanut plants (Peralta et al., 2020). This legume symbiotically interacts with slow-growing *Bradyrhizobium* sp. strains that invade root cells intercellularly, forming a nitrogen-fixing nodule (Ibañez et al., 2016). This process requires ATP, reducing power and a low O_2 level inside the nodule, mediated by leghemoglobin activity to guarantee high rate of bacteroid respiration and avoid inhibition of nitrogenase activity due to oxidation (Fabra et al., 2010; Matamoros et al., 2017). While rice has served as a model crop to understand the

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interaction between flooding and As uptake, the physiological response of peanut to this combined stress remains largely unexplored.

Forage legumes show considerable variation in their tolerance to waterlogging. Although some plants have the ability to induce aerenchyma and/or adventitious roots formation, by reprogramming root architecture to allow longitudinal oxygen transport within roots to shoots (Herzog et al., 2016), others must also switch to fermentative metabolism to sustain minimal ATP production and regenerate NAD^+, leading to lactate and ethanol accumulation (Kumar et al., 2018). To assure cellular energy homeostasis, fermentation must proceed at a much higher rate than aerobic respiration, implying a higher demand for C supply in the submerged roots.

Nodulation and symbiotic N_2 fixation are relatively tolerant to flooding, with functional nodules observed even in flooding-sensitive *Medicago sativa,* despite a marked reduction in nodule number and shoot nitrogen (N) concentrations (Arrese-Igor et al., 1993). Arsenic strongly affects nodulation and the expression of nodulation-related genes (Lafuente et al., 2015; Wevar Oller et al., 2025). However, the inoculated bacterial strain plays an important role in As tolerance, as shown in soybean and peanut, where the selection of the best symbiotic partner can either enhance or reduce As effects (Bianucci et al., 2018; Peralta et al., 2019, 2020).

The interaction of waterlogging and As toxicity in legume species remains poorly investigated. Both stresses, either individually or in combination, cause oxidative stress in sensitive plants (Mascher et al., 2002; Kumar et al., 2018; Manghwar et al., 2024). Under flooding, three mechanisms have been identified as responsible for ROS accumulation: activation of NADPH oxidase, mitochondrial dysfunction, and the accumulation of redox sensitive metal(loid) ions (Iron (Fe), Manganese (Mn), Copper (Cu), As, Antimony (Sb)) to toxic concentrations (Shabala et al., 2014). Similarly, ROS production is also an immediate consequence of As uptake (Finnegan and Chen, 2012). Like flooding, As enhances NADPH oxidase activity and causes damage to both mitochondria and chloroplasts. A specific source of ROS in plants exposed to arsenate is the intracellular reduction of $As^{(V)}$ to $As^{(III)}$ via the arsenate reductase enzyme. This reduced form readily interacts with sulfhydryl (SH-) groups of key molecules in antioxidant defense, such as glutathione (GSH), phytochelatins (PCs), and ROS detoxifying enzymes, thereby disrupting redox homeostasis (Mishra et al., 2019; Peralta et al., 2022).

In summary, both stress factors individually induce oxidative stress, metabolic alterations, and alterations in root architecture. We hypothesize that simultaneous exposure to arsenic and flooding results in either additive or synergistic effects, with the combined impact being equal to or greater than the sum of the individual stress responses. To test this hypothesis, in our study we used peanut plants growing in symbiosis with a N_2- fixing *Bradyrhizobium* sp. SEMIA6144 strain under controlled conditions applying a low, environmentally relevant, As concentration (3 μM) and waterlogging (8 days). Besides growth, nodulation, and mineral concentration analysis, redox metabolism, oxidative damage, organic acid levels and hormone concentrations were also determined. This approach was taken considering the impact of ROS on vital molecules, the role of plant carbon (C) metabolism and nutrient responses, and the involvement of phytohormones in plant adaptations to either waterlogging or As stress (Yeung et al., 2018; Peralta et al., 2021).

2. Materials and methods

2.1. Growth conditions

Peanut seeds cv. Granoleico (El Carmen S.A, Córdoba, Argentina) were surface sterilized and germinated at 28 °C. Pre-germinated seeds were transferred to pots filled with a mixture of sterilized perlite: sand (1:2) placed on trays (Peralta et al., 2020). Trays were filled with nitrogen-free Hoagland solution (Hoagland and Arnon, 1950) containing 3 μM As supplemented as $Na_2HAsO_4.7H_2O$. Plants without As addition were maintained as controls. Seven days after the radicle emergence, plants were inoculated with *Bradyrhizobium* sp. SEMIA6144, a strain able to limit As translocation to the shoot (Peralta et al., 2019). For this purpose, the bacterial culture suspension (3 mL, containing 10^8 CFU mL^{-1}) was placed into the sterile substrate close to the crown root. The plants were grown in a controlled-environment chamber (light intensity of 200 μmol $m^{-2}s^{-1}$, 16 h day/8 h night cycle, a constant temperature of 28 °C, and a relative humidity of 50 %). The plants were maintained for 30 days post-inoculation, corresponding to the phenological state R1, flowering, according to Boote (1982). At this stage, symbiosis is already established, and the nodules are visible and completely formed. Then, plants were randomly distributed into four separate groups, two of them continued with the same water system irrigation, and the other two were exposed to flooding conditions (saturated pots by capillary rise to 20 cm), in the presence or absence of As. The experimental design was as follows: 1- Control (C); 2- Flooding (F); 3- Arsenic (As); 4- combination of arsenic and flooding stress (As + F). After 8 days of treatment, leaves, roots and nodules were harvested and stored at −80 °C until used. Growth and nodulation variables were determined both in fresh and dry plants. Nodules were transversely cut, and the brownish-red internal color was taken as a signal of the presence of leghemoglobin, indicating nodule activity and potential N-fixation (Virtanen et al., 1947).

2.2. Arsenic concentration

Arsenic concentrations were determined in the leaves, roots, and nodules of peanut plants. Dry plant material (0.1 g for leaves and roots; 0.05 g for nodules) was digested in an acid mixture containing 0.3 mL HNO_3, 0.5 mL H_2O and 0.2 mLH_2O_2 (Ortega-Villasante et al., 2005). The digestion was performed under high-pressure conditions in an autoclave (121 °C, 20 min), and the digests were filtered through a PVDF filter and diluted in Milli-Q water to 5 mL. The As concentrations were measured using inductively coupled plasma mass spectroscopy (ICP-MS) NexION 300 (PerkinElmer Sciex, San Jose, CA, USA).

2.3. Arsenate reductase

The activity of arsenate reductase (AR) was determined following the Anderson and Cook (2004) technique with modifications. Plant material (0.1 g for leaves and roots) was homogenized with a sodium phosphate (50 mM, pH 7.5) extraction buffer and centrifuged at 12,000 *g* for 15 min. The supernatant was used to determine the enzyme activity. The reaction mix contained: 50 mM Buffer MOPS/MES pH 6.5, 2 mM DTT, 2 mM sodium arsenate, 2 mM NADPH, and 0.02 mg protein plant extract (final volume of 500 μL). Measurements were recorded at 340 nm, following NADPH oxidation. One unit of AR was defined as the quantity of enzyme needed to reduce 1 μmol arsenate min^{-1}.

2.4. Concentrations of nitrogen and other essential mineral nutrients

To determine N concentration, 25 mg of dried shoot material was analyzed following the Kjeldahl method (Nelson and Sommers, 1973). The concentrations of other selected essential mineral nutrients, Calcium (Ca), Sulfur (S), Magnesium (Mg), Mn, Potassium (K), Fe, Phosphorus (P) and Zinc (Zn), were determined in dried leaves or roots (0.1 g of each) digested with concentrated HNO_3 and placed in Pyrex tubes in a hot-block digestion system (an open-air digestion) at 110 °C for 5 h (SCI54-54-Well Hot Block, Environmental Express, Charleston, SC, US). Extracts were analyzed by ICP-MS (PerkinElmer Ink., ELAN 6000, MA, USA) or ICP-OES (Thermo Jarrell-Ash, model 61 E Polyscan, UK).

2.5. Determination of stress markers

2.5.1. Root cell viability

To detect viable or dead root cells, vital staining was performed in

roots using fluorescein-diacetate (FDA) and propidium iodide (PI) (Jones and Senft, 1985). Root tips of approximately 1 cm length were incubated for 3 min in FDA (5 mg mL^{-1} acetone) and rinsed with Dulbecco's Phosphate-Buffered Saline (DPBS) buffer (6 mM Na_2HPO_4; 4 mM KH_2PO_4; 140 mM NaCl; pH 7.4). Then, roots were submerged for 10 min in PI (0.02 mg mL^{-1} DPBS buffer) and rinsed with the same buffer. Root fluorescence was observed in an epifluorescence microscope equipped with an excitation filter ranging from 450 to 490 nm and an emission filter of 520 nm (Nikon Optiphot 2, Tokio, Japan).

2.5.2. Callose

Root segments were stained for 15 min with aniline blue 0.05 %, prepared in potassium phosphate buffer (PiK 0.15 mM, pH 9) following the method described by Gunsé et al. (2000). After proper rinsing with the same buffer, the segments were observed in a fluorescence microscope (Nikon Optiphot 2, Hg lamp, filter UV-1A, excitation filter 365/10 nm, dichroic mirror 400 nm, barrier filter 400 nm).

2.5.3. Chlorophyll concentrations and photosynthetic efficiency (Fv Fm^{-1})

Leaf chlorophyll concentrations were obtained using a SPAD chlorophyll meter (CCM-200, Opti-Science, USA). The maximum PSII quantum yield determination was performed in fully expanded second nodal leaves previously dark-adapted for at least 30 min and measured with a Mini-PAM II (Heinz Walz GmbH, Effeltrich, Germany).

2.5.4. NADPH oxidase

Fresh plant material (0.1 g for leaves and roots) was homogenized in PiK extraction buffer (50 mM, pH 7.5) and centrifuged at 12,000 *g* for 15 min at 4 °C. The reaction mix, with a final volume of 500 μL contained: Tris- HCl 50 mM, Nitroblue Tetrazolium (NBT) 0.6 mM, 0.02 mg protein plant extract, and NADPH 0.2 mM (final volume of 500 μL). The NADPH activity was determined spectrophotometrically measuring the reduction of NBT at 560 nm. One unit of NADPH oxidase was defined as the quantity of enzyme needed to reduce 1 μmoL NADPH min^{-1} (Sagi and Fuhr, 2001).

2.5.5. Hydrogen peroxide quantification

Plant material (0.1 g for leaves and roots) was homogenized in 0.1 % (w/v) of trichloroacetic acid (TCA) and centrifuged at 10,000 *g* for 15 min. Then, an aliquot of the supernatant was mixed with a reaction buffer containing 100 mM PiK, and 1 M of potassium iodide (KI). The reaction was allowed to proceed for 1 h in dark conditions, and then the absorbance at 390 nm was measured. The amount of H_2O_2 was calculated using a standard calibration curve prepared with known H_2O_2 concentrations (Alexieva et al., 2001).

2.5.6. Histochemical and fluorescence microscopy in situ observation of ROS

2.5.6.1. O_2^- histochemical in situ detection in roots and leaves. Selected fragments of peanut lateral roots were incubated for 45 min in Tris-HCl buffer 10 mM at pH 7.4 at 37 °C, with 10 μM dihydroethidium (DHE). The samples were washed 3 times for 10 min with the same buffer and observed in a fluorescence microscope (Nikon Optiphot 2, excitation at 488 nm, emission at 520 nm) (Fink et al., 2004). Since fluorescence interferes with chlorophyll, the DHE probe cannot be used in leaves. Therefore, they were incubated in 1 mM NBT previously prepared in sodium citrate buffer (10 mM, pH 6), in darkness at room temperature on a shaker for 8 h. Then the leaves were blanched with ethanol 96° (Frahry and Schopfer, 2001). NBT reacts with O_2^- to form an insoluble dark blue formazan compound.

2.5.6.2. H_2O_2 histochemical in situ detection in roots and leaves. Selected fragments of peanut roots were incubated for 45 min in Tris-HCl buffer 10 mM, pH 7.4 at 37 °C, with 25 μM 2′-7′-dichlorofluorescein diacetate (DCF-DA). The samples were washed 3 times for 10 min with the same buffer and then were observed in a fluorescence microscope (Nikon Optiphot 2, excitation at 485 nm, emission at 530 nm) (Romero-Puertas et al., 2004). Leaves were incubated with 1 mg mL^{-1} of 3,3-diaminobenzidine (DAB)-HCl at pH 3.8 and room temperature, on a shaker for 8 h. Then, leaves were exposed to light for 1 h and blanched in ethanol 96° (DAB reacts with H_2O_2 in the presence of peroxidases, forming a brown deposit) (Orozco-Cárdenas and Ryan, 1999).

2.6. Analysis of selected phytohormone and organic acid concentrations

The phytohormones jasmonic acid (JA), salicylic acid (SA), methyl jasmonate (MeJA), and 1-aminocyclopropane 1-carboxylic acid (ACC) were extracted and determined by HPLC/ESI-MS/MS with multiple reaction monitoring (MRM) according to Llugany et al. (2013). The deuterated forms of the compounds d5-JA, d4-SA, and d4-ACC were added as internal standards. Quantification was done by the creation of a calibration curve including each of the unlabelled analyte compounds (JA, SA, MeJA, and ACC). Organic acids (OA) were extracted following the modified procedure from Tolrà et al. (2005). Briefly, 250 mg of fresh material was homogenized with 2 mL of 0.025 M hydrochloric acid and centrifuged at 1000 *g* for 15 min at 4 °C. The resulting supernatant was passed through Sep- Pack C18 cartridges (Waters, USA) previously activated with methanol and 0,025 M hydrochloric acid. Samples were filtered and injected into an HPLC-UV system (Shimazu, Japan on a YMC-Pack ODS-A HPLC column 5 μm 120 Å 250 × 4.6 mm (YMC, Germany). The injected samples were eluted for 15 min with an isocratic and constant flow of 50 mM potassium phosphate (KH_2PO_4) adjusted at a pH of 2.8. Acetic acid, cis-aconitic acid, trans-aconitic acid, ascorbic acid, citric acid, isocitric acid, formic acid, glycolic acid, glyoxylic acid, lactic acid, maleic acid, malic acid, malonic acid, oxalic acid, pyruvic acid, and succinic acid were measured. The detected peaks' retention times were compared with the retention times of commercial standards injected under the same conditions, and the identification was confirmed by standard enrichment injection of samples. Only organic acids detected above the quantification limit in at least one treatment were considered for quantification and reporting. Compounds below detection thresholds in all samples of a given organ were excluded from the analysis.

2.7. Statistical analysis

Experiments were performed in a random design and repeated three times. The data were analyzed using the *InfoStat* software (Di Rienzo et al., 2020). Differences among treatments were analyzed using ANOVA, taking $P < 0.05$ as significant according to the Duncan test. Before significance testing, the normality and homogeneity of variance were verified using Shapiro-Wilk and Levene tests, respectively.

3. Results

3.1. Fitness characterization

Flooding affected neither the length nor the dry weight of the shoots

Table 1
Plant growth variables on peanut exposed to As and flooding.

Treatments	Shoot length (cm)	Shoot dry weight (g)	Root length (cm)	Root dry weight (g)
C	12.15 ± 0.49^{a}	1.13 ± 0.12^{a}	26.75 ± 0.88^{a}	0.17 ± 0.02^{ab}
F	12.18 ± 0.47^{a}	0.98 ± 0.05^{a}	26.44 ± 0.93^{a}	0.17 ± 0.01^{b}
As	9.46 ± 0.39^{b}	0.71 ± 0.03^{b}	22.22 ± 0.70^{b}	0.18 ± 0.01^{ab}
As + F	9.88 ± 0.43^{b}	0.64 ± 0.04^{b}	21.69 ± 0.78^{b}	0.22 ± 0.03^{a}

Values represent the mean ± SE (n = 7). Different letters indicate significant differences among treatments according to the Duncan's test ($P < 0.05$).

(Table 1). Contrastingly, both As and combined As + F treatments considerably reduced shoot growth and root length (Table 1). Arsenic exposure decreased shoot length by 22 % and shoot dry weight by 37 % compared to the control, while the As + F treatment caused a 43 % reduction in shoot dry weight. The number and dry weight of peanut nodules were significantly reduced in plants exposed to As and As + F compared to control (Table 2). Arsenic reduced nodule number by 57 % and nodule dry weight by 27 %, while the combined treatment decreased them by 52 % and 37.5 %, respectively. Nitrogen content was reduced in all treatments, with the most pronounced reduction (79 %) under As + F. This result was consistent with the severe decline of active nodules. The percentage of red nodules, indicating the presence of leghemoglobin, dropped from 94 % in control plants to 74 % under As, 57.5 % under flooding, and 46 % under the combined treatment, highlighting the strong impact of dual stress on symbiotic efficiency (Table 2). Root morphology analysis showed slight darkening under all tested treatments, being most evident under combined stress followed by arsenic and flood applied individually (Supplementary Fig. 1–I) In plants exposed to waterlogging the formation of adventitious roots was evident (Supplementary Fig 1-II). Vital staining of roots revealed red spots, indicative of dead cells in the elongation zone under all the tested conditions but the control. Roots receiving the As + F treatment were most affected (Fig. 1-I, 10*X*). In the mature zone these spots were also seen in all the treatments and were most evident under the As and As + F conditions (Fig. 1-I, 40*X*). These treatments also caused the highest accumulation of callose in the root tissues (Fig. 1-II).

3.2. Ionomic profile

All stress treatments had a strong impact on root ionomic profiles, being As and As + F treatments the ones that caused a pronounced depletion of key macronutrients (Fig. 2, Supplementary Fig. 2). Root P concentration was reduced by nearly half under As + F, while K decreased by about 63 % relative to the control. Manganese levels dropped more than 65 % under combined stress. Zinc concentrations were consistently lower across all stress conditions, with As + F reducing Zn levels by ~35 % compared to control values. In contrast, Ca and Fe concentrations increased under As + F stress, with rises of approximately 44 % and 31 %, respectively. Conversely, sulfur levels dropped by nearly 45 % under both As and As + F treatments, suggesting a marked disruption in S metabolism and its potential implications in antioxidant responses. The ionomic profile of leaves was less affected. In comparison to control, both As and As + F caused reductions of ~30 % in S and Mg, and ~20 % in K concentrations. The negative effect on Zn concentrations was evident in As + F, where they declined to 40 % of control values. The combined As + F stress also caused the strongest decrease of P concentrations, with a 65 % reduction compared to control, while the individually applied stress factors had a less impact (Fig. 2, Supplementary Fig. 3).

3.3. As distribution in peanut plant and arsenate reductase enzyme activity

Roots accumulated higher As levels followed by nodules and shoots (Table 3). Although no differences were observed in the As concentration found in shoots and roots between the applied treatments, nodules of plants exposed to the combined stress presented the highest level (50 % increase) compared to As alone suggesting additive uptake and retention in symbiotic tissues. Arsenate reductase (AR) activity displayed tissue-specific responses (Fig. 3). The AR enzyme activity in shoots significantly increased by 75 % under the combined treatment compared to As alone, indicating enhanced arsenate detoxification demand. In contrast, the AR activity in roots was decreased by ~18 % under combined stress compared to As alone, suggesting possible enzyme inhibition or tissue-specific downregulation under dual stress (Fig. 3).

3.4. Reactive oxygen species

All treatments tested increased NADPH oxidase activity and O_2^- immunofluorescence detection in peanut roots compared to the control (Fig. 4A–B). NADPH oxidase activity rose by approximately 70 % under F, As, and As + F treatments. Histochemical detection of O_2^- corroborated these findings. Roots exposed to the applied treatments significantly increased H_2O_2 content, with levels rising more than 100 % compared to control condition. In agreement, the most intense bright green H_2O_2 immunofluorescence was observed in plants exposed to As, followed by flooding and the combined stress (Fig. 4C and D). In shoots, an increase of NADPH oxidase activity was observed in As and As + F treatments, showing more than 200 and 300 % increases, respectively, compared to the control (Fig. 5A). Superoxide (O_2^-) production, visualized by NBT staining, followed a similar trend, with dark blue precipitates intensifying in the order As + F ≥ As > F, relative to control (Fig. 5B). Leaf H_2O_2 content increased significantly only under As and As + F treatments, with increases of approximately 220 % and 140 %, respectively, compared to the control. This quantitative pattern was consistent with DAB staining (Fig. 5C–D).

3.5. Photosynthetic pigments and lipid peroxidation

Total chlorophyll concentration was strongly reduced by As + F and, to a lesser extent, by As, both showing statistically significant differences compared to the control. Compared to the control, only As + F significantly decreased the photosynthetic efficiency (Fig. 6A–B). The level of lipid peroxidation in roots was increased approximately 67 % with F, and 27 % under As and the combined treatment. In contrast, lipid peroxidation in leaves was significantly decreased (~60 %) under all stress treatments (Fig. 7A–B).

3.6. Concentrations of selected phytohormones

Arsenic and flooding, alone or in combination, had different effects on the endogenous concentrations of the selected phytohormones in roots and leaves (Fig. 8). All stress treatments decreased the concentrations of JA in roots, while the levels in leaves were unaffected (Fig. 8A–B). Flooding caused a ~80 % reduction, As a ~52 % decrease, and As + F a ~70 % drop in root JA levels compared to the control. Only minor changes in MeJA concentrations were observed in leaves, with As + F slightly, but in a significant way (~18 %), decreasing the hormone level compared to the control (Fig. 8C–D). The most remarkable change was observed in the SA concentrations. A strong decrease of the hormone level was observed in roots under F (60 %), while in leaves it

Table 2
Effect of As and flooding on peanut nodulation.

Treatments	Nodule number (N° plant^{-1})	Nodule dry weight (g plant^{-1})	Nitrogen content (mg N plant^{-1})	% of active nodules
C	41.64 ± 1.93^a	0.048 ± 0.003^a	36.91 ± 2.61^a	94.00 ± 2.45^a
F	38.75 ± 2.26^a	0.044 ± 0.006^a	13.84 ± 2.3^b	57.50 ± 4.79^c
As	17.91 ± 2.79^b	0.035 ± 0.002^b	15.37 ± 0.96^b	74.00 ± 5.10^b
As + F	20.09 ± 2.79^b	0.030 ± 0.004^b	7.65 ± 0.77^c	46.00 ± 5.00^c

Values represent the mean ± SE (n = 10). Different letters indicate significant differences among treatments according to the Duncan's test ($P < 0.05$).

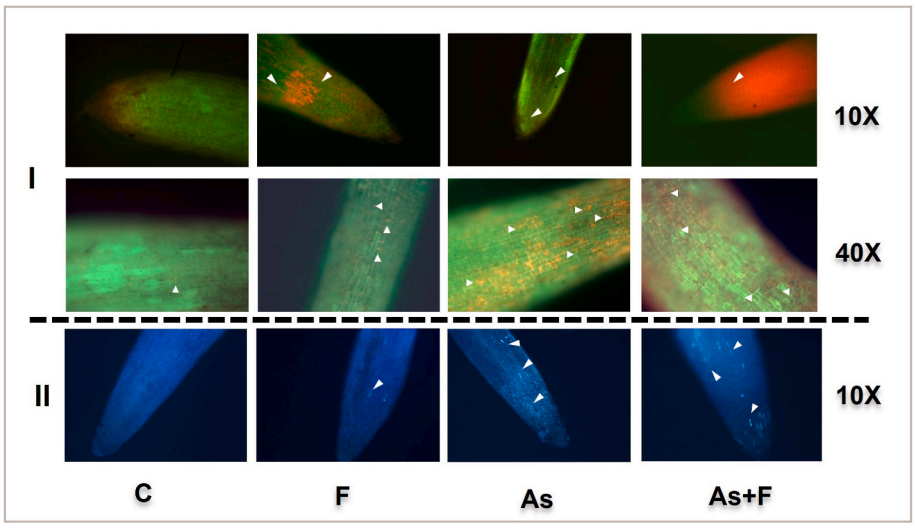

Fig. 1. Vital staining and callose deposition in peanut root under different treatments: control, flooding, arsenic (As), and combined arsenic + **flooding (As + F).** Panel I: Fluorescein diacetate (FDA) staining to assess cell viability, shown at 10*X* and 40× magnification. Green fluorescence indicates viable cells, while orange/red regions (white arrowheads) indicate cell death. Panel II: Aniline blue staining for callose deposition, observed at 10× magnification. White arrowheads indicate areas of callose accumulation. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

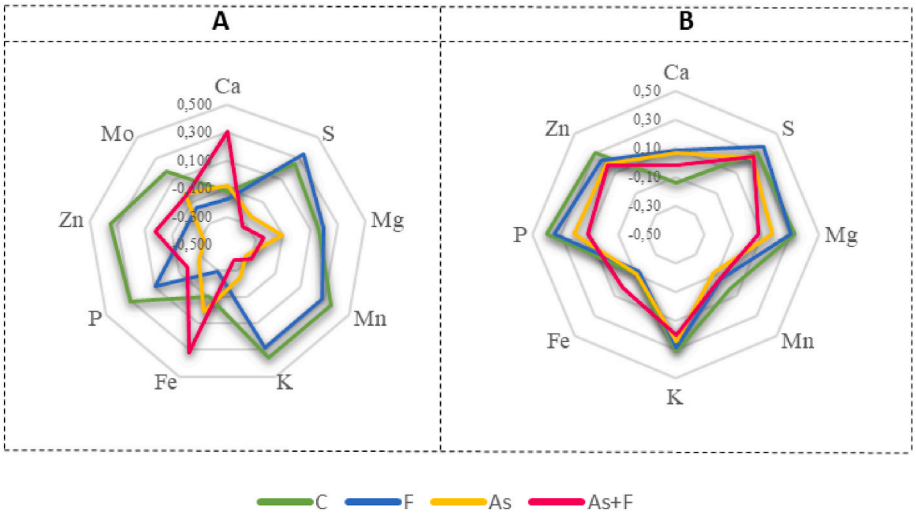

Fig. 2. Radar plots showing the ionomic profiles of peanut plants under different treatments: control (C), flooding (F), arsenic (As), and combined arsenic + flooding (As+F). Panel A: Mineral element concentrations in roots. Panel B: Mineral element concentration in leaves.

Table 3
Arsenic distribution in peanut plant.

Treatments	Arsenic content (μg g^{-1})		
	Shoots	Roots	Nodules
As	13.19 ± 1.10[a]	141.12 ± 17.5[a]	19.06 ± 1.42[b]
As + F	12.16 ± 0.95[a]	162.71 ± 19.6[a]	28.84 ± 1.23[a]

Values represent the mean ± SE (n = 5). Different letters indicate significant differences between treatments according to the Duncan's test ($P < 0.05$). Abbreviations: nd: not detected.

increased by more than 150 % (Fig. 8E–F). In contrast, As substantially increased SA in roots (~250 %), while exposure to the metalloid alone slightly but significantly decreased leaves SA concentrations compared to control. The combined As + F treatment caused an intermediate response with no significant differences in roots compared to control (Fig. 8E), while in leaves it led to an increase of approximately 40 % (Fig. 8F). Concentrations of ACC were significantly increased in the roots being the highest levels observed under the combined As + F treatment (200 %), followed by As (100 %) and F (60 %) compared to control (Fig. 8G). In leaves this hormone was unaffected by the applied treatments (Fig. 8H).

3.7. Organic acid concentrations

The organic acid concentrations showed major differences between shoots and roots (Tables 4 and 5). It is worth noting that several organic acids were not detected in roots or leaves under any treatment and thus were not included in the tables. This accounts for the different numbers of acids reported in both organs. These differences in compound detection reflect organ-specific metabolic adjustments, as roots and leaves activate distinct biochemical responses to cope with stress-induced energetic and redox imbalance. Under control conditions, formic acid and isocitrate were the most abundant organic acids in roots (Table 4) and shoots (Table 5), respectively.

In roots, no pyruvic acid, malonic acid, isocitrate acid, lactate,

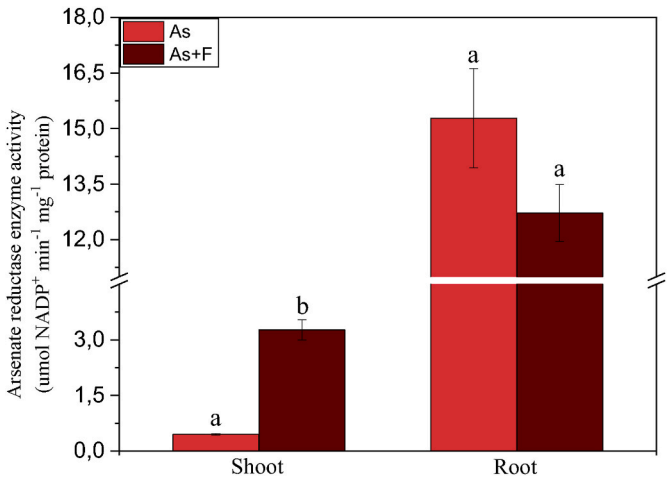

Fig. 3. Arsenate reductase enzyme activity in shoots and roots of peanut plants exposed to arsenic (As) and combined arsenic + flooding (As + F) treatments. Bars represent the mean ± SE (n = 5). Different letters indicate statistically significant differences between treatments within each organ, as determined by one-way ANOVA followed by Duncan's post hoc test ($p < 0.05$).

citrate, succinic acid, and aconitic acid were detected, independently of the applied treatments (Table 4). Oxalacetic acid showed no significant differences between control and F; however, a significant decrease in As and As + F treatments was observed, with arsenic alone causing the greatest reduction. The glycolic acid concentrations were unchanged among treatments. Formic acid was enhanced in the F treatment (by ~72 %) but was reduced under As (by ~87 %) and As + F exposure (~36 %) compared to control. Malic acid was only detected in the combined treatment and ascorbic acid was also observed in As, being this last one significantly higher. Acetic acid concentrations were significantly reduced in As and As + F treatments compared to control (~70 % and ~61 % reduction, respectively), while flooding alone increased its concentration more than 300 %.

In shoots (Table 5), oxalic acid was only detected in the F treatment, isocitrate, lactate and aconitic acids were observed only in control and in the combined stress, being significantly decreased in As + F compared to the control. Malonic, acetic, and citrate acids were only detected in control and F conditions, showing a contrasting response between them since malonic and citrate acids showed significantly higher levels in control, while acetic acid peaked in F treatment. Pyruvic acid was highest under As + F, followed by F. Malic acid was not detected in the F treatment and its level was significantly higher in As (by ~605 % compared to control) followed by the combined treatment (by ~223 %) in comparison to the control. Succinic acid was not detected in the combined treatment but was detected in control and F, which showed similar values, while As significantly reduced its level.

4. Discussion

Peanut (*Arachis hypogaea*) is widely cultivated in regions frequently affected by As contamination and seasonal waterlogging. Despite not being classified as a flood-tolerant species, recent evidence (Wu et al., 2024) and our observations suggest that peanut plants can partially endure short-term waterlogging with limited physiological damage. However, several studies have reported detrimental effects of prolonged flooding on peanut growth, photosynthesis, pod filling stage, and ultimately yield (Zeng et al., 2022; Bucior et al., 2025), highlighting the need for a better understanding peanut responses to these abiotic stresses. Here, we explored for the first time the responses of peanut plants to As and waterlogging, both individually and in combination, under controlled-environment conditions, using a low As concentration resembling a realistic field scenario. Additionally, we inoculated *Bradyrhizobium* sp. SEMIA6144, a strain previously shown to limit As translocation to peanut shootsts (Peralta et al., 2019).

4.1. Growth, nodulation, nitrogen, and arsenic concentrations

Legumes' growth and nodulation widely differ in their tolerance to waterlogging (Pucciariello et al., 2019). While *Medicago truncatula* maintains nodulation even after 4 weeks of flooding (El Msehli et al., 2016), other species like pea and white lupin show a 50 % reduction in nodule number after just 5 days (Pampana et al., 2016). In our study, 8-day waterlogging treatment did not affect peanut plant growth or nodulation (Supplementary Fig. 1, Tables 1 and 2), suggesting that peanut could be relatively tolerant to short-term flooding. This aligns with previous findings showing no negative effects in peanut plants exposed to short waterlogging periods of 3–5 days (Wu et al., 2024). Nevertheless, flooding in our experiment significantly decreased the

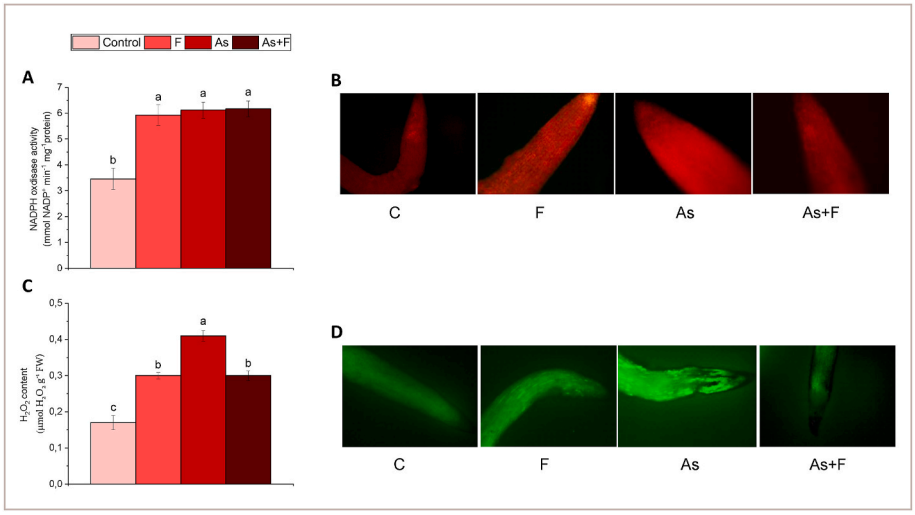

Fig. 4. Reactive oxygen species (ROS) production in peanut roots under control (C), flooding (F), arsenic (As), and combined arsenic + flooding (As + F) treatments. (A) NADPH oxidase enzyme activity. (B) Histochemical detection of O_2^- through DHE-dependent fluorescence in lateral roots, magnification: 10*X*. (C) Quantification of hydrogen peroxide (H_2O_2) in peanut root plants. (D) Visualization of H_2O_2 accumulation using DCF-DA dependent fluorescence in lateral peanut roots, magnification: 10*X*. Bars represent the mean ± SE (n = 5). Different letters indicate significant differences among treatments according to Duncan's test ($P < 0.05$).

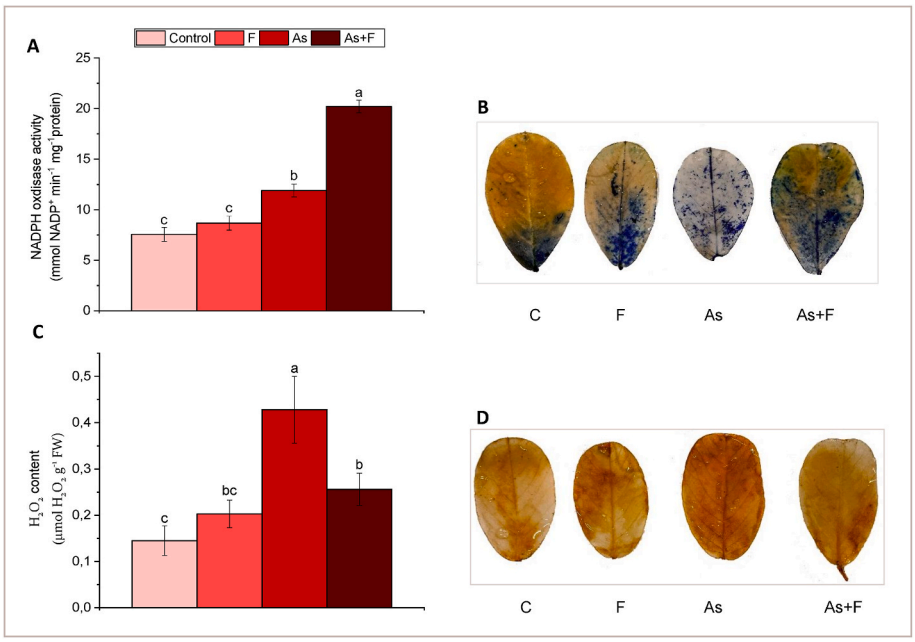

Fig. 5. Reactive oxygen species (ROS) production in peanut leaves under control (C), flooding (F), arsenic (As), and combined arsenic + flooding (As + F) treatments. (A) NADPH oxidase enzyme activity. (B) Detection of superoxide anion ($O_2^{\cdot-}$) by Nitroblue tetrazolium (NBT) staining. Blue formazan deposits indicate superoxide production. (C) Quantification of hydrogen peroxide (H_2O_2) in peanut leaves. (Visualization of H_2O_2 by 3,3′-diaminobenzidine (DAB) staining. Brown precipitate indicates H_2O_2 accumulation in the presence of peroxidase activity. Bars represent the mean ± SE (n = 5). Different letters indicate statistically significant differences among treatments according to Duncan's test ($P < 0.05$). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

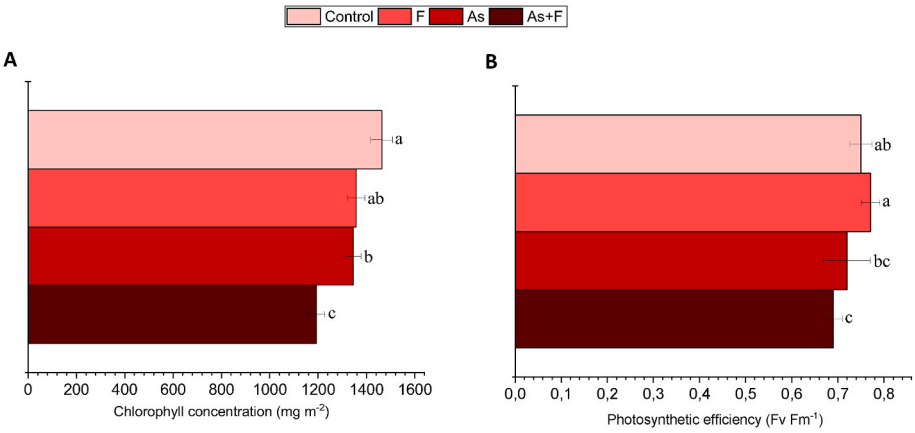

Fig. 6. Effects of individual and combined stress treatments on photosynthetic parameters in peanut plants. (A) Total chlorophyll concentration (mg m^{-2}). (B) And photosynthetic efficiency (Fv/Fm). Bars represent the mean ± SE (n = 5). Different letters indicate statistically significant differences among treatments according to Duncan's test ($P < 0.05$).

nitrogen content in peanut leaves to levels well below the sufficiency threshold of 3.5 % (Balota, 2014), indicating that nodule activity was impaired. Although growth was unaffected during the experiment, prolonged exposure could potentially compromise yield (Zeng et al., 2022; Bucior et al., 2025).

The significant drop in leaf nitrogen content under flooding suggests a decline in nodule activity, despite the unchanged number of nodules (Table 2). In fact, while the number of nodules was unaffected, the percentage of active nodules decreased by almost 40 % under waterlogging conditions (Table 2). Although nitrogenase functions under low-oxygen conditions, aerobic respiration in nodulated roots is essential to meet the high ATP demand of nitrogen fixation (Layzell et al., 1993). Thus, oxygen diffusion into roots and nodules must be tightly regulated. Waterlogging impairs this regulation, triggering adaptive responses such as adventitious root and aerenchyma formation to facilitate internal oxygen transport (Yamauchi et al., 2013; Zhang et al., 2025). In our study, both flooding and As + F treatments induced adventitious roots (Supplementary Fig 1-II), as an adaptive response to support growth and nodulation. However, the significant decline in active nodules and tissue N concentrations suggests that O_2 availability remained insufficient for optimal nodule function.

Arsenic exposure, even at the low environmentally relevant concentration of 3 µM used here, proved to be more detrimental to peanut growth than flooding (Table 1). This aligns with previous findings reporting strong phytotoxic effects of As in legumes, including peanut (Alam et al., 2019; Bianucci et al., 2020; Peralta et al., 2020). Arsenic distribution followed previously reported patterns, with the highest accumulation in roots and lower levels in nodules and shoots (Bianucci

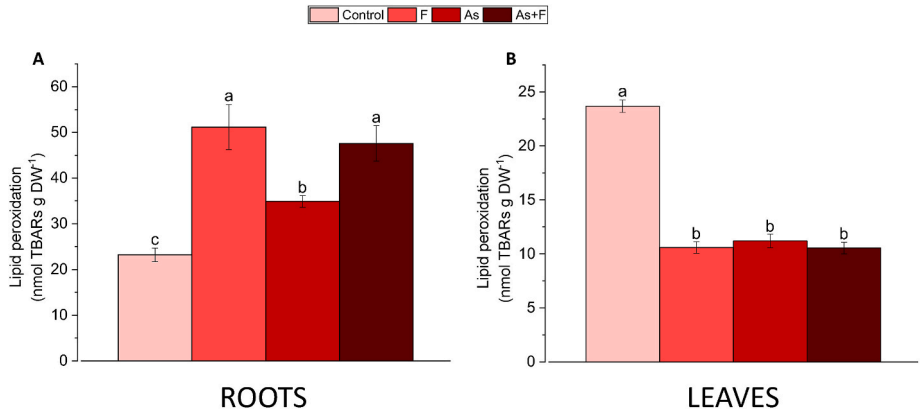

Fig. 7. Lipid peroxidation in peanut roots (A) and leaves (B) under control (C), flooding (F), arsenic (As), and combined arsenic + flooding (As + F) treatments. Lipid peroxidation was measured as malondialdehyde (MDA) equivalents (nmol TBARS g^{-1} DW). Bars represent the mean ± SE (n = 5). Different letters indicate statistically significant differences among treatments according to Duncan's test ($P < 0.05$).

et al., 2020), likely due to arsenate reductase activity limiting As translocation (Fischer et al., 2021). Despite the lower shoot As concentrations, shoot biomass was more severely reduced than root biomass (Tables 1 and 2), likely due to chloroplast damage and associated ROS production (Fig. 6; see section 4.3). This inhibitory effect in the shoots did not reduce the assimilate export to the roots, as indicated by the maintenance of root dry weight. However, root length was negatively impacted by the As treatment yielding a stunted architecture, a key trait for rhizobial infection and nodule formation. The combined treatment did not significantly change these variables in comparison to As alone, suggesting that the effect on growth and nodulation was mainly due to the presence of the metalloid rather than synergistic effects. Notably, the As concentration used mimics levels found in Córdoba's groundwater (Bécher Quinodóz et al., 2019), emphasizing that even low As exposure can severely compromise peanut development. Symbiotic nitrogen fixation, the sole nitrogen source in this experiment, is known to be highly sensitive to both As and flooding when applied individually (El Msehli et al., 2016; Pucciariello et al., 2019; Peralta et al., 2019). Until now, no information was available on the responses of inoculated peanut plants exposed simultaneously to both stress factors. Our results reveal a strong negative impact of both As and As + F treatments on nodulation and N accumulation. The reduced nodule number observed under these treatments may result from altered root morphology and direct toxicity to the microsymbiont, as was previously reported in peanut and lupin (Carpena et al., 2006; Bianucci et al., 2017, 2018; Peralta et al., 2019). Interestingly, the percentage of active nodules and leaf N content declined even when total nodule number was similar between both treatments, suggesting compromised N fixation efficiency. However, the combined treatment was the most detrimental. Moreover, the decline in the brownish red deposits in the infection zone (representing leghemoglobin presence in active nodules) confirmed a reduction in nodules' effectiveness (Table 2). These effects likely reflect inhibited nodule development, impaired nitrogenase activity, and/or compromised microbial infection under As and combined stress, which may exceed the protective capacity of *Bradyrhizobium* sp. under hypoxic conditions associated with flooding. Thus, the impact of the applied treatments could be attributed not only to a direct effect on microbial infection and nodule development, but also to alterations in the nitrogenase enzyme complex that limit N fixation (Souza et al., 2016). Given that biological N fixation was the sole N source, these findings underscore the vulnerability of this process to even low As concentrations commonly found in groundwater (Bécher Quinodóz et al., 2019).

4.2. Concentrations of other essential elements

Numerous studies have revealed that the uptake and transport of ions from root to shoot is highly disturbed under As and flooding conditions (Zhang et al., 2025; Kumar et al., 2018). In concordance with our results, a decline of P, S, K, Zn and Mg concentrations was observed in peanut plants exposed to As and the combined treatment, indicating a severe disorder of nutritional status. It is widely known that As (as arsenate) is a chemical analogue of phosphate and uses the same transporter to cross the plasma membrane of roots thus, competition between these two elements could be observed (Abbas et al., 2018). In this sense, the significant reduction of P levels of peanuts shows the prevalence that As has over P, leading to a disruption of phosphate metabolism. Regarding S metabolism, both low oxygen and the presence of As, can directly affect the availability of this element. Moreover, As reacts with SH- groups of proteins and enzymes reducing the antioxidant defense system and increasing oxidative damage (Finnegan and Chen, 2012). Other macronutrients such as K and Mg, also decreased in peanut. Low concentrations of these macronutrients could severely restrict shoot growth as was observed in *Arabidopsis thaliana* exposed to As + F treatment (Kumar et al., 2018). Potassium is an important regulator of growth in plants under flooding (Kumar et al., 2018; Wang et al., 2022). Magnesium, as a cofactor of phosphorylating enzymes and a core component of chlorophyll, is especially critical for energy metabolism and light harvesting. Thus, the marked Mg depletion in As + F plants may partly explain the decline in shoot biomass and chlorophyll integrity observed under these treatments (Fig. 6). Thus, both stresses compromise nutrient uptake and growth.

4.3. Oxidative stress markers

Cell death is the most impactful consequence of the low O_2 availability under flooding. Mostly linked to ROS production, a reprogramming of root architecture and programmed cell death is activated as a regulatory process to adapt to this unfavorable growth condition (O'Lexy et al., 2018). In our study, the vital staining of the peanut roots revealed clear root tip damage mostly under both F and As + F exposure, while in As-treated plants, cell death was also evident in the mature zone (Fig. 1-I). Additionally, As + F exposure induced substantial callose deposits in roots; mainly attributable to arsenic, and to a lesser extent to waterlogging (Fig. 1-II). Detailed analyses of root architecture changes, cell viability and transcriptomics induced in *A. thaliana* exposed to either or both As and flooding also revealed the strong impact of these treatments on root tip meristem viability and callose deposition (Kumar et al., 2020). The authors concluded that the changes in root architecture under the combined stress were induced by nutrient deficiencies and redox regulation.

The production of ROS in plants growing under unfavorable growth conditions, such as flooding, has a key role in the acclimation process

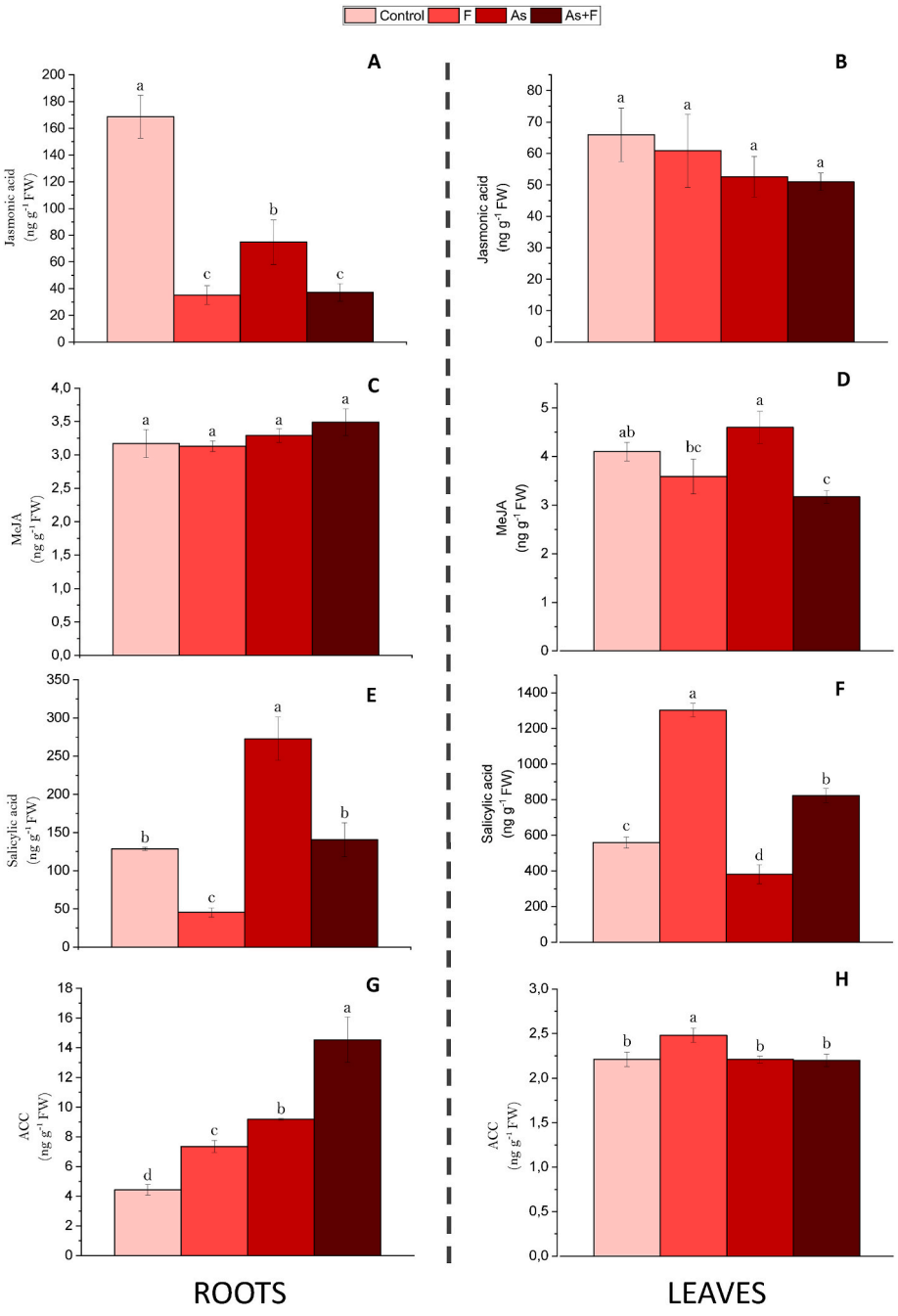

Fig. 8. Hormone content in peanut plant roots and leaves under control (C), flooding (F), arsenic (As), and combined arsenic + flooding (As + F) treatments. Bars represent levels of Jasmonic acid (JA), Salicylic acid (SA), Methyl jasmonate (MeJA) and 1-aminocyclopropane 1-carboxylic acid (ACC) in each organ. Values represent the mean ± SE (n = 5). Different letters indicate statistically significant differences among treatments according to Duncan's test ($P < 0.05$).

Table 4
Root organic acids composition in peanut plants exposed to flooding, As and combined stress.

	OXALACETIC ACID		GLICOLIC ACID		FORMIC ACID		MALIC ACID		ASCORBIC ACID		ACETIC ACID	
	mg ml^{-1}											
C	0.122	±0.0016^{a}	4.870	±0.79^{a}	9.12	±1.06^{b}	nd		nd		0.443	±0.032^{b}
F	0.124	±0.0019^{a}	4.590	±0.24^{a}	15.72	±1.06^{a}		nd	nd		1.968	±0.115^{a}
As	0.108	±0.0013^{c}	4.590	±0.24^{a}	1.20	±0.32^{d}	nd		0.015	±0.002^{a}	0.132	±0.021^{c}
As + F	0.115	±0.0027^{b}	6.090	±0.47^{a}	5.85	±0.29^{c}	0.396	±0.001	0.009	±0.0002^{b}	0.173	±0.065^{c}

Values represent the mean ± SE (n = 5). Different letters indicate significant differences among treatments according to the Duncan's test ($P < 0.05$). Abbreviations: nd: not detected.

Table 5
Leaves organic acids composition in peanut plants exposed to flooding, As and combined stress.

	OXALIC ACID		PIRUVIC ACID		MALIC ACID		MALONIC ACID		ISOCITRATE ACID		LACTATE		ACETATE		CITRATE		SUCCINIC ACID		ACONITIC ACID	
	mg ml^{-1}																			
C	nd		nd		1.45	±0.06^{c}	2.79	±0.28^{a}	6.92	±0.22^{a}	4.06	±0.45^{a}	1.77	±0.33^{b}	0.92	±0.14^{a}	2.41	±0.22^{a}	1.48	±0.09^{a}
F	0.0424	±0.0028	0.159	±0.01^{a}	nd		1.24	±0.1^{b}	nd		nd		4.5	±0.2^{a}	0.19	±0.01^{b}	2.00	±0.14^{a}	nd	
As	nd		nd		10.23	±0.11^{b}	nd		nd		nd		nd			nd	1.32	±0.06^{b}		
As + F	nd		0.264	±0.018^{b}	4.69	±0.41^{a}	nd		1.07	±0.22^{b}	1.29	±0.09^{b}	nd		nd		nd		0.31	±0.01^{b}

Values represent the mean ± SE (n = 5). Different letters indicate significant differences among treatments according to the Duncan's test ($P < 0.05$). Abbreviations: nd: not detected.

(Zhang et al., 2025), being the participation of NADPH oxidases outstanding in ROS production. In *Arabidopsis*, it was observed that the NADPH oxidase gene *RbohD* is upregulated at both the transcript and protein levels, leading to an oxidative burst when exposed to hypoxic stress (Baxter-Burrell et al., 2002). It is well established that As induces ROS production; furthermore, the formation of As complexes with SH-containing molecules such as GSH favors ROS accumulation by reducing the antioxidant capability of cells (Finnegan and Chen, 2012; Bianucci et al., 2017; Peralta et al., 2020, 2021). The oxidative response of peanut plants exposed to combined As + F stress is poorly understood. Peanut plants exposed to F and As treatments increased NADPH oxidase activity and ROS levels. Especially, under the combined treatment, enhanced enzyme activity was detected in leaves, but a reduction of H_2O_2 content in roots and leaves was observed, indicating a clear activation of ROS signaling and antioxidant mechanisms to preserve cells from severe and irreversible damage.

4.4. Photosynthetic pigments and lipid peroxidation

Photosynthesis is a reliable metabolic indicator of plant health. Both O_2 depletion and As affect this vital process by altering the respiratory electron transport, carbon fixation, and the production of ATP (Finnegan and Chen, 2012). The chlorophyll content of peanut plants was severely affected by As and combined treatments while the photosynthetic efficiency was only reduced in the As + F treatment, compared to the control suggesting a functional C fixation after the applied treatment. This alteration could be explained by ROS production and the observed mineral imbalances, particularly in Mg, P, and S concentrations. The reduction of Mg directly impacts the photosynthetic process, as it is a key component of the molecule of chlorophyll. In addition, the decreases in P and S alter the plant metabolism by increasing As uptake and reducing the antioxidant capacity. In the combined treatment both variables were severely affected, indicating a synergistic response probably due to alterations in the plant's oxidative status. In contrast to our results, no changes in chlorophyll content were observed in red clover plants under low As concentrations, in inoculated *M. truncatula* plants exposed to flooding, or in *A. thaliana* exposed to As + F stress (Mascher et al., 2002; El Msehli et al., 2016; Kumar et al., 2018).

The oxidative burst observed in peanut roots was followed by an increase in lipid peroxidation, indicating oxidative stress, independently of the treatment applied. In contrast, this response was not detected in leaves. These results indicate that a signaling response is activated mainly attributable to the plant defense system being more efficient in leaves, even though a reduction in photosynthetic variables was observed.

Despite the observed oxidative stress responses and the functional impairment of photosynthesis, mineral concentrations in leaves were comparatively less affected. This likely reflects intrinsic physiological and biochemical regulation. As the primary site of arsenic uptake and ion exchange, roots are more directly impacted by As-induced imbalances and waterlogging-related hypoxia, explaining their stronger ionomic shifts. In contrast, nutrient concentrations in leaves tend to be more tightly controlled to preserve vital functions like photosynthesis under stress. For instance, Mishra et al. (2019) highlighted that plants exposed to arsenic activate defense mechanisms in aerial tissues that help to sustain the ionic equilibrium despite oxidative damage. Similarly, Lin et al. (2023) reported transcriptional regulation of genes related to nutrient transport and metal homeostasis in arsenite-exposed rice, reinforcing the idea that nutrient stability in leaves is prioritized to prevent metabolic collapse. Together, these findings suggest that the limited ionomic changes observed in peanut leaves may represent a protective strategy to maintain metabolic function.

4.5. Organic acids and phytohormones

Hypoxic conditions under waterlogging have a severe impact on

plants' energy and carbon metabolism. Anaerobic respiration in waterlogged roots causes ATP deficit, alteration of C and N metabolism, and toxin accumulation (Rengel, 2023; Rengel et al., 2023). Moreover, As can further reduce ATP biosynthesis by uncoupling both mitochondrial and photosynthetic electron transport chains and increasing fermentative processes (Finnegan and Chen, 2012). So, it may be expected that simultaneous exposure to waterlogging and As would have synergistic or at least additive detrimental effects.

The ability to shift from aerobic to anaerobic metabolism provides, at least temporarily, an alternative low-energy supply to maintain vital processes (Manghwar et al., 2024). Fermentative metabolism implies a high demand for carbohydrates in submerged roots. A major consequence is the accumulation of ethanol, acetaldehyde, and organic acids like formate, acetate, and or lactate that may become toxic to the plant. Transport to leaves and volatilization may help reduce the accumulation of ethanol, acetaldehyde and acetate in the roots (Jardine and McDowell, 2023). Recently, it has been shown that in flooding-tolerant soybean lactate, the main fermentation product is exported to the leaves where it is metabolized to malate and succinate (Posso et al., 2025).

In the roots of our flooded experimental plants formate was the most abundant organic acid (Table 4), while acetate was the most abundant in the leaves (Table 5), suggesting a possible root-to-shoot transport of acetate indicating the fermentative shift from pyruvate. High root exudation of formate into waterlogged soil has been reported in flood tolerant plants (Meng et al., 2022). The exact pathway of fermentative formate production in plants is still under debate. Root accumulation may be further enhanced by the inhibition of either or both ATP-driven formate conversion and formate oxidation to CO_2. Under flooding conditions, the induced anaerobic state in the root system alters the levels of TCA cycle intermediates in roots and leaves (especially oxalic, formic, and acetic acids). These changes are consistent with a shift from aerobic respiration to fermentation-based metabolism. This adaptation activates fermentation to sustain at least a limited supply of ATP and NADH. Additionally, the lack of oxygen stimulates photorespiration, ultimately affecting plant growth but supporting N demand via glutamate. Arsenic exposure reduced the levels of both formate in roots and acetate in leaves, while strongly enhancing the accumulation of malic acid in the leaves. Arsenic is highly toxic to the mitochondrial respiration process, and besides ATP deficit, causes the accumulation of TCA cycle intermediates. Malate dehydrogenase is particularly sensitive, and even the expression of this protein can be completely suppressed (Requejo and Tena, 2006). Therefore, the impact on growth and oxidative status in peanut plants exposed to As can be attributed to a significant disruption of the TCA cycle, regardless of the organ analyzed but especially in leaves. This disturbance is evident in the reduction of organic acid production with a marked accumulation of malic acid and a reduction of succinic acid (Table 5), ultimately resulting in decreased ATP and NADH production and the alteration of the oxidative status (Fig. 5). Additionally, an activation of the defense response was observed through increased ascorbate accumulation in the roots. Our results agree with those in rice exposed to arsenate (Saha et al., 2017). The combined stress appears to have an intermediate response by accumulating TCA intermediates mainly from the flooding condition, but also ascorbic and malic acid from the As treatment. However, the impact on plant growth and nodulation was detrimental. This fermentative response under combined arsenic and flooding stress likely reflects an adaptive mechanism through which peanut plants attempt to sustain a minimal ATP supply and mitigate redox imbalance under unfavorable conditions. The accumulation and organ-specific distribution of organic acids such as formate, acetate, pyruvate, and malate suggest a metabolic reprogramming aimed at coping with energetic and oxidative stress. Although insufficient to prevent growth inhibition, this response highlights the metabolic plasticity of peanut under dual stress and contributes to our understanding of the physiological modifications involved. Altogether, these metabolic shifts reveal a fermentative adjustment intended to mitigate energy and redox imbalance, although ultimately insufficient to sustain growth and nodulation under combined stress. Phytohormones play a crucial role in regulating plant responses to adverse environmental conditions. In our study, As and F, either alone or in combination (As + F), differentially affected the concentrations of JA, SA, and ACC in peanut roots and leaves (Fig. 8). Jasmonic acid and its derivatives as MeJA play a key role in regulating physiological and molecular processes by participating in complex signal transduction pathways, allowing plants to counteract the negative effects of abiotic stress (Rehman et al., 2023). The reduction of this phytohormone by heavy metals or metalloids leads to a significant negative effect on growth and photosynthetic pigments; however a recovery of the mentioned variables could be achieved by MeJA foliar application (Mousavi et al., 2020). JA levels significantly decreased in roots under all stress treatments, suggesting a reduced signaling capacity for stress response activation. Since JA is involved in the regulation of defense mechanisms against biotic and abiotic stresses, this reduction could indicate a diminished ability of the plants to counteract the negative effects of As and F, particularly under prolonged flooding conditions. It is well known that JA biosynthesis depends on the presence of oxygen in plant tissues (Lee et al., 2020). In our study, JA accumulation in roots was primarily affected by flooding, with As having a lesser impact. In contrast, JA concentrations in leaves remained unchanged, suggesting that the root system is the main site of hormonal regulation under stress conditions (Fig. 8A–B). Therefore, the observed negative changes in plants exposed to As, F, and the combined As + F treatment could be attributed to a reduced cellular capacity to counteract the adverse effects, particularly under flooding conditions. Salicylic acid plays a crucial role in plant response to flooding (Hasanuzzaman et al., 2022). Also, SA has been shown to help plants exposed to heavy metal or metalloid exposure (Sharma et al., 2020). In our work, SA revealed a contrasting response in roots and leaves of peanut plants exposed to F or As (Fig. 8E–F). In this regard, the increment of SA levels in leaves, but the significant decrease in roots of plants exposed to flooding indicates a clear signaling activation from roots to shoot probably caused by the high levels of oxidative stress observed in roots in order to maintain vital processes in leaves as photosynthesis variables (Fig. 6A–B and 7A). The opposite behavior was observed under As treatment where a clear accumulation of SA was detected in roots to modulate plant responses to this abiotic stress. These findings underscore the deleterious effects of As, as reflected in reduced leaf SA, impaired photosynthesis, and elevated oxidative damage. In the combined treatment As + F, roots maintained the level of SA, meanwhile in leaves an increase was observed. However, an increased oxidative stress in roots was found together with reduced growth and photosynthesis variables, indicating a synergistic negative effect of the treatments. This contrasts with the results found by Kumar et al. (2018) in *A. thaliana* since treatment with As, F, and As + F maintained SA levels in roots and leaves similar to control plants. The direct precursor of ethylene, ACC, is a key phytohormone in plant responses to flooding. Under this condition, ACC transformation into ethylene can be inhibited and the transport of ACC from root to shoot in flooded plants serves as an important signal to improve their tolerance (Vanderstraeten and Van Der Straeten, 2017). In line with previous findings showing that arsenic elevates ethylene levels (Singh et al., 2021), we observed an accumulation of ACC in peanut roots exposed to As and As + F, with notably higher concentrations in roots than in leaves (Fig. 8G–H). However, this ACC could not be efficiently converted into ethylene, likely due to hypoxic conditions in the roots caused by limited oxygen availability. The increase of both ACC and SA observed in peanut roots exposed to arsenic suggests an important activation of the signaling mechanisms to improve plant tolerance under the unfavorable growth conditions tested. In the combined stress, although ACC levels remained high in roots, the SA accumulation observed under As alone was no longer present, suggesting a disrupted hormonal induction caused by flooding.

5. Conclusion

This study demonstrates that both arsenic and flooding compromise growth, nodulation efficiency, nutrient homeostasis, and oxidative balance in peanut plants. Their combined effect (As + F) was not always additive, but it showed synergistic effects in several key parameters, particularly evident in oxidative stress, photosynthetic damage, and disruption of carbon and nitrogen metabolism. Arsenic toxicity primarily triggers oxidative stress and disrupts essential biochemical processes, whereas flooding-induced hypoxia shifts the metabolism toward fermentation. Under combined As + F exposure, peanut plants exhibited unique physiological signatures not seen under individual treatments, including altered phytohormonal regulation, increased accumulation of malic acid and ascorbate, and severe reductions in nitrogen fixation and photosynthesis. These findings confirm our initial hypothesis of additive or synergistic stress interactions and underscore the importance of metabolic plasticity as a temporary coping strategy in peanut, albeit insufficient to fully mitigate stress damage. Understanding this complexity is crucial for guiding crop management and biotechnological interventions aimed at improving stress tolerance in legumes under increasingly variable environmental conditions.

CRediT authorship contribution statement

Eliana Bianucci: Writing – review & editing, Writing – original draft, Supervision, Resources, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Ana Laura Furlan:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis. **Mercè Llugany:** Writing – review & editing, Methodology, Investigation. **Charlotte Poschenrieder:** Writing – review & editing, Writing – original draft, Supervision, Resources, Funding acquisition, Conceptualization. **Roser Tolrà:** Writing – review & editing, Writing – original draft, Methodology, Investigation.

Availability of data and material

All data and materials support their published claims and comply with field standards.

Code availability

Not applicable.

Ethics approval

Not applicable.

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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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2025.110266.

Data availability

Data will be made available on request.

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