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Exogenous curcumin mitigates As stress in spinach plants: A biochemical and metabolomics investigation

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ABSTRACT

The spinach (*S. oleracea* L.) was used as a model plant to investigate As toxicity on physio-biochemical processes, exploring the potential mitigation effect of curcumin (Cur) applied exogenously at three concentrations (1, 10, and 20 μM Cur). The employment of Cur significantly mitigated As-induced stress in spinach photosynthetic performance (F_v/F_m, F_o/F_m, and F_v/F_o). Moreover, the co-incubation of Cur with As improved physiological processes mainly associated with plant water systems affected by As stress by recovering the leaf's relative water content (RWC) and osmotic potential (ψπ) nearly to the control level and increasing the transpiration rate (E; 39–59%), stomatal conductivity (g_s; 86–116%), and carbon assimilation rate (A; 84–121%) compared to As stressed plants. The beneficial effect of Cur in coping with As-induced stress was also assessed at the plant's oxidative level by reducing oxidative stress biomarkers (H_2O_2 and MDA) and increasing non-enzymatic antioxidant capacity.

Untargeted metabolomics analysis was adopted to investigate the main processes affected by As and Cur application. A multifactorial ANOVA discrimination model (AMOPLS-DA) and canonical correlation analysis (rCCA) were employed to identify relevant metabolic changes and biomarkers associated with Cur and As treatments. The results highlighted that Cur significantly determined the accumulation of glucosinolates, phenolic compounds, and an increase in glutathione redox cycle activities, suggesting an overall elicitation of plant secondary metabolisms. Specifically, the correlation analysis reported a strong and positive correlation between (+)-dihydrokaempferol, L-phenylalanine (precursor of phenolic compounds), and serotonin-related metabolites with antioxidant activities (ABTS and DPPH), suggesting the involvement of Cur application in promoting a cross-talk between ROS signaling and phytohormones, especially melatonin and serotonin, working coordinately to alleviate As-induced oxidative stress. The modulation of plant metabolism was also observed at the level of amino acids, fatty acids, and secondary metabolites synthesis, including N-containing compounds, terpenes, and phenylpropanoids to cooperate with As-induced stress response.

1. Introduction

The uncontrolled increases in industrialization and urbanization have caused the excessive accumulation of heavy metals in agricultural systems, adversely impacting plant germination, growth, and reproduction (Ma et al., 2022b). There are different natural and anthropogenic sources of arsenic (As) contamination, which is a carcinogenic element and highly toxic. Among these sources, mining, the erosion of rocks, geothermal waters, volcanic activities, industrial wastes, and the use of pesticides can be counted (Alsafran et al., 2022). Pollution hampers cell division, chromosomal structure, and the proteins related to the signaling mechanisms in the cell cycle, thus inhibiting the growth of plants. Acute As poisoning also disrupts the nutrient uptake, photosynthetic pigments, electron transfer system (ETS) in chloroplast and mitochondria, glutathione (GSH) consumption, and glycolate oxidase reaction in plants (Alvarenga et al., 2020). The impairment of the

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oxidation/reduction process, especially in photosynthesis and respiration, promotes reactive oxygen species (ROS) in plants exposed to excessive As toxicity. As-mediated oxidative stress causes enzyme inactivation, disrupted senescence, lipid peroxidation, disordered membrane functionality, and protein oxidation (Bhat et al., 2022). To eliminate disturbances on redox balance induced by stress, one of the main alterations in the biological systems is the activation of antioxidant mechanisms, including superoxide dismutase (SOD), peroxidase (POX), and catalase (CAT), ascorbate peroxidase (APX) and glutathione reductase (GR) (Khan et al., 2023a). The same study showed that producing compounds derived from secondary metabolisms is another defensive response to coping with As stress.

The plants with stress treatments exhibit the induced biosynthesis of secondary metabolites, a marker of tolerance to adverse conditions (Benjamin et al., 2020). Inorganic acids, carbohydrates, terpenes, alkaloids, dipeptides, and phytohormones as metabolome contribute to osmotic adjustments, antioxidant system, ROS scavenging, and membrane stabilization (Benjamin et al., 2019). Since these compounds have important roles, metabolomic profiling is a powerful tool in giving more detail about tolerance responses against stress conditions. Among these metabolites, curcumin (Cur, 1,7-bis-(4-hydroxy-3-methoxyphenyl)-1, 6-heptadiene-2,5-dione), is a polyphenol compound, has anti-cancer, anti-angiogenic and anti-inflammatory properties (Rafiee et al., 2019). There are few reports about the metal-chelating effects of Cur under heavy metal toxicity (Park et al., 2021). Liu et al. (2023) reported that Cur had a dominant role in As detoxification by providing less bioaccumulation of metals in human systems. The findings obtained from pharmacological studies that Cur extracted from *Curcuma longa* eliminates oxidative stress by decreasing lipid peroxidation and inhibiting radical production (Tang et al., 2021). Also, Cur can scavenge radicals as a potent antioxidant. This property of Cur depends on the presence of methoxy, ketone aldehyde, and phenolic groups (Priyadarsini, 2014). Mekkara and Bukkan (2021) indicated that Cur application (10 μM) prevents protein degradation and membrane stability against oxidative stress. As-mediated reduced germination is removed in Cur-applied *Vigna radiata* by increasing alpha-amylase activity (Khan et al., 2023b). Since the interaction of Cur with other metabolites has been scarcely studied in plants, the investigation of Cur-triggered metabolic profiling in the plants co-exposed to Cur and As pollution constitutes the originality of this study. Besides, limited information has been conducted on the effects of Cur on antioxidant activity, ROS accumulation, and chlorophyll fluorescence in As-stressed plants.

One cultivated vegetable crop that can withstand excessive As concentration is spinach (*Spinacia oleracea* L.), belonging to Chenopodiaceae (Tang et al., 2020). *S. oleracea* contains vitamins (C, A, and B), antioxidants, phenolics, and minerals (Roberts and Moreau, 2016). As well as the responses to heavy metals, its properties, such as high growth rate, strong antioxidant system, and induced biomass production, make spinach a good candidate for studies on defense performance against various heavy metals (Hussain et al., 2022; Zaheer et al., 2020a). In recent years, some findings have documented the negative impacts on *S. oleracea* under heavy metal toxicity (Ma et al., 2022a; Saleem et al., 2023; Sun et al., 2023; Zafar et al., 2022). However, there is no information on how Cur-mediated repair mechanisms in the presence of As pollution interact with photosynthesis, the elimination activities of ROS, and metabolite accumulation in *S. oleracea*. On this basis, we hypothesized that the exogenous application of curcumin may alleviate the detrimental effects on plants associated with As pollution. To provide data on this postulation, the objectives of the current study were to explain the changes in terms of growth, chlorophyll fluorescence, antioxidant machinery, ROS content, lipid peroxidation, and metabolite profile in *S. oleracea*.

2. Material and methods

2.1. Experimental methodology

Spinach seeds (*Spinacia oleracea* L.) were sterilized with 10% bleach and allowed to germinate. Curcumin (C1386) was purchased from Sigma-Aldrich (Darmstadt, Germany). Seedlings were grown in hydroponic Hoagland solution (1M KNO_3, 1M $CaNO_3$, 0.4 M $MgSO_4$, 0.2 M KH_2PO_4, 0.03 M H_3BO_3, 0.002 M $CuSO_4$, 0.004 M $ZnSO_4$, 0.005 M $MnCl_2$, 0.001 M $(NH_4)Mo_7O_{24}$ and 0.2 M Fe-EDTA) under controlled conditions (12-h light/12-h dark regime; 24 °C; 70% relative humidity; 350 μmol m^{-2} s^{-2} photon flux density). Thirty healthy three-week-old seedlings for each group were used in curcumin (Cur1, 1 μM; Cur2, 10 μM; Cur3, 20 μM) treatments with or without As stress (100 μM, $Na_2HAsO_4{\cdot}7H_2O$). The As and phenolic treatment doses applied to spinach plants were chosen by previous studies (Parvin et al., 2020; Saleem et al., 2023). Environmentally detectable As pollution is considered 5 mg kg^{-1} on average, while the amount of As in the soil is up to 15 mg kg^{-1} in Germany and 626 mg kg^{-1} in China (Fatoki and Badmus, 2022). 100 μM sodium arsenate corresponds to a concentration of about 7 mg L^{-1} As in the hydroponic culture of spinach seedlings. After 7 days of the treatment, gas exchange and chlorophyll-*a* fluorescence parameters were measured in the experimental groups, and samples were collected for physiological and biochemical analyses.

2.2. Analysis of physiological parameters

After seven days, six leaves were harvested from each group, and their fresh weight (FW) was measured. After being soaked in de-ionized water for 6 h, the turgid weight (TW) was determined. The leaves were dried in the oven at 70 °C, their dry weight (DW) was measured, and the relative water content (RWC) was calculated by the formula stated by Maghsoudi et al. (2019). Vapro Vapor Pressure Osmometer 5600 was used to determine leaves osmotic potential (Ψ_Π). Ψ_Π was converted to MPa as reported by Santa-Cruz et al. (2002). Carbon assimilation rate (A), stomatal conductance (g_s), intercellular CO_2 concentration (C_i), and transpiration rate (E) as gas exchange parameters were measured with a portable gas exchange system on leaves of three plants per treatment group (LCpro+; ADC).

2.3. Recording data for chlorophyll-a fluorescence parameters

A portable fluorometer (Handy PEA, Hansatech Instruments Ltd., Norfolk, UK) was used to determine the maximal quantum yield of PSII photochemistry (F_v/F_m), physiological state of the photosynthetic apparatus (F_o/F_m) and potential photochemical efficiency (F_v/F_o).

2.4. Oxidative stress biomarker assays

H_2O_2 content in leaves was evaluated using the method described by Velikova et al. (2000), and lipid peroxidation level (TBARS content) was computed using the method described by Rao and Sresty (2000).

2.5. Examination of antioxidant and enzyme inhibitory effects

For the assessment of antioxidant potential in the extracts, a battery of six complementary *in vitro* spectrophotometric assays was conducted. These included the ABTS and DPPH assays, evaluating the extracts' ability to neutralize free radicals. Additionally, we utilized the FRAP and CUPRAC assays to measure the extracts' reduction capabilities, along with the metal chelating ability (MCA) and phosphomolybdenum (PBD) assays. All assays, except for MCA, were standardized using the Trolox standard. The comparison for MCA was expressed in terms of equivalent EDTA per gram of extract. Detailed procedures for each assay are outlined in our previous work (Uysal et al., 2017). To evaluate the inhibitory effects of the tested extracts on various enzymes,

acetylcholinesterase (AChE), butyrylcholinesterase (BChE), tyrosinase, amylase, and glucosidase were employed. Comprehensive information on the experimental procedures can be found in our previous publication. (Uysal et al., 2017). AChE and BChE inhibitions were quantified in milligrams of galanthamine equivalents (GALAE) per gram of extract, tyrosinase inhibition in milligrams of kojic acid equivalents (KAE) per gram of extract, and α-amylase and α-glucosidase inhibition in millimoles of acarbose equivalents (ACAE) per gram of extract.

2.6. Metabolomics and multivariate statistical analysis

The metabolomics profile of *S. oleracea* extract, following a specific extraction method (supplementary material), was performed using an ultra-high performance liquid chromatography-quadrupole time-of-flight mass spectrometry (UHPLC-QTOF-MS) instrument. Specifically, we used a 1290 liquid chromatograph coupled to a G6550 mass spectrometer detector via a Dual Electrospray Jet Stream ionization system (all from Agilent Technologies, Santa Clara, CA, USA). The instrumental conditions for untargeted analysis are reported in detail as supplementary materials. Datasets normality and homogeneity were assessed using Shapiro's and Levene's tests in R studio (v. R 4.2.3). Analysis of Variance (ANOVA) associated with the Tukey post hoc test was also conducted in R studio.

3. Results

3.1. Photosynthetic performance parameters

The assessment of spinach leaf photosynthetic performance under various treatments involved the examination of key parameters, including the maximal quantum yield of PSII photochemistry (F_v/F_m), the physiological state of the photosynthetic apparatus (F_o/F_m) determined by the rate of the opened reaction center relative to the closed reaction center, and the potential photochemical efficiency (F_o/F_m). The latter parameter was derived from the ratio of the photochemical and nonphotochemical utilization rates of reaction center light energy, equaling the ratio of the rate constants for the primary photochemical reaction and the total rate of nonphotochemical loss. The increased concentration of Cur application reported no statistically significant difference in photosynthetic performance parameters compared to the control (Fig. 1A); nevertheless, it mitigated the adverse effects of As stress (Fig. 1B).

3.2. Gas exchange parameters

The effect of As stress alone and treatments with increasing concentrations of Cur with or without As stress was assessed considering various gas exchange parameters (Table S2), including intercellular CO_2 concentration (C_i), transpiration rate (E), stomatal conductance (g_s), and carbon assimilation rate (A), strictly correlated to the efficiency of the photosynthetic performance and carbon assimilation process.

The treatment with Cur significantly modulated gas exchange parameters under both As stress and un-stress conditions. Specifically, the application of Cur at 1 μM significantly increased E, g_s, and A values compared to the control. In contrast, Cur at 10 μM produced the opposite effect, significantly reducing these parameters. As stress increased significantly C_i and reduced E, g_s, and A values compared to the control. Nevertheless, the co-exposure of As with increased Cur concentration led to the modulation of these parameters to the levels of control or even higher.

3.3. Physiological and oxidative stress parameters

The effect of Cur treatment alone and combined with As stress on spinach was also evaluated considering other physiological parameters, including leaf relative water content (RWC; Fig. 2A and B) and osmotic potential (ψπ; Fig. 2C and D), assess the water preservation potential as well as membrane capacity. Moreover, the quantification of oxidative molecules such as hydrogen peroxide (H_2O_2; Fig. 2E and F) and malondialdehyde (MDA; Fig. 2G and H) contents was performed to determine the overall oxidative stress status.

Specifically, the application of Cur at different concentrations significantly increased leaf RWC (1 μM Cur) and osmotic potential (ψπ; 10 and 20 μM Cur) under non-stressed conditions (Fig. 2A–C). On the other hand, As stress reduced by 20–30% of the spinach leaf RWC and osmotic potential (Fig. 2B–D). Interestingly, Cur treatments, especially at 10 μM, significantly recovered these parameters at the level of the control (Fig. 2B).

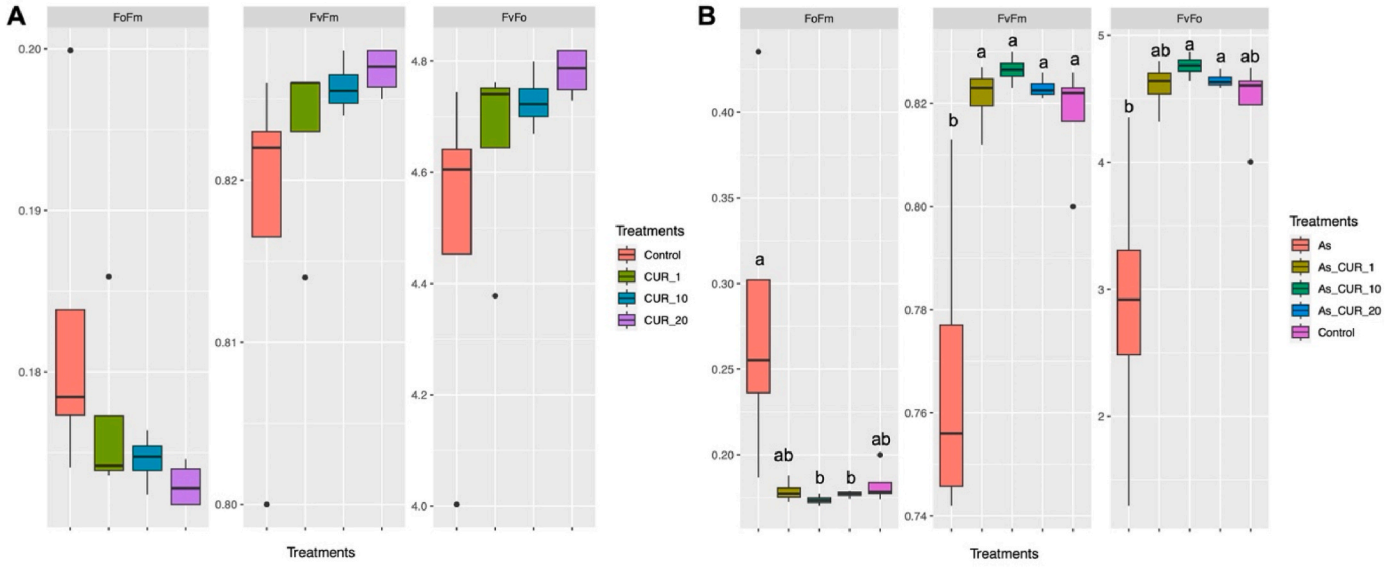

Fig. 1. Photosynthetic performance parameters of spinach leaf considering maximal quantum yield of PSII photochemistry (F_v/F_m), physiological state of the photosynthetic apparatus (F_o/F_m), and potential photochemical efficiency (F_v/F_o). (**A**) shows the effect of increased concentration of curcumin application compared to the control: 1 μM of curcumin (CUR_1), 10 μM of curcumin (CUR_10), and 20 μM of curcumin (CUR_20). (**B**) shows the effect of increased concentration of curcumin application under arsenic stress (As; 100 μM).

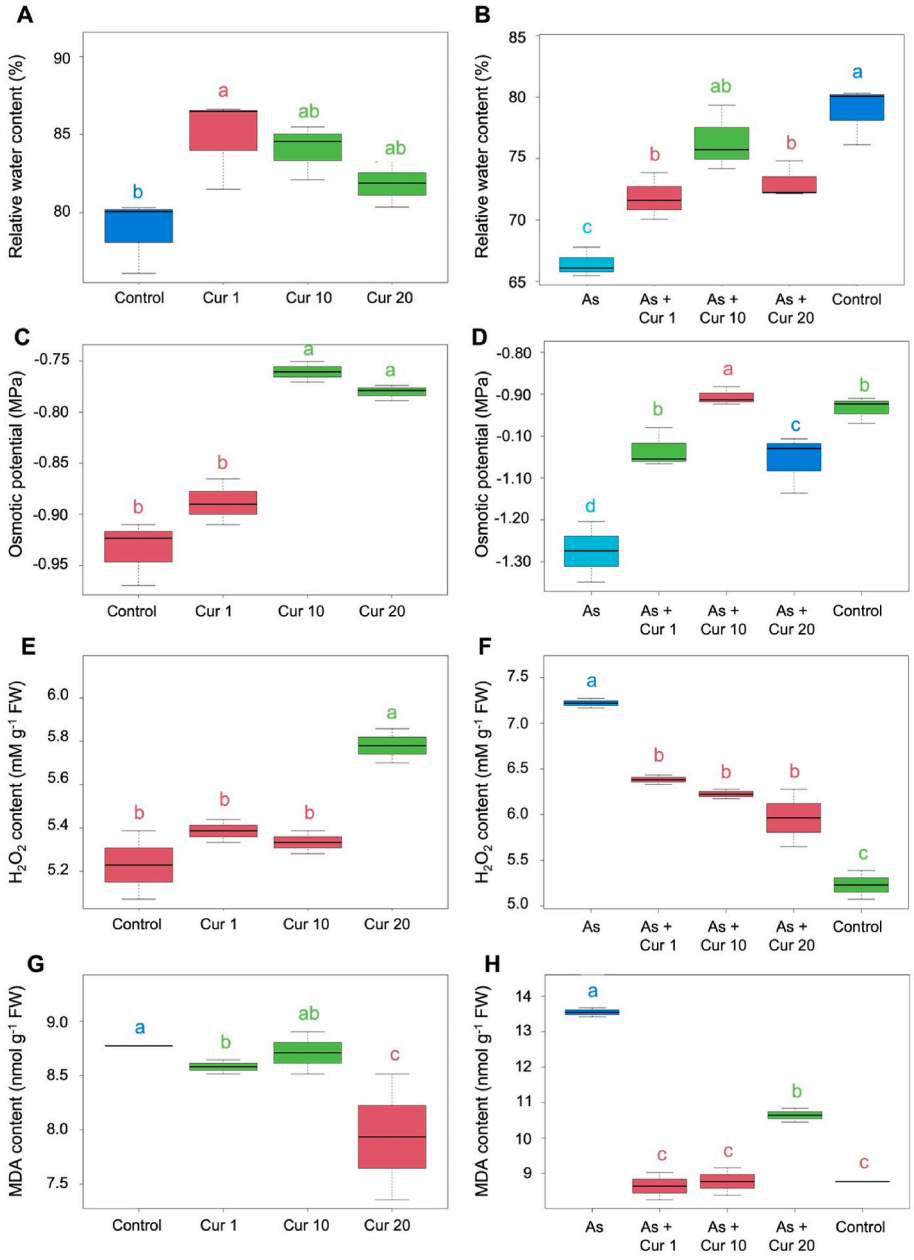

Fig. 2. Physiological parameters of [**A-B**] leaf relative water content (RWC), [**C-D**] Osmotic potential, [**E-F**] hydrogen peroxide content (H_2O_2), and [**G-H**] malondialdehyde content (MDA), evaluating the effect of increased concentration of curcumin application (1 μM Cur, 10 μM Cur, and 20 μM Cur) under both As stress (100 μM) or control conditions.

Regarding oxidative stress biomarkers, the higher dosage of Cur (20 μM) and As stress triggered a substantial accumulation of oxidative markers, such as H_2O_2 and MDA content, compared to the control (Fig. 2E–H), indicating a significant dysregulation in tissue oxidative status. Cur application exhibited a specific mitigation effect on the lipid peroxidation process, with a lesser extent on H_2O_2 species than the control (Fig. 2F–H).

3.4. Non-enzymatic antioxidant capacity in spinach leaves treated with increased concentration of curcumin under As stress and no-stressed conditions

The non-enzymatic antioxidant properties of leaf extract were investigated using several antioxidant assays, including DPPH (2,2-diphenyl-1-picrylhydrazyl; mg TE/g), ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid; mg TE/g), CUPRAC (cupric reducing antioxidant capacity; mg TE/g), FRAP (ferric reducing antioxidant power; mg TE/g), PMD (phosphomolybdenum; mmol TE/g), MC (metal chelating; mg EDTAE/g) activities, to comprehensively determine the radical scavenging activity after Cur treatment in the absence (Fig. 3A) and presence (Fig. 3B) of As stress. The employment of Cur at 1 μM dosage under no-stressed conditions reported a significantly increased antioxidant activity in terms of DPPH, ABTS, CUPRAC, and FRAP. The As-induced stress condition determined a general reduction of antioxidant capacity compared to the control; however, the co-application with Cur reverted the negative trend in the case of CUPRAC and FRAP assays. Different behaviors were reported in the DPPH and ABTS potential under As stress, where the co-application with Cur at 10 μM was the only dosage able to revert into the control status.

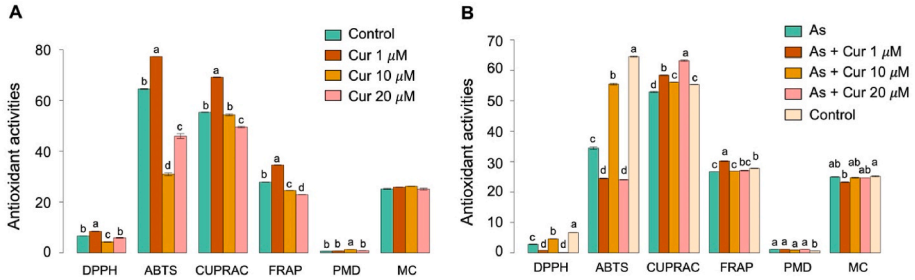

Fig. 3. [**A-B**] DPPH (2,2-diphenyl-1-picrylhydrazyl; mg TE/g), ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid; mg TE/g), CUPRAC (cupric reducing antioxidant capacity; mg TE/g), FRAP (ferric reducing antioxidant power; mg TE/g), PMD (phosphomolybdenum; mmol TE/g), MC (metal chelating; mg EDTAE/g) activities of spinach leaves treated with increased concentration of curcumin (1 μM Cur, 10 μM Cur, and 20 μM Cur) in absence [A] and presence [B] of As stress (100 μM).

3.5. Leaf metabolomics profile and multivariate statistical analysis

The metabolic profiling of spinach leaves subjected to three concentrations of Cur under both As-stressed and non-stressed growing conditions was evaluated using an untargeted metabolomics approach, leading to the putative annotation of over 3000 metabolites (Table S3). The entire dataset was inspected for similarities and dissimilarities across treatments, incorporating both Cur treatments and As stress factors. A hierarchical clustering analysis (HCA), produced from the fold-change of each annotated compound, was employed (Fig. 4A), revealing As stress as the most discriminant factor. The resulting two distinctive clusters highlighted the precise metabolic regulation influenced by As stress compared to non-stressed conditions. Based on these considerations, two principal component analyses (PCAs) were conducted to assess the metabolic profiles among treatments. As reported in Fig. 4B, the model significantly explained over 52% of all metabolic changes influenced by the increasing application of Cur, with Cur at 10 μM showing the most impactful one. Considering the As stress effect, the PCA model distinctly differentiated between As and control compared to As + Cur (Fig. 4C). Both unsupervised multivariate statistics were used as a data reduction approach to identify the compounds that better discriminate between stress and Cur treatment factors on spinach.

3.6. Multifactorial metabolomics analysis through ANOVA multiblock OPLS (AMOPLS) to isolate biomarkers attributed to various factors and their interaction

To evaluate the contribution of multiple factors in the differential modulation of metabolic compounds, the annotated metabolites were subjected to multivariate data analysis using the AMOPLS approach (Fig. S1). This supervised approach allowed us to evaluate the contribution of main factors, such as As-induced stress and Cur treatments, and their potential interactions. The predictive component values of the AMOPLS model and their scores outlined that the two main factors contributed significantly to the total observed variability, reporting 13% and 26% as stress and treatments, respectively. In contrast, the interaction between the two factors (stress × treatments) contributed to 29% of the total variability (Table 1). Finally, the remaining 33% represented residual variability, suggesting that other sources of variation contributed to the data variability.

Given the high predictivity of the model, the (Variable Importance in the Projection) VIP^2 markers were selected based on various experimental factors and their interaction, representing the most discriminant compounds of the model (Fig. 5). The total VIP^2 score is valuable for prioritizing compounds and identifying metabolites that mostly discriminated the treatments. The comprehensive list of 50 discriminant

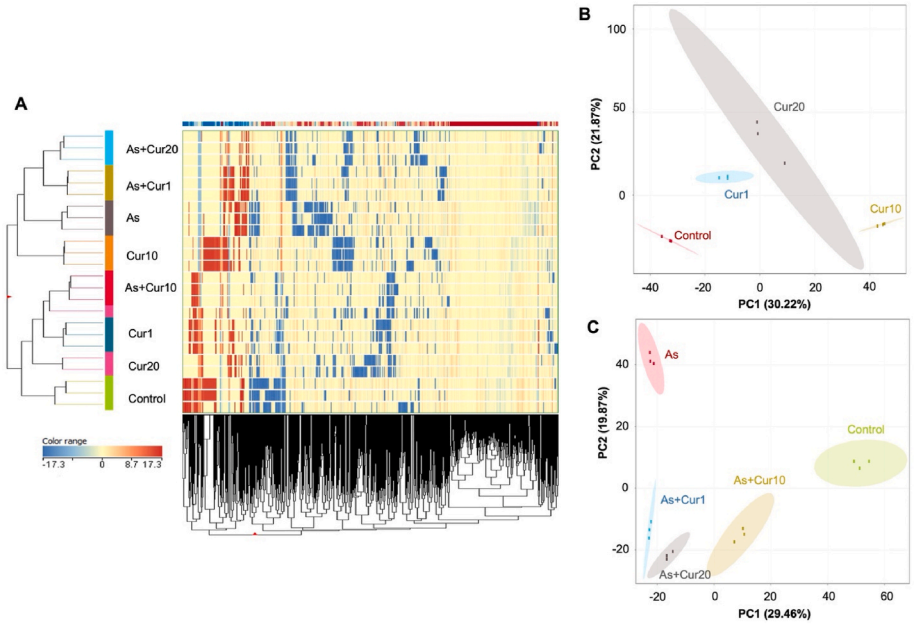

Fig. 4. Metabolomics analysis of spinach leaf metabolic profiles treated with increased concentration of curcumin (Cur) application (Cur1: 1 μM Cur; Cur10: 10 μM Cur; and Cur20: 20 μM Cur) under both As stress (100 μM) and control no stress. [**A**] Hierarchical Cluster Analysis of the entire dataset, considering both stressed and no-stressed conditions. Principal Component (PC) Analysis of [**B**] no-stress and [**C**] As stressed conditions.

Table 1
Relative variability and block contributions of the AMOPLS analysis of spinach metabolomic data exposed to different concentrations of curcumin under stress and no-stress conditions.

Effect Name	RSS	p-value	Block contributions						
			Tp1	Tp2	Tp3	Tp4	Tp5	Tp6	To
Stress	13%	0.01	**0.999**	0.001	0.000	0.000	0.001	**0.004**	0.226
Treatment	26%	0.01	0.000	**0.998**	0.000	0.000	**0.997**	0.985	0.193
Stress × Treatment	29%	0.01	0.000	0.000	**0.999**	**0.998**	0.001	0.003	0.180
Residuals	33%	NA	0.000	0.001	0.000	0.001	0.001	0.008	0.401

RSS: Relative sum of squares, tp1-6: predictive components, to: orthogonal component. The highest contribution for each component is reported in bold.

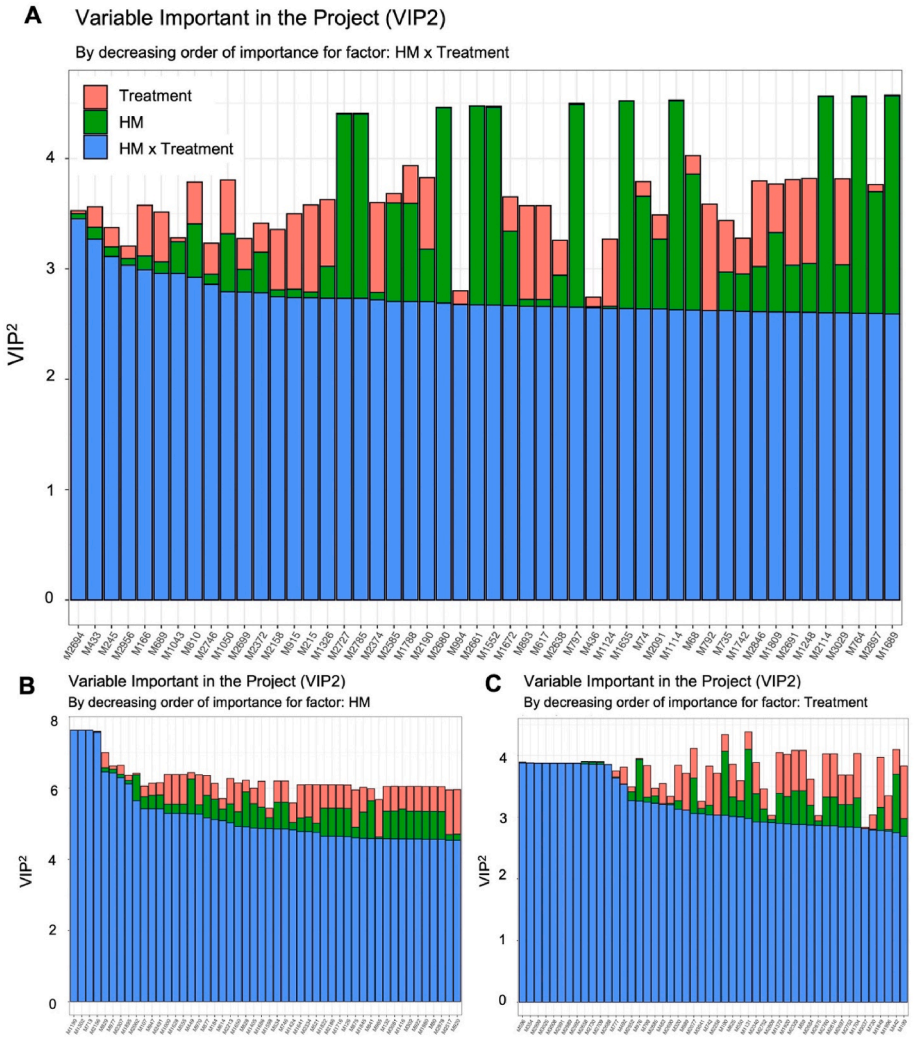

Fig. 5. Total and factor-specific VIP^2 values for the 50 compounds showing the most significant contribution to the [**A**] heavy metal (HM) x Treatment, [**B**] HM, and [**C**] Treatment factor. The names of metabolites are reported in the supplementary material (Table S4).

compounds for each factor is reported in the supplementary materials (Table S4), including compound pathway classification, LogFC values, and VIP^2 scores for each experimental factor. Considering VIP^2 markers for As stress factor, several metabolites were reported as down-accumulated with respect to unstressed control, mainly involved in plant growth and energetic pathways, as well as phytohormonal signal regulation, such as brassinosteroid, abscisic acid, and gibberellin (Table S4). Regarding the influence of Cur treatments, the AMOPLS model highlighted the accumulation of metabolites responsible for the biosynthesis of glucosinolates and phenolic compounds. Furthermore, an increase in glutathione redox cycle activities has been detected, suggesting an overall elicitation of plant metabolism and secondary metabolites (Table S4). Finally, the effect of As stress combined with Cur treatment was discriminated by metabolites contributing to the synthesis of glucosinolates, phenolic compounds, and alkaloids. The Stress x Treatment factor was also characterized by the modulation in lipids biosynthesis metabolism, demonstrated by a decrease in ergosterol metabolites biosynthesis, fatty acids and very log fatty acids biosynthesis, and glycolipid biosynthesis (Table S4).

3.7. Regularized canonical correlation analysis of integrated metabolic discrimination markers and leaf bioassay activities

The regularized canonical correlation analysis (rCCA) was

performed to extract latent features shared between VIP[2] discriminant metabolites dataset, outcoming from the AMOPLS model, which represents the most differentially modulated metabolites affected by treatments, and the biological assays dataset, including photosynthetic performance index (i.e., F_o/F_m, F_v/F_m, F_v/F_o), gas exchange parameters (i.e., C_i, A, E, and Gs), leaf oxidative stress biomarkers (i.e., MDA, H_2O_2, ψπ, and RWC), and overall antioxidant potential of the leaf extracts, to finding the linear combinations of features-referred to as canonical variables (CVs)-within each assay that achieve maximal across-assay correlation. The first two rCCA CVs maximized the datasets' correlation and discrimination between treatments (Fig. S2), reporting As-treated samples in an isolated cluster with respect to other clusters.

The resulting rCCA scores were represented as a heat map correlation plot (Fig. 6A), plotting the most correlated latent features based on their correlation coefficients. Moreover, these metabolites were filtered to include only those having a high correlation score ($r > 0.7$) and represented in the network plot (Fig. 6B). The rCCA highlighted PMD, ABTS, and DPPH activities as the most correlated assays with the VIP[2] biomarkers, reflecting those affected mainly by treatments. Network analysis underscored a positive correlation between ABTS and DPPH activities with (+)-dihydrokaempferol, 2-(cystein-S-yl)-2-(1H-indol-3-yl)-acetonitrile (indole phytoalexin), D-glucaro-1,5-lactone (hydroxycinnamate sugar acid), L-phenylalanine (precursor for the synthesis of phenolics compounds), and serotonin (Table S5). Accordingly, the biosynthetic pathway of serotonin and melatonin was elicited by Cur application, especially Cur at 10 μM, under As stress (Fig. 6C).

3.8. Leaf metabolomics response treated with increased concentration of curcumin under As stress and no-stressed conditions

Pathway analysis has been conducted to underscore differences in metabolic modulation under Cur treatments in the presence or absence of As stress. The most significant metabolites, deriving from Volcano analysis (p-values corrected with Bonferroni <0.05 + LogFC >2), were used to build metabolic pathways using the omics pathway tool from PlantCyc (Figs. 7 and 8). All the metabolites are reported in the supplementary materials (Table S6).

In the optimal condition, without As stress, a total of 286 significant metabolites were considered for pathways interpretation. The increasing Cur application significantly influenced secondary metabolism, phytohormone, lipids, and amine biosynthesis, highlighting an intrinsic metabolic modulation profile, reporting that the Cur dosage of 10 μM had a differential behavior compared to other concentrations (Fig. 7A). Principal pathways in secondary metabolite modulation induced by Cur treatments covered were N-containing compounds, terpene, and phenylpropanoid biosynthesis. Specifically, Cur 10 μM

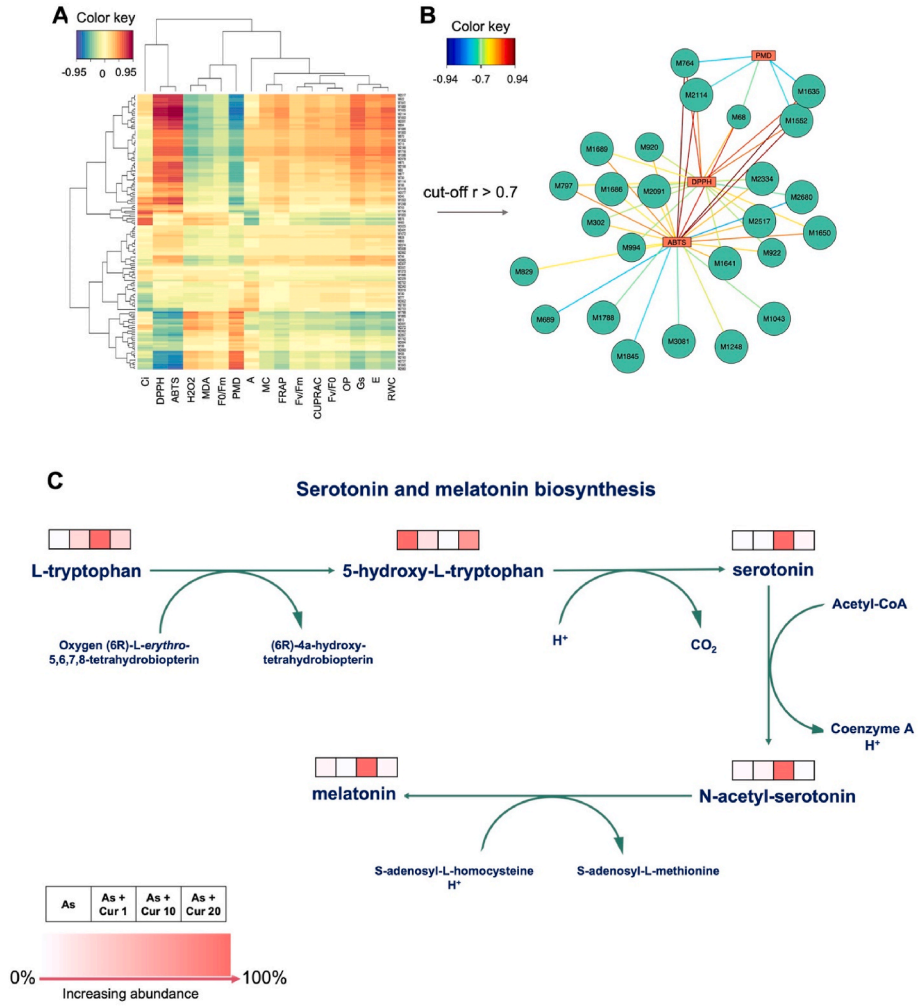

Fig. 6. [**A**] Heatmap correlation plot between the discriminant metabolites and bioassays, and [**B**] relevance network plot of the most correlated features ($r > 0,7$) between the discriminant metabolites and bioassays to evaluate the canonical correlation between discriminant metabolites and physiological and antioxidant activities of spinach leaves treated with increased concentration of curcumin (1 μM Cur, 10 μM Cur, and 20 μM Cur) applied alone and under As stress (100 μM). The metabolite list is provided in the supplementary material (Table S6). [C] Representation of the serotonin and melatonin biosynthetic pathways with relative abundances expressed as a pink scale gradient.

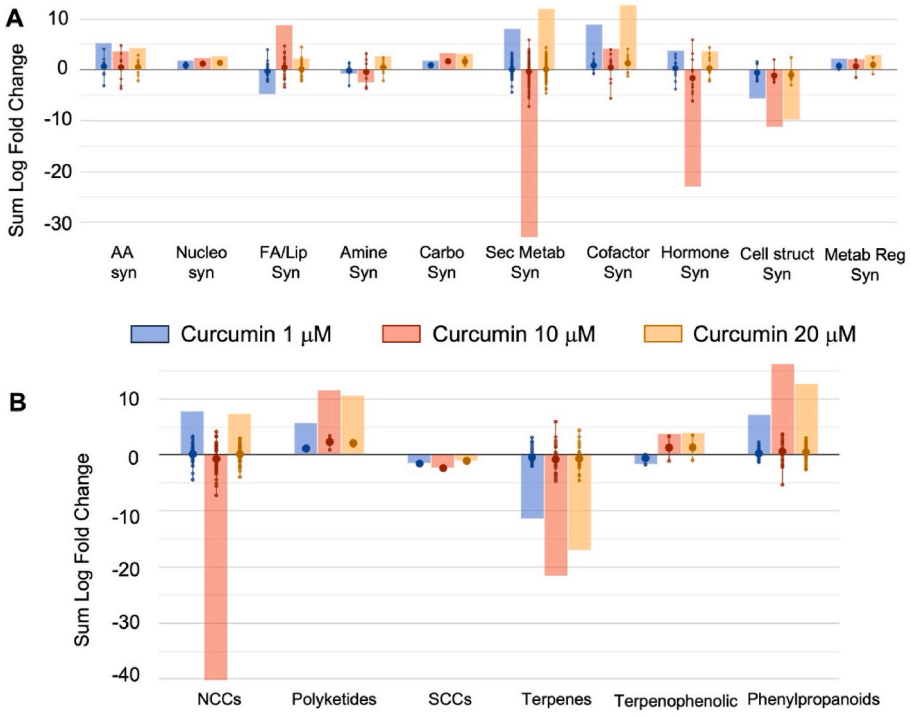

Fig. 7. Biosynthesis [**A**] and secondary metabolite synthesis [**B**] pathways of spinach leaves modulated by different concentrations of curcumin (Cur) treatment, such as 1 μM Cur, 10 μM Cur, and 20 μM Cur. The large dots represent the average log FC for the different metabolites in the class, while the small dots represent the individual log FC. Abbreviated: AA: amino acids; Nucleo: nucleosides and nucleotides; FA/lipids: fatty acids and lipids; Amine: amines and polyamines; Carbo: carbohydrates; Sec metab: secondary metabolism; Cofactor: cofactors, prosthetic groups, electron carriers, and vitamins; Cell struct: plant cell structures; Metab Reg: Metabolic Regulator; NCCs: nitrogen-containing compounds; SCCs: sulfur-containing compounds; syn: synthesis.

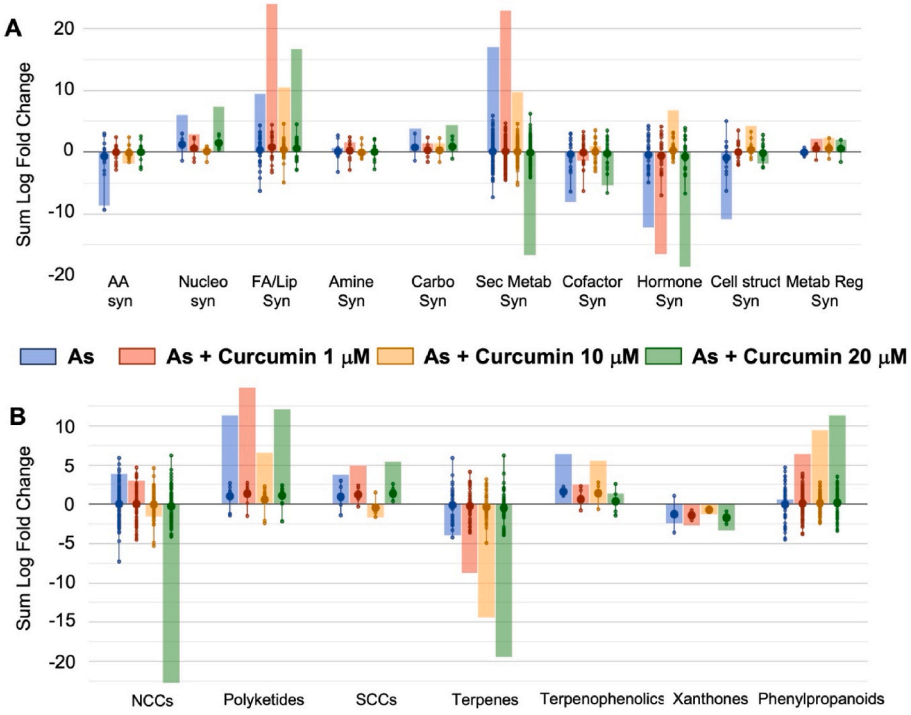

Fig. 8. Biosynthesis [**A**] and secondary metabolite synthesis [**B**] pathways of spinach leaves modulated by different concentrations of curcumin (Cur) treatment under As stress, such as As, As + 1 μM Cur, As + 10 μM Cur, and As + 20 μM Cur. The large dots represent the average log FC for the different metabolites in the class, while the small dots represent the individual log FC. Abbreviated: AA: amino acids; Nucleo: nucleosides and nucleotides; FA/lipids: fatty acids and lipids; Amine: amines and polyamines; Carbo: carbohydrates; Sec metab: secondary metabolism; Cofactor: cofactors, prosthetic groups, electron carriers, and vitamins; Cell struct: plant cell structures; Metab Reg: Metabolic Regulator; NCCs: nitrogen-containing compounds; SCCs: sulfur-containing compounds; syn: synthesis.

produced the down-regulation of secondary metabolites synthesis, mainly within N-containing compounds, including cyanogenic glucoside, isoquinoline, benzylisoquinoline alkaloids, and other alkaloids (Fig. 7B). Conversely, all evaluated Cur concentrations exhibited a down-modulation of terpenoids, represented by diterpenes (phytol salvage, steviol, and steviol glucoside biosynthesis) and triterpenes (saponin biosynthesis), coupled with an up-modulation of phenylpropanoids, represented by flavonoids (isoflavonoids, anthocyanins, and flavonol), coumarins, quinone, and lignans. Furthermore, in response to Cur treatments, phytohormonal pathways such as auxin, cytokinins, abscisic acid, brassinosteroids, and melatonin and serotonin exhibited substantial modulation. Regarding lipids and fatty acids biosynthesis, Cur treatments significantly induced phospholipids, fatty acids, and glycolipids while inhibiting sterol biosynthesis.

Under As stress conditions, the analysis considered a total of 428 metabolites for pathway interpretation. The effect of As stress in spinach leaves produced a strong down-modulation in the amino acids, cofactors, prosthetic groups, electron carriers, vitamins, phytohormone, and cell structure synthesis, coupled with an up-modulation in the lipids and secondary metabolite biosynthesis (Fig. 8A). The combined exposure to As and increasing concentrations of Cur modulated differentially in a dose-response effect in amino acids and secondary metabolite, including N-containing compounds, terpenes, and phenylpropanoids, compared to As alone (Fig. 8B). In detail, the increased application of Cur under As stress determined a specific down-modulation of glucosinolates biosynthesis (as N-containing class) and mono-, di-, tri-terpenes and phytosterols (as terpenoids class). Considering phenylpropanoids, Cur treatments positively modulated the biosynthesis of polyphenols and flavonoids, such as flavonoids and isoflavonoids, coupled with cinnamate, coumarins, lignans, and quinone.

4. Discussion

Arsenic is a metalloid element that occurs naturally. Arsenite (III) and arsenate (V) are toxic anions of As that plants can absorb through their roots from contaminated soil. Indeed, the presence of As in agricultural soil severely threatens crop plant growth and may affect human and animal health. Elevated As forms in plant tissues can disrupt plant functions, affecting metabolic pathways at physio-biochemical and molecular levels (Ulhassan et al., 2022). This study has considered spinach (*S. oleracea* L.) as a plant model because of its remarkable tolerance to heavy metal stress due to its strong antioxidant defense system and other physiological mechanisms (Zaheer et al., 2020b, 2020c). Furthermore, it has investigated the exogenous application of Cur as a strategy for As-induced stress remediation.

4.1. Curcumin application remediated physio-biochemical parameters induced by As stress in spinach

The As-induced stress significantly affected the physio-biochemical parameters of spinach plants, including leaf photosynthetic performance (F_v/F_m, F_o/F_m, F_v/F_o), the gas exchange parameters (C_i, E, g_s, and A), physiological processes (leaf RWC and $\psi\pi$), and oxidative stress biomarkers content (H_2O_2 and MDA), compared to the control. Similar observations have been reported by various authors, emphasizing that elevated As concentrations in the soil influenced spinach morpho-physiological traits, including plant growth and biomass, photosynthetic pigments, and gas exchange attributes (Saleem et al., 2023; Sun et al., 2023). Interestingly, our results corroborate an intricately interconnected response among these biochemical parameters. The exogenous application of Cur at increasing dosages remediated the toxic effects induced by As stress, reporting both photosynthetic performance and gas exchange parameters being restored, comparable to, and in some cases, even surpassing, the control samples. Furthermore, the application of Cur under As stress significantly increased leaf relative water content and osmotic potential, compared to As-stressed samples, suggesting a potential amelioration in the plant nutrient uptake and growth and developmental processes by stabilizing membrane integrity structures, respectively (Ahmad et al., 2018; Irshad et al., 2020; Lessl et al., 2015). Interestingly, these latest effects were also observed under optimal conditions, without As stress, in which the application of Cur improved water and nutrient assimilation capacity.

Exposure to As induces plant stress response system by producing reactive oxygen species (e.g., H_2O_2) involved in initiating antioxidant cascade responses that can potentially disturb plant morpho-physiological parameters, lipid peroxidation, cellular metabolism, and homeostasis. In this sense, two direct oxidative biomarkers were considered to assess spinach plants' general oxidative stress status, including H_2O_2 and MDA. In this study, our results collaborated with several other works reporting an accumulation of these two oxidative biomarkers after As-induced stress in spinach (Saleem et al., 2023; Sun et al., 2023). Surprisingly, the exogenous application of Cur significantly reduced the H_2O_2 and MDA content compared to the As-stressed plants. Specifically, Cur reduced MDA content as the control plant, suggesting a mechanism of action mostly faced to avoid lipid peroxidation and membrane structure and function preservation following as accumulation in plant tissues (Arora et al., 2002; Popov et al., 2023). Moreover, the positive effect of Cur application was reported even under optimal conditions, in the absence of As stress, to maintain the ROS homeostasis physiologically produced by metabolic processes such as the photosynthetic electron transport system, occurring in metal-free environments (Arora et al., 2002).

4.2. Curcumin application enhanced the endogenous antioxidative defense system's capacity through the crosstalk between ROS and phytohormones to cope with As-induced stress in spinach

Oxidative stress is a tightly regulated process where the balance between oxidative and antioxidative capacities determines the plant's vitality. Our results reported that applying Cur on spinach plants under As-stress and non-stress conditions enhanced the endogenous antioxidative defense system's capacity against active oxygen and free radicals generated during regular cell activity or induced by As exposure. Usually, plants respond to oxidative stress through two main mechanisms: enzymatic and non-enzymatic antioxidative systems (Dominic et al., 2022). In the case of non-redox heavy metals like As, the non-enzymatic antioxidative response is preferred and involves the activity of multiple bioactive secondary metabolites, including ascorbic acid, glutathione, tocopherol, carotenoids, and phenolic compounds, that can participate in ROS scavenging to maintain cellular redox balance (Thakur et al., 2022). Accordingly, the most discriminant metabolites derived from the AMOPLS-DA model by characterizing Cur treatment factor were phenolic compounds such as 2-hydroxynaringenin, salvianin, (4S)-2,3-dehydroleucopelargonidin, and 1-O-sinapoyl--beta-D-glucose, 2′-hydroxy 3,7,3′,4′-tetramethylquercetin, apigenin, and curcumin, which were reported to be highly accumulated in Cur treated plants compared to the control. These samples were also associated with increased non-enzymatic antioxidant potential, particularly considering ABTS and DPPH assays. This correlation was also confirmed by the canonical correlation analysis between the biological activity assays and the most discriminant metabolites, outcoming from AMOPLS-DA multivariate analysis, which highlighted a strict and positive correlation between increased ABTS and DPPH activities with different products of secondary metabolisms, including polyphenols and their precursors of the shikimate pathway, indole phytoalexin, and phytohormones (e.g., serotonin and melatonin). The multi-omics correlation approach suggests an interplay of various secondary metabolites and plant hormones in response to Cur treatment in As-driven oxidative stress. Specifically, a clear positive correlation was established between ABTS and several metabolites involved and antioxidant properties, including (+)-dihydrokaempferol (Yin et al., 2024), D-glucaro-1, 5-lactone (precursors for the biosynthesis of a hydroxycinnamate sugar)

(Shahidi and Chandrasekara, 2010), and an L-cysteine-S-conjugate compounds (involved in glutathione-mediated detoxification pathway) (Cooper and Hanigan, 2018). Interestingly, serotonin, the precursor for synthesizing melatonin, was reported to be correlated. It is important in plant defense and adaptation mechanisms against biotic and abiotic stresses. Several studies have characterized melatonin as a strong antioxidant compound that scavenges ROS and free radicals by enhancing antioxidant enzyme activity and non-enzymatic antioxidants (Hodzic et al., 2021; Hoque et al., 2021). The specific role of melatonin was clearly explained by Bose and Howlader (2020), which revealed that its biosynthesis and accumulation are mainly regulated under stress conditions to increase the tolerance against environmental stresses through the up-regulation of hormones, including salicylate, gibberellin, and abscisic acid biosynthetic pathways, as also confirmed by our finding.

4.3. Curcumin application modulated secondary metabolites biosynthesis as plant response to As-induced stress in spinach

As-driven stress modulates spinach metabolism, influencing both primary and secondary metabolite biosynthesis. As-induced stress reported the down accumulation of amino acids, cofactors for enzyme activities, and cell structure, which were mitigated by the treatment of Cur. A possible explanation could be attributed to the interaction of Cur with As to avoid its binding to sulfhydryl groups of peptides and forming an arsenic-phytochelatin complex (Kofroňová et al., 2019). This complex determines the sequestration of protein in the cell vacuoles and remains in tissues such as roots and stems (Kofroňová et al., 2019).

Moreover, lipid and fatty acid metabolism were modulated by As stress. The fatty acid imbalance is characterized by an overall increase in concentrations of polyunsaturated fatty acids, phospholipids, and glycolipids, suggesting an activated lipid synthesis and turnover as a response to the damage caused by As stress (Chaffai et al., 2009). Notably, our results revealed a marked decrease in cutin lipid synthesis induced by As stress, a trend reverted following Cur treatments. The plant cuticle, comprising cutin and cuticular wax, serves as a barrier regulating the movement of gas, water, and solute within the plant. Our results align with the membrane stability data discussed earlier regarding lipid peroxidation indices, gas exchange attributes, and, consequently, the impairment of the photosynthesis apparatus induced by As stress in spinach plants. These findings contribute valuable insights into the mechanism of action carried out by Cur applications in mitigating As-stress in spinach.

Considering plant secondary metabolism, the employment of Cur under As determined a progressive increase in the biosynthesis of phenylpropanoids and the decrease of glucosinolates and terpenoids, indicating the activation of an overall plant stress response mechanism. The progressive down-accumulation of glucosinolates and terpenoids after increasing the application of Cur under As-stress supported the mitigation potential via modulation of secondary metabolism. Indeed, glucosinolates were highly discussed for their role in plant response to abiotic stress (Del Carmen Martínez-Ballesta et al., 2013), as well as terpenoids (Gonzalez-Burgos and Gomez-Serranillos, 2012). Moreover, the application of Cur specifically enhanced the synthesis of phenylpropanoid compounds, recognized as non-enzymatic metabolites (Roy et al., 2022), through dose-response mechanisms. The most elicited phenolic metabolites were cinnamates (sinapoyl-(S)-malate), anthocyanins (delphinidin-3-O-(6″-O-malonyl)-β-glucoside-3′-O-β-glucoside), flavonoids (hesperidin), and lignans ((+)-piperitol) (Nguyen et al., 2021). These flavonoids and lignin are well recognized for their beneficial antioxidant capacity and for scavenging harmful active oxygen species induced by heavy metal stress (Sakihama et al., 2002). The specific modulation of the phenylpropanoid pathway resulting from the exogenous application of Cur was further supported by a concurrent decrease in the accumulation of glucosinolate and terpenoid compounds, indicating the activation of plant mechanisms associated with enhanced resiliency.

5. Conclusions

In conclusion, this study sheds light on the detrimental effects of As on spinach metabolism, emphasizing the impact on the physio-biochemical parameters of the plant, including leaf photosynthetic performance (F_v/F_m, F_o/F_m, F_v/F_o), the gas exchange parameters (C_i, E, g_s, and A), physiological processes (leaf RWC and ψπ), and oxidative stress biomarkers content (H_2O_2 and MDA). Furthermore, metabolic processes were highly implicated, especially primary and secondary metabolite synthesis, lipid metabolism, and hormonal regulation. The application of Cur emerges as a promising strategy for mitigating As-induced stress, as evidenced by its positive impact on physiological parameters, antioxidant defense systems, and metabolite synthesis. The study suggests that Cur counteracts As-induced damage and contributes to redistributing metabolic resources, promoting the synthesis of bioactive metabolites involved in As-induced oxidative stress mitigation. The findings contribute valuable insights into the potential use of Cur to enhance plant resilience under As stress, highlighting its multifaceted role in regulating plant physiology and metabolism. For future perspectives, conducting similar experiments in open fields could shed light on how environmental conditions influence metabolic responses. Additionally, exploring sustainable methods for extracting curcumin from industrial byproducts deriving from the production of turmeric or curry powders might be used as sources of curcumin for large field studies and industrial exploitation. These latter points, including costs, would information about the potential of curcumin in a real agricultural context.

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CRediT authorship contribution statement

Leilei Zhang: Writing – review & editing, Writing – original draft, Methodology, Investigation, Data curation, Conceptualization. **Gokhan Zengin:** Writing – review & editing, Conceptualization, Data curation, Methodology, Writing – original draft. **Ceyda Ozfidan-Konakci:** Investigation, Conceptualization, Methodology, Writing – original draft. **Evren Yildiztugay:** Conceptualization, Investigation, Methodology, Writing – original draft. **Busra Arikan:** Conceptualization, Data curation, Investigation, Methodology, Writing – original draft, Writing – review & editing. **Rumeysa Ekim:** Investigation, Methodology, Visualization. **Buket Koyukan:** Investigation, Methodology, Visualization. **Fevzi Elbasan:** Writing – review & editing, Conceptualization, Data curation, Methodology, Writing – original draft. **Luigi Lucini:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Conceptualization, Data curation.

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.

Data availability

Data will be made available on request.

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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.2024.108713.

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