



The regulatory roles of a plant neurotransmitter, acetylcholine, on growth, PSII photochemistry and antioxidant systems in wheat exposed to cadmium and/or mercury stress[☆]

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ABSTRACT

Heavy metals increase in nature due to anthropogenic activities and negatively impact the growth, progress, and efficiency of plants. Among the toxic metal pollutants that can cause dangerous effects when accumulated by plants, mercury (Hg) and cadmium (Cd) were investigated in this study. These metals typically inhibit important enzymes and halt their functioning, thereby adversely affecting the capability of plants to achieve photosynthesis, respiration, and produce quality crops. Acetylcholine (ACh) serves as a potent neurotransmitter present in both primitive and advanced plant species. Its significant involvement in diverse metabolic processes, particularly in regulating growth and adaptation to stress, needs to be further elucidated. For this aim, effects of acetylcholine (ACh1, 10 μ M; ACh2, 100 μ M) were surveyed in *Triticum aestivum* under Hg and/or Cd stress (Hg, 50 μ M; Cd, 100 μ M). Wheat seedlings exhibited a growth retardation of about 24% under Hg or Cd stress. Combined stress conditions (Cd + Hg) resulted in a decrease in RWC by approximately 16%. Two different doses of ACh treatment to stressed plants positively affected growth parameters and regulated the water relations. Gas exchange was limited in stress groups, and the photochemical quantum competency of PSII (F_v/F_m) was suppressed. Cd + ACh1 and Cd + ACh2 treatments resulted in approximately 2-fold and 1.5-fold improvement in stomatal conductance and carbon assimilation rate, respectively. Similarly, improvement was observed with ACh treatments in wheat seedlings under Hg stress. Under Cd and/or Hg stress, high levels of H_2O_2 accumulated and lipid peroxidation occurred. According to our results, ACh treatment upon Cd and Hg stresses improved the activities of SOD, POX, and APX, thereby reducing oxidative damage. In conclusion, ACh treatment was found to ensure stress tolerance and limit the adverse effects caused by heavy metals.

1. Introduction

Numerous external stresses are encountered by plants frequently. Of all the abiotic stressors, heavy metal stress stands out as a major ecological hazard, affecting many aspects of plant physiology, soil health, and ecosystem sustainability negatively (Mousavi, 2022; Pandey et al., 2022). The persistence of heavy metals in soil creates a serious risk to ecosystems and organisms alike, as they cannot be readily degraded (Sharma et al., 2022). Heavy metals consist of metal groups whose atomic densities are five times higher than that of water. The global prevalence of heavy metal pollution stems from a confluence of human

activities and environmental factors, resulting in high levels of heavy metals (Feng et al., 2021). Heavy metal intoxication has a severe effect on plant structure, which also lowers biomass accumulation, germination ability, and photosynthetic pigment biosynthesis (Singh et al., 2020). Moreover, this factor hinders cellular processes like photosynthesis and respiration, macro and micronutrient uptake, and overall plant functions, ultimately retarding plant growth (Dhalaria et al., 2020).

Cd and Hg are non-essential heavy metals that can cause serious toxicity to all organisms, including plants. Moreover, they can adversely affect the growth and reproduction of various plant species, either alone

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or in combination (Ronzan et al., 2018). Cd is a toxic, silver-white metallic element (Zhao et al., 2021) and, in particular, is highly mobile and toxic even at low concentrations (El Rasafi et al., 2022). The hazards of Cd include two aspects: (1) interference with the plant's life cycle, resulting in reduced yield; and (2) absorption and accumulation by plants, allowing it to enter the food chain and adversely affect animals (Wang et al., 2022). Physiologically, Cd stress is characterized by enzyme inactivation, substantial accumulation of ROS, and metabolic imbalance (Meng et al., 2022). Cd²⁺ ions can be readily absorbed by crop plants, leading to the build-up of Cd in plants. This can cause stomatal closure, lipid and protein oxidation, reduction in photosynthetic potential, and subsequent disruption of plant development. Hg is considered one of the most toxic heavy metals due to its persistence in the environment and high mobility. It can cause harmful effects on plant growth and functions. Elevated levels of Hg in agricultural lands may decrease plant productivity. Even at low concentrations, Hg exerts significant detrimental effects on plant growth and metabolism, disrupting fundamental physiological processes such as germination, nutrient absorption, photosynthesis, and proliferation (Ibrahim et al., 2022). Its high lipid solubility facilitates accumulation in biological membranes, promoting biomagnification in the food chain (Robas Mora et al., 2022). Furthermore, Hg disrupts cellular functions by interfering with the electron transport chain and causing oxidative stress (Ghori et al., 2019). Clarifying the combined effects of Cd and Hg stress on plants has received less attention than studying the multifaceted effects of heavy metal pollution on plant development in isolation. This knowledge gap underscores the need for comprehensive investigations aimed at unraveling the interactive effects of these two pollutants on plant physiology and biochemistry, particularly in economically important crops.

Heavy metal ions instigate the production of reactive oxygen species (ROS) via autoxidation and the Haber-Weiss cycle (Li et al., 2022). Excessive ROS generation disrupts redox homeostasis and damages cellular components. Furthermore, redox-active heavy metals exacerbate ROS-mediated damage by directly interacting with ROS, converting them into more reactive and harmful forms (Nowicka, 2022). Simultaneously, hydrogen peroxide (H₂O₂), superoxide radical (O₂^{•-}), and hydroxyl radical (•OH) react further with organelles, leading to cellular and DNA damage, inhibiting adenosine triphosphate (ATP) production, and causing lipid peroxidation (Sachdev et al., 2023). Enzymatic and non-enzymatic components of a comprehensive defense system against oxidative stress have been developed by plants. Among enzymatic antioxidants, APX (ascorbate peroxidase), CAT (catalase), SOD (superoxide dismutase), and GPX (glutathione peroxidase) are present, non-enzymatic systems include glutathione (GSH), ascorbate (AsA), phenolic compounds, tocopherols, and carotenoids (Mansoor et al., 2022).

Neurotransmitters were initially discovered in fungi, and later it became known that they function as signaling molecules and regulators in mammals. Acetylcholine (ACh), an ester of choline and acetic acid, is the best-known neurotransmitter (Ewins, 1914). Predominantly synthesized from choline and acetyl-coenzyme A (acetyl-CoA) in animals, it occurs at synaptic junctions, nerves, and the motor endplates of vertebrate muscles (Roychoudhury, 2020). The synthesis of acetylcholine is limited by the transport of choline, which is facilitated by the acetylcholine transferase enzyme (Robas Mora et al., 2022). Beyond its canonical roles in neuronal signaling, ACh is also found in non-neuronal cells of species lacking nervous systems, including bacteria, algae, and plants (Su et al., 2020b). In plants, ACh, along with other neurotransmitters such as melatonin, dopamine, glutamate, serotonin, and gamma-aminobutyric acid, serves diverse functions in modulating responses to environmental cues (Su et al., 2020b). ACh, in particular, participates in photosynthesis, root development, seed germination, growth regulation, osmoregulation, and defense mechanisms against environmental stresses such as harsh weather conditions, heavy metal toxicity, and drought (Qin et al., 2021). It is involved in signaling

processes between roots and shoots (Braga et al., 2017). Additionally, ACh cooperates with plant hormones including indoleacetic acid (IAA), ethylene (ET), and gibberellic acid (GA), affecting antioxidants, osmo-protectants, and the expression of stress-response genes, thus influencing plant growth and development (Di Sansebastiano et al., 2014). Recent studies have highlighted the potential of exogenous ACh application in mitigating the adverse effects of stressors on plant physiology. For instance, foliar spraying or exogenous application of ACh has been shown to alleviate salinity-induced damages in *Nicotiana benthamiana*, enhancing photosynthetic efficiency, antioxidant enzyme activity, and osmotic regulation (Qin et al., 2021). Yang et al. (2023) explored into the effects of Cd stress on endogenous ACh levels in duckweed (*Lemna turionifera*) in a different study. Under Cd stress, ACh supplementation was demonstrated to lower root abscission rates, relieve leaf etiolation, and enhance chlorophyll fluorescence characteristics.

Worldwide, wheat (*Triticum aestivum* L.) is one of the most extensively grown cereal crops (Wei et al., 2022). It is widely acknowledged that wheat is more sensitive to heavy metals than other crops (Ilyas et al., 2022). Wheat plants possess a remarkable capacity to uptake and accumulate significant levels of heavy metals, primarily through their fibrous root system. Consequently, these metals are translocated to aerial plant parts, including grains, posing potential risks to human health (Qin et al., 2020). Despite the growing recognition of ACh's pivotal role in mediating plant responses to environmental stresses, investigations into its potential efficacy in alleviating heavy metal stress in wheat plants remain limited. Therefore, the present study aims to elucidate the effects of exogenous ACh treatment on the growth, gas exchange parameters, photochemical reactions, and antioxidant system in wheat leaves under Cd and/or Hg stress conditions. By unraveling the mechanistic insights into ACh-mediated tolerance against heavy metal stress, this research seeks to contribute to the development of sustainable strategies for enhancing the resilience of wheat plants to environmental challenges.

2. Materials and methods

2.1. Experimental methodology

Under carefully monitored conditions, three-week-old wheat seedlings (*Triticum aestivum* L. cv. Taner) were cultivated hydroponically in Hoagland solution. Acetylcholine (ACh1, 10 μM; ACh2, 100 μM) treatments were administered to the seedlings with or without Hg and/or Cd stress (Hg, 50 μM; Cd, 100 μM), as per previous studies (Jawad Hassan et al., 2020; Pirzadah et al., 2018; Qi et al., 2023). After one week, samples were harvested for physiological and biochemical analysis.

2.2. Determination of physiological parameters

Six plants were used for the control group and each treatment group. Fresh weights (FW) of the leaves were measured. After the samples were dried, dry weights (DW) were measured. RGR values were calculated according to the following formula by Hunt et al. (2002):

$$\text{RGR} = [\ln(\text{DW}_2) - \ln(\text{DW}_1)] / (t_2 - t_1),$$

where DW₁ = dry weight (g) at t₁; DW₂ = dry weight (g) at t₂, t₁; initial harvest and t₂; final harvest.

After one week of the treatment period, six leaves were harvested and their fresh weight (FW) was determined. The leaves were floated on de-ionized water for 6 h and the turgid tissue was blotted dry prior to determining turgid weight (TW). Dry weight (DW) was determined after oven drying at 70°C. The leaf relative water content (RWC) was calculated by the following formula (Smart and Bingham, 1974):

$$\text{RWC} (\%) = [(\text{FW}-\text{DW}) / (\text{TW}-\text{DW})] \times 100$$

Proline levels were determined in 0.5 g of fresh specimens,

employing the protocol outlined by Chandrakar et al. (2016).

2.3. Analysis of photosynthesis related parameters with OJIP test

A portable fluorometer (FMS-2, Hansatech, King's Lynn, UK) was used to determine the maximal quantum yield of PSII photochemistry (F_v/F_m). The descriptions for the estimated parameters are included in Supplementary Table S1. The radar plots depict the mean parameter values of different treatments in leaves.

Carbon assimilation rate (A), stomatal conductance (g_s), intercellular CO_2 concentration (C_i), and transpiration rate (E) were measured with a portable gas exchange system (LCpro+; ADC, Hoddesdon, UK). Gas exchange parameters were measured in 6 replicates by selecting leaves of similar size from each treatment group at the end of the seven-day experimental setup. The stomatal limitation value (L_s) was measured as $1 - C_i/C_a$ (Ma et al., 2011).

2.4. Analysis of H_2O_2 and TBARS contents

The quantification of H_2O_2 content in leaves was performed according to the methodology elucidated by Velikova et al. (2000), and while the determination of lipid peroxidation levels (TBARS content) was conducted following the protocol described by Rao and Sresty (2000). Visualization of H_2O_2 concentration in guard cells was accomplished using 2,7-dichlorofluorescein diacetate ($H_2DCF-DA$), as previously detailed (Ahmed et al., 2020; Gao et al., 2024).

2.5. Identification of isozyme and/or enzyme compositions

0.5 g leaf samples were extracted in Tris-HCl (25 mM Tris, 1% Triton-X100, pH: 7.4) and centrifuged at 14000 g for 30 min. Supernatants were collected, and total protein contents were measured by the Bradford (1976) method.

For SOD (EC 1.15.1.1) isozyme activity, samples were subjected to non-denaturing polyacrylamide gel electrophoresis (PAGE) as described by Laemmli (1970). Total SOD activity assay was based on the method of Beauchamp and Fridovich (1971). CAT isozymes were detected according to Woodbury et al. (1971). Total CAT (EC 1.11.1.6) activity was estimated according to the method of Bergmeyer (1970). The isozymes and enzyme activity of POX (EC 1.11.1.7) were based on the method described by Seevers et al. (1971) and Herzog and Fahimi (1973), respectively. NADPH oxidase (NOX) isozymes were identified as described by Sagi and Fluhr (2001). NOX (EC 1.6.3.1) activity was measured according to Jiang and Zhang (2002). The enzyme/isozyme activities of glutathione S-transferase (GST, EC 2.5.1.18) and glutathione peroxidase (GPX, EC 1.11.1.9) were determined (Hossain et al., 2006; Ricci et al., 1984).

2.6. Identification of enzyme/non-enzyme compositions related to the AsA-GSH cycle

Electrophoretic APX separation was performed according to Mittler and Zilinskas (1993). APX (EC 1.11.1.11) enzyme activity was measured according to Nakano and Asada (1981). GR (EC 1.6.4.2) activity was measured according to Foyer and Halliwell (1976). Isozymes compositions of GR were determined by native PAGE analysis (Hou et al., 2004).

Monodehydroascorbate reductase (MDHAR; EC 1.6.5.4) activity was assayed by the method of Miyake and Asada (1992). Dehydroascorbate reductase (DHAR; EC 1.8.5.1) activity was measured according to Dalton et al. (1986). Total and reduced ascorbate (AsA) contents were done according to the method of Dutilleul et al. (2003) with modifications. The oxidized form of ascorbate (DHA, dehydroascorbate) was measured using the formula $DHA = Total\ AsA - Reduced\ AsA$. The glutathione (GSH) was assayed according to Paradiso et al. (2008). Oxidized glutathione (GSSG) was determined after the removal of GSH by 2-vinylpyridine derivatization. GSH redox state (%) was determined

by calculating the ratio of GSH to total glutathione (GSH + GSSG) according to Shi et al. (2013).

Gels stained for SOD, CAT, POX, APX, GR, GST, and NOX activities were photographed with the Gel Doc XR + System and then analyzed with Image Lab software v4.0.1 (Bio-Rad, California, USA). Known standard amounts of enzymes (0.5 units of SOD and 0.2 units of CAT and POX) were loaded onto gels. For each isozyme set/group, the average values were significantly different at $p < 0.05$ using Tukey's post-test.

2.7. Statistical analysis

The experiments were carried out in triplicates and each data was expressed as the means of six replicates. ANOVA was completed using SPSS 20 for Windows (IBM; Armonk, NY, USA) and significance was performed using Student's t-test or Duncan's multiple range test. Comparisons with $p < 0.05$ were considered significantly different.

3. Results

3.1. Effects of ACh treatments on growth characteristics of wheat seedlings under Cd and/or Hg

While no change was observed in RGR of ACh1-treated wheat plants, a slight increase was detected in the ACh2 group (Fig. 1A). Cd stress caused a 24% decrease in RGR. According to Cd stress, approximately a 30% increase was noted in the Cd + ACh1 group and 40% in the Cd + ACh2 group. Hg stress reduced the RGR by 24% compared to control plants. In the Hg + ACh1 and Hg + ACh2 groups, a 25% and 50% increases in RGR were detected respectively, compared to Hg stress. Cd + Hg + ACh1 and Cd + Hg + ACh2 treatments resulted in 2.4-fold and 3.1-fold improved RGR, respectively, compared to Cd + Hg. ACh1 treatment resulted in a 9% increase in relative water content (RWC) compared to the control group (Fig. 1B). Under Cd stress, RWC decreased by 5% compared to the control group. Meanwhile, the Cd + ACh1 and Cd + ACh2-treated plants experienced an increase of 8% and 11%, respectively. RWC decreased by 7% under Hg stress compared to control. Hg + ACh1 and Hg + ACh2 groups showed 13% and 7% improvement compared to the stress group. In the combined stress (Cd + Hg), there was a 16% decrease in RWC. Cd + Hg + ACh1 and Cd + Hg + ACh2 groups showed an increase in RWC of 17% and 7%, respectively. Treatment of ACh1 and ACh2 increased the proline content (Pro) by approximately 4-fold and 3-fold, respectively, under control conditions (Fig. 1C). Likewise, an increase was found in Pro content at all stress applications. Pro content further increased with Cd + ACh2, Hg + ACh1, and Cd + Hg + ACh2 treatments in stressed leaves.

3.2. Effects of ACh treatments on gas exchange and photosynthesis parameters of wheat leaves under Cd and/or Hg

Under stress conditions (Cd, Hg, Cd + Hg), there was a decrease of carbon assimilation rate (A) approximately 40%, 50% and 70%, respectively (Table 1). An enhancement of 71% and approximately 50% was observed in the Cd + ACh1 and Cd + ACh2 groups, respectively. In Hg + ACh1 and Hg + ACh2 treatments, A increased 2.4-fold and 2.3-fold in wheat leaves. In the combined groups (Cd + Hg + ACh1, Cd + Hg + ACh2), this ratio rose by 2.2 and 3 times, respectively. While stomatal conductance (g_s) increased by 50% in the ACh1 group, no significant change was observed in ACh2. g_s decreased by approximately 70% in the stress groups (Cd, Hg, Cd + Hg). However, in the Cd + Hg + ACh2 group, a 2.7-fold higher effect in g_s was observed compared to the combined stress group (Cd + Hg). In the ACh1 treatment group, the intercellular CO_2 concentration (C_i) increased by 4% compared to the control conditions. Compared to the Cd group, there was a 12% increase under Cd + ACh1 and an 18% increase at Cd + ACh2. C_i decreased by 21% and 31% in the Hg and Cd + Hg groups. A 23% and 30% increase in Hg + ACh1 and Hg + ACh2 were reported compared to the Hg group. C_i

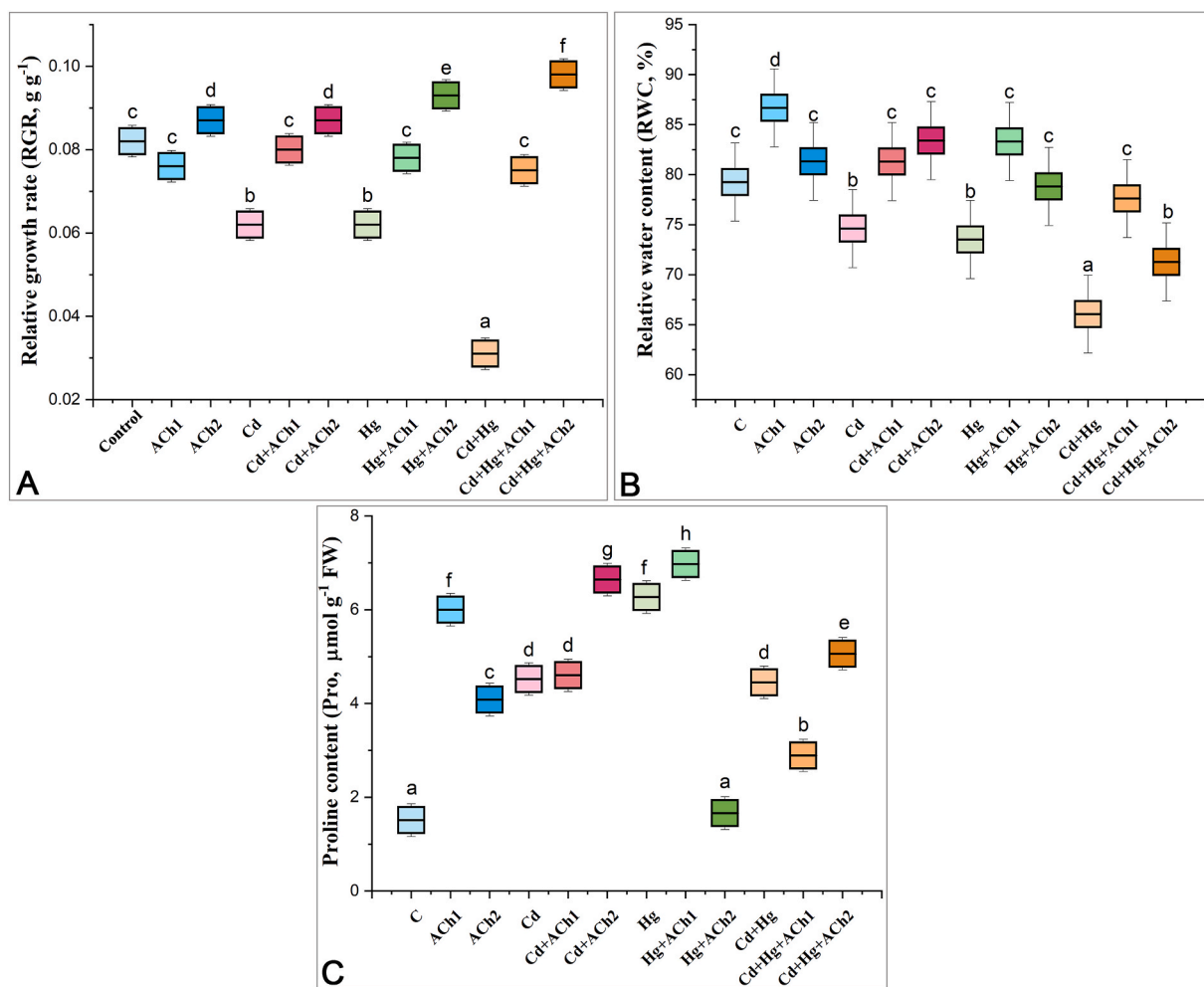


Fig. 1. The relative growth rate (RGR, A), relative water content (RWC, B) and proline content (Pro, C) in acetylcholine (ACh1, 10 μM ; ACh2, 100 μM) treated wheat leaves under Hg and/or Cd stress (Hg, 50 μM ; Cd, 100 μM). All data obtained were subjected to a one-way analysis of variance (ANOVA). Differences were considered to be significant at $p < 0.05$.

Table 1

Carbon assimilation rate (A), stomatal conductance (g_s), intercellular CO_2 concentrations (C_i), transpiration rate (E), stomatal limitation rate (L_s) and carboxylation efficiency (A/C_i) in acetylcholine (ACh1, 10 μM ; ACh2, 100 μM) treated wheat leaves under Hg and/or Cd stress (Hg, 50 μM ; Cd, 100 μM).

Groups	Carbon assimilation rate (A, $\mu\text{mol m}^{-2}\text{s}^{-1}$)	Stomatal conductance (g_s , $\mu\text{mol m}^{-2}\text{s}^{-1}$)	Intercellular CO_2 concentration (C_i , $\mu\text{mol mol}^{-1}$)	Transpiration rate (E, $\mu\text{mol m}^{-2}\text{s}^{-1}$)	Stomatal limitation value (L_s)	Carboxylation efficiency (A/C_i)
C	9.20 ± 0.09^c	81.25 ± 1.14^d	514 ± 7.20^d	1.74 ± 0.017^f	0.358 ± 0.0029^b	0.0179 ± 0.00027^e
ACh1	13.81 ± 0.17^g	122 ± 3.42^e	539 ± 15.09^e	2.31 ± 0.029^g	0.312 ± 0.0037^a	0.0256 ± 0.00064^h
ACh2	11.01 ± 0.08^f	80 ± 1.48^d	498 ± 9.21^d	1.71 ± 0.013^f	0.404 ± 0.0034^c	0.0221 ± 0.00028^g
Cd	5.59 ± 0.08^c	24.95 ± 0.25^a	509 ± 4.07^d	1.24 ± 0.017^d	0.582 ± 0.0058^f	0.0110 ± 0.00011^b
Cd + ACh1	9.58 ± 0.24^c	62 ± 0.78^c	571 ± 6.85^f	1.38 ± 0.039^e	0.438 ± 0.0055^d	0.0168 ± 0.00021^e
Cd + ACh2	8.27 ± 0.10^d	38.75 ± 0.29^b	603 ± 5.13^g	1.00 ± 0.019^e	0.439 ± 0.0033^d	0.0137 ± 0.00010^c
Hg	4.30 ± 0.03^b	26.8 ± 0.40^a	404 ± 4.04^b	0.47 ± 0.004^a	0.612 ± 0.0092^f	0.0106 ± 0.00015^b
Hg + ACh1	10.60 ± 0.13^f	43.3 ± 1.08^b	500 ± 6.25^d	0.91 ± 0.011^b	0.515 ± 0.0129^e	0.0212 ± 0.00059^g
Hg + ACh2	10.24 ± 0.09^f	38.3 ± 0.48^b	529 ± 3.97^e	0.82 ± 0.007^b	0.487 ± 0.0061^e	0.0194 ± 0.00036^f
Cd + Hg	2.78 ± 0.04^a	21.25 ± 0.17^a	350 ± 5.25^a	0.56 ± 0.008^a	0.404 ± 0.0040^c	0.0079 ± 0.00027^a
Cd + Hg + ACh1	6.12 ± 0.17^c	19 ± 0.23^a	396 ± 9.90^b	0.63 ± 0.016^a	0.606 ± 0.0076^f	0.0155 ± 0.00019^d
Cd + Hg + ACh2	8.46 ± 0.16^d	58.33 ± 0.50^c	453 ± 5.66^c	0.99 ± 0.012^c	0.316 ± 0.0024^a	0.0187 ± 0.00016^f

increased by 13% and 29% in plant leaves treated with Cd + Hg + ACh1 and Cd + Hg + ACh2, respectively. No difference in transpiration rate (E) was observed in the ACh2 group compared to the control conditions. The most significant increase in E was found in the Hg + ACh1 group, with 93%. While the Cd + ACh1 group increased by 11% compared to Cd stress conditions, the Cd + ACh2 group decreased by 19%. Compared

to the control conditions, the stomatal limitation value (L_s) decreased by 12% under ACh1, while an increase of 12% was observed at ACh2. After stress exposure (Cd, Hg, Cd + Hg), this value increased by 62%, 70%, and 12%, respectively. All ACh treatments under stress resulted in the attenuation of L_s except the Cd + Hg + ACh1 group. The carboxylation efficiency (A/C_i) of ACh1 and ACh2-applied wheat leaves increased by

43% and 23%, respectively, compared to control conditions. There was a decrease in stress groups (Cd, Hg, Cd + Hg) compared to the control. No statistical difference was detected in the F_v/F_m , F_v/F_o , and F_o/F_m in the ACh1 and ACh2-applied groups compared to the control (Fig. 2). F_v/F_m decreased by 5% in the single stress groups (Cd, Hg) and by 3% in the combined stress exposure (Cd + Hg). Cd and Hg applications decreased the F_v/F_o by 21%. F_o/F_m increased by 19% in the Cd and Cd + Hg groups and by 33% in the Hg group. Radar plots created by chlorophyll a fluorescence parameters are presented in Fig. 3. Increased VI and VJ parameters were detected in all stress treatments, indicating reaction centers with low photochemical activity. The groups that achieved the highest success in raising the overall performance indexes PI_{ABS} and PI_{total} were those who received ACh1 treatment in single stress applications (Cd and Hg) and ACh2 treatment in combined stress applications (Cd + Hg).

3.3. Effects of ACh treatments on oxidative stress markers of wheat seedlings under Cd and/or Hg

Fig. 4A presents the ROS accumulation in guard cells of wheat leaves from all treatment groups. H_2O_2 accumulation increased significantly in plant leaves exposed to Cd and/or Hg. Consistent with the H_2O_2 content results, ACh treatments limited H_2O_2 accumulation in stressed plants. There was no change in the H_2O_2 and TBARS contents of wheat leaves between the control and ACh alone applications (Fig. 4B and C). However, H_2O_2 accumulation increased by 22%, 20%, and 48% in the Cd, Hg, and Cd + Hg groups, respectively. ACh treatments were successful in preventing radical accumulation under stress conditions. All stress + ACh application groups resulted in a decrease in H_2O_2 and TBARS contents and alleviation of oxidative stress.

3.4. Effects of ACh treatments on antioxidant enzyme profiles of wheat leaves under Cd and/or Hg

Three isozymes for the SOD enzyme have been defined in wheat leaves (Mn-SOD1-2 and Fe-SOD) (Fig. 5A). The treatment groups (ACh1 and ACh2) exhibited an increase in SOD activity compared to the control conditions (Fig. 5B). Cd + ACh1 treatment increased the total SOD activity by 30% compared to the Cd conditions. ACh1 treatment was successful in triggering SOD activity under Cd and Cd + Hg stress conditions, and both ACh treatments boosted SOD activity under Hg exposure. Only one isozyme band was detected for CAT (Fig. 5C).

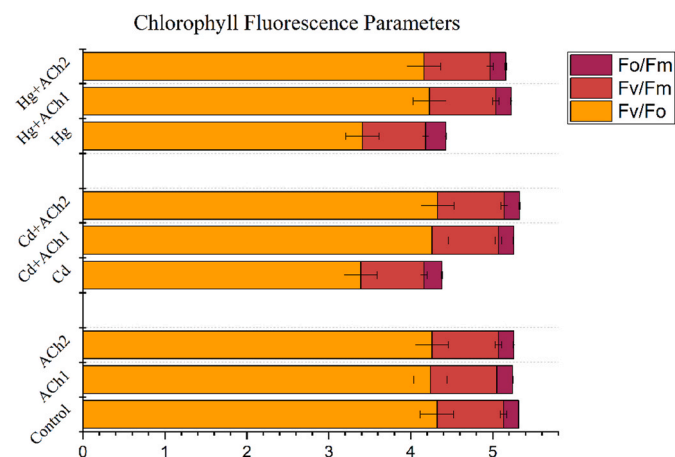


Fig. 2. The maximal quantum yield of PSII photochemistry (F_v/F_m), potential photochemical efficiency (F_v/F_o) and physiological state of the photosynthetic apparatus (F_o/F_m) in acetylcholine (ACh1, 10 μ M; ACh2, 100 μ M) treated wheat leaves under Hg and/or Cd stress (Hg, 50 μ M; Cd, 100 μ M). All data obtained were subjected to a one-way analysis of variance (ANOVA). Differences were considered to be significant at $p < 0.05$.

Although the ACh1-applied plants showed only a slight increase, the ACh2-treated leaves exhibited an approximately 24% increase (Fig. 5D). The application of Cd, Hg, and Cd + Hg resulted in a decrease in CAT activity by 24%, 14%, and 21%, respectively, compared to the control. CAT activity also decreased in the Hg + ACh1 and Hg + ACh2 groups. High CAT activity was observed in the Cd and combined stress groups treated with ACh1 and ACh2. Eight bands were detected for POX isoenzyme in wheat seedlings (POX1-8) (Fig. 6A). It was found that the treatment of ACh1 and ACh2 resulted in an increase in POX activity of more than 2-fold compared to the control conditions (Fig. 6B). While POX activity increased 2.4-fold under Cd conditions, ACh treatments increased the activity 25% further. Hg + ACh1 treatment increased POX activity 2.3-fold. Combined stress resulted in 70% higher POX activity and ACh1 treatment provided approximately 70% further increase. The presence of nine isoenzymes in GST has been proven (Fig. 6C). When compared to control conditions, ACh1 and ACh2 treatments increased the total GST activity by 40% (Fig. 6D). It was seen that the Cd and Hg stress groups increased GST activity by 65% and 49%, respectively. All ACh treatments under stress exposure exhibited high levels of GST activity. Eight isozymes have been reported for the NOX enzyme in wheat leaves (Fig. 7A). Compared to control, ACh1 and ACh2 treatments increased the NOX activity by 15% and 26%, respectively (Fig. 7B). NOX activity decreased by 16%, 22%, and 32% in Cd, Hg, and Cd + Hg groups. The Cd + ACh1 group, showed the highest value by increasing NOX activity by 2-fold. However, NOX activity decreased by 20% in the Hg + ACh2 condition. Two isozymes have been identified in native PAGE analysis for GPX enzyme (Fig. 7C). Compared to control conditions, ACh1 and ACh2 treatments alone showed higher GPX activity (Fig. 7D). Under Cd, Hg, Cd + Hg conditions, GPX activity decreased by 20%, 20% and 36%, respectively. Compared to Cd stress, Cd + ACh1 increased by 40% and Cd + ACh2 increased by 28% in GPX activity. According to the Hg, no statistical change occurred between ACh treatment groups. Compared to the Cd + Hg group, the effect of the ACh was seen in GST activity by 21% at Cd + Hg + ACh1 and approximately 30% at Cd + Hg + ACh2.

3.5. Effects of ACh treatments on AsA-GSH cycle of wheat seedlings under Cd and/or Hg

Three APX isozymes were detected in the APX gel image (Fig. 8A). Compared to control, the ACh1 and ACh2 treatments in wheat leaves increased the total APX activity by 53% and 70%, respectively (Fig. 8B). Cd stress decreased APX activity by 41%, Hg stress increased it by 16%. Compared to the stress-alone treatments, all ACh applications increased APX activity, and the highest activity was determined in the Hg + ACh1 group with a 42% increase. Nine isozymes for GR were determined in the wheat leaves (Fig. 8C). While Cd stress did not cause a change in GR activity, it decreased by more than 20% compared to the control in Hg and Cd + Hg applications (Fig. 8D). All ACh treatments increased GR activity under stress conditions. Hg and Cd + Hg conditions decreased MDHAR activity by 27% and 37% (Fig. 9A). Compared to Cd, a 40% increase in Cd + ACh1 and an approximately 80% increase in Cd + ACh2 were detected. Compared to stress groups, Hg + ACh1 treatment resulted in a 2-fold, and Cd + Hg + ACh2 treatment provided an 84% increased MDHAR activity. Under control conditions, ACh treatments did not cause changes in DHAR activity (Fig. 9B). Similarly, Cd stress did not cause any difference in DHAR activity compared to control. However, 57% and 76% increased DHAR activity was determined in the Cd + ACh1 and Cd + ACh2 groups, respectively. Hg + ACh1 and Hg + ACh2 treatments increased DHAR activity by 36% and 22%, respectively. In the combined groups (Cd + Hg + ACh1 and Cd + Hg + ACh2), 25% high activity in DHAR occurred compared to Cd + Hg. Compared to control, tAsA content increased by 12% with ACh1 application and approximately 50% with ACh2 (Fig. 9C). Cd, Hg and Cd + Hg conditions reduced tAsA content by 11%, 36% and 51%, respectively. ACh treatments under stress groups increased the tAsA content. Stress exposure

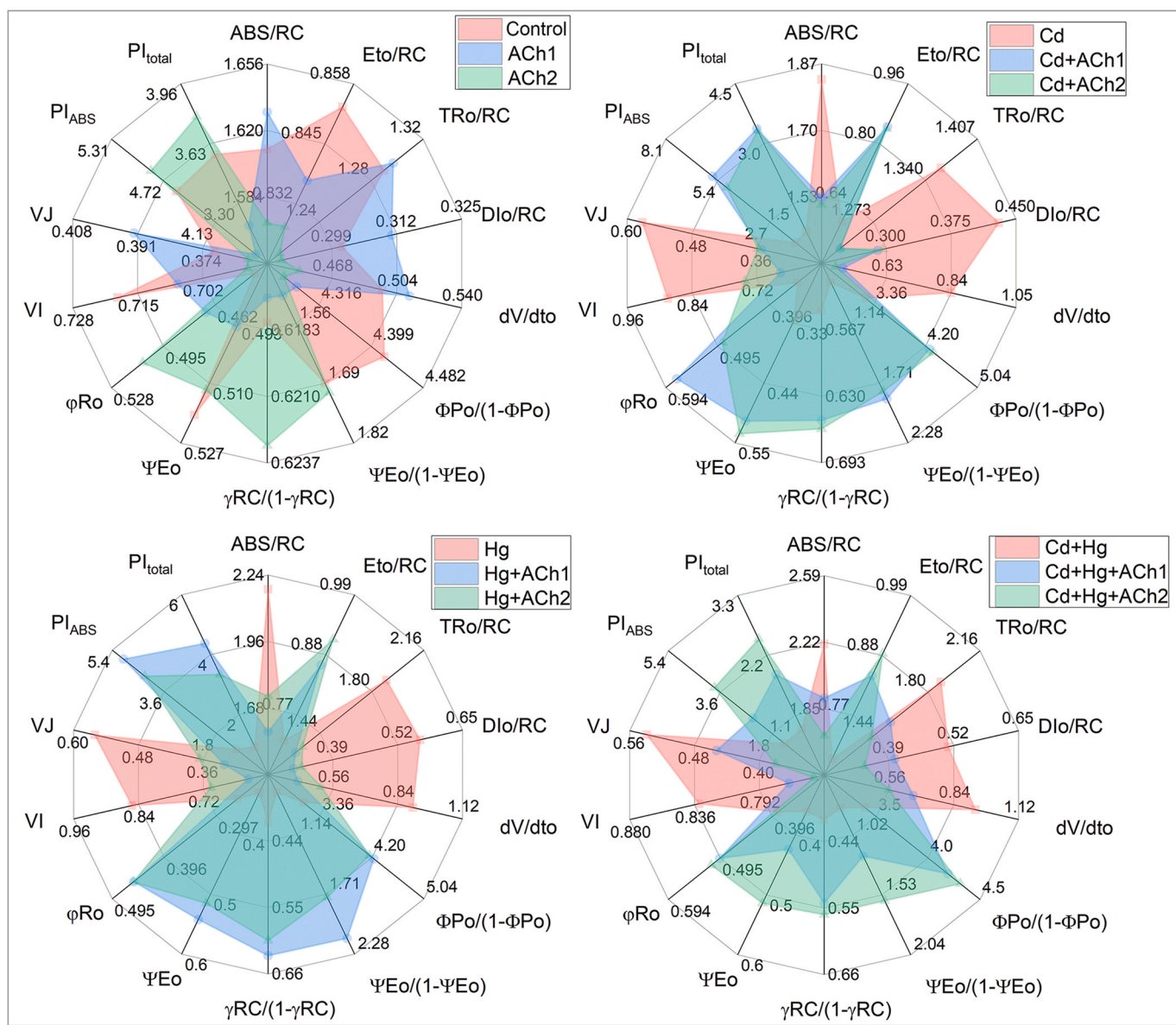


Fig. 3. The effects of acetylcholine (ACh1, 10 μ M; ACh2, 100 μ M) treatments to wheat leaves under Hg and/or Cd stress (Hg, 50 μ M; Cd, 100 μ M) on quantum efficiencies, structural indicators, and performance indices. The parameters derived from the OJIP transient and their definitions were given in [Supplementary Table 1](#).

(Cd, Hg, Cd + Hg) increased DHA content in wheat leaves (Fig. 9D). Likewise, according to the combined stress (Cd + Hg) condition, there was a decrease in the DHA content of Cd + Hg + ACh1 and Cd + Hg + ACh2 by 21% and approximately 40%, respectively. GSH content increased with ACh1 application by 16% compared to the control (Fig. 9E). All stress conditions resulted in decreased GSH content. However, Cd + ACh1 and Hg + ACh1 treatments provided 48% and 29% increased GSH levels compared to stress groups. In the combined stress application, the Cd + Hg + ACh2 group provided a 36% increased GSH content. All stress + ACh treatments decreased GSSG accumulation in wheat leaves (Fig. 9F). ACh1 and ACh2 treatments increased the tAsA/DHA by approximately 30% and 56%, respectively, but a decrease was observed in the stress groups (Cd, Hg, Cd + Hg) (Fig. 9G). Cd + ACh1 and Cd + ACh2 groups resulted in a 76% and 33% increased tAsA/DHA compared to the Cd condition. Hg + ACh1 and Hg + ACh2 treatments increased the tAsA/DHA by 2.3-fold and approximately 3-fold. Cd + Hg + ACh1 and Cd + Hg + ACh2 caused a 2.3-fold and 4.2-fold increase in tAsA/DHA, respectively. There was no statistical difference in the GSH

redox state of the ACh1 and ACh2 treatments compared to the control (Fig. 9H). While all stress exposures reduce GSH redox state, ACh treatments have been successful in attenuating stress-induced effects on GSH.

4. Discussion

Heavy metal pollution in soil can cause morphological damage, metabolic imbalances, decreased growth, and reduced yields in plants (Alsafran et al., 2023). Simultaneously, high levels of heavy metal uptake initially affect cellular organelles and directly impact DNA and protein synthesis (Gupta et al., 2023). Water generally constitutes more than half of the fresh weight of plants. Water deficiency occurs when the plant's needs, affecting factors such as growth, photosynthesis rate, and stomatal conductance, are not fully met. From a different perspective, water deficiency occurs when the rate of transpiration exceeds water uptake by the roots (dos Santos et al., 2022). In this study, a reduction in RWC was observed in wheat plants exposed to Cd, Hg, and Cd + Hg

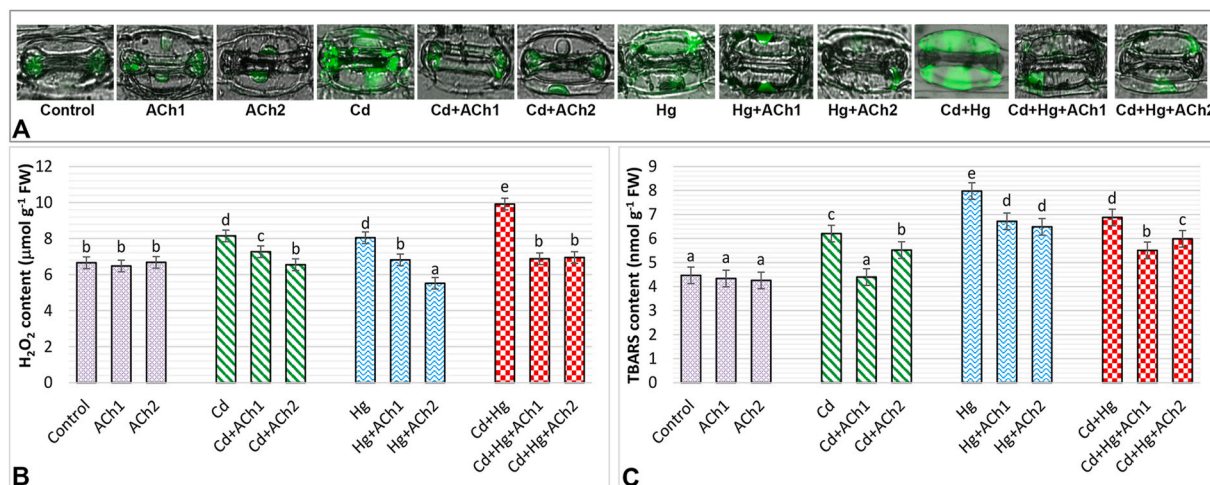


Fig. 4. ROS accumulation (A), hydrogen peroxide (H₂O₂, B) and lipid peroxidation (TBARS, C) contents in acetylcholine (ACh1, 10 μM; ACh2, 100 μM) treated wheat leaves under Hg and/or Cd stress (Hg, 50 μM; Cd, 100 μM). All data obtained were subjected to a one-way analysis of variance (ANOVA). Differences were considered to be significant at p < 0.05.

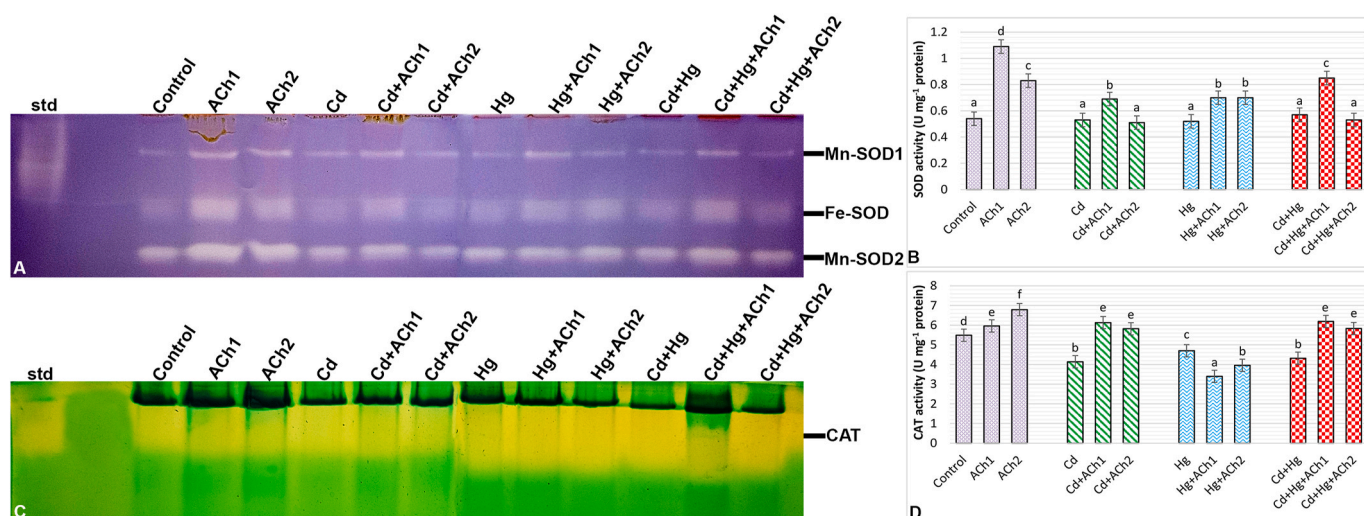


Fig. 5. Relative band intensity of different types of superoxide dismutase isoenzymes (SOD, A) and SOD activity (B), relative band intensity of different types of catalase isoenzymes (CAT, C) and CAT activity (D) in acetylcholine (ACh1, 10 μM; ACh2, 100 μM) treated wheat leaves under Hg and/or Cd stress (Hg, 50 μM; Cd, 100 μM).

stress. RGR is also reduced under Cd and/or Hg conditions. The over-accumulation of Cd and Hg in plants results in the inhibition of growth and metabolic dysfunction (Yuan et al., 2021). Our results are consistent with the previous study of Rellán-Álvarez et al. (2006), where they detected disruptions in growth parameters and water relations of *Zea mays* upon Cd and Hg pollution. However, we found that ACh1 and ACh2 treatments alleviated the decrease caused by heavy metal stress, resulting in increased RWC and RGR under Cd and/or Hg stress conditions. Similar positive outcomes were observed in tobacco plants subjected to Cd stress and treated with ACh, suggesting that ACh's capacity to enhance water absorption and nutrient efficiency might contribute to the reduction of heavy metal uptake (Su et al., 2020b). Furthermore, Saleh (2024) demonstrated that nickel (Ni) application to wheat plants resulted in decreased growth parameters. However, the application of ACh effectively alleviated the detrimental effects induced by stress to a significant extent. In addition, the study by Wang et al. (2003) shows that ACh can affect cell division and differentiation in root tissues. Qi et al. (2022) further confirms the role of ACh in regulating root cell division and growth under various conditions. Therefore, the ACh-promoted growth regulation we observed in stressed plants may

also be achieved by promoting root development.

Under stress conditions, proline serves as an indicator of its role in mitigating oxidative stress damage. Proline functions by scavenging free radicals, preserving osmotic balance, and supporting PSII (Hayat et al., 2021). In this study, an increase in proline content was observed under stress conditions induced by Cd, Hg, and their combination (Cd + Hg). Consequently, elevated levels of proline indicate the antioxidant potential in wheat plants, as it contributes to the detoxification of reactive oxygen species (ROS) accumulation (Pandian et al., 2020). Our findings are in agreement with those of Nazir et al. (2019), who suggested that Cu stress increased proline content in tomato leaves compared to the control. Similarly, Cd + ACh2, Hg + ACh1, and Cd + Hg + ACh2 conditions showed an increase in Pro content. This increase plays a crucial role in protecting cells from harmful effects by maintaining RWC, stabilizing protein structure and function, enhancing tolerance mechanisms, and expelling ROS. Saleh (2024) also reported that ACh supplementation increased Pro and other soluble sugar content in wheat leaves under nickel toxicity and suggested that the reason for this may be the induction of biosynthesis pathways. ACh can modulate stress signaling pathways, potentially altering the plant's response to stress. Depending

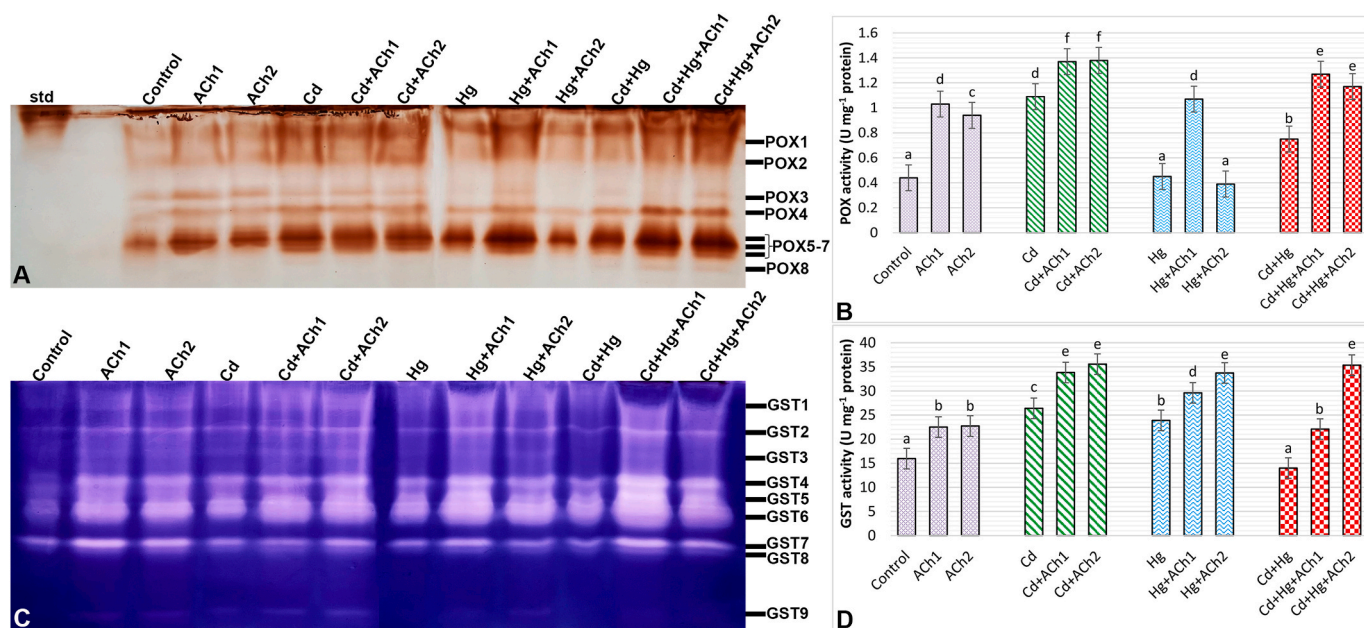


Fig. 6. Relative band intensity of different types of peroxidase isoenzymes (POX, A) and POX activity (B), relative band intensity of different types of glutathione S-transferase isoforms (GST, C), GST activity (D) in acetylcholine (ACh1, 10 μ M; ACh2, 100 μ M) treated wheat leaves under Hg and/or Cd stress (Hg, 50 μ M; Cd, 100 μ M).

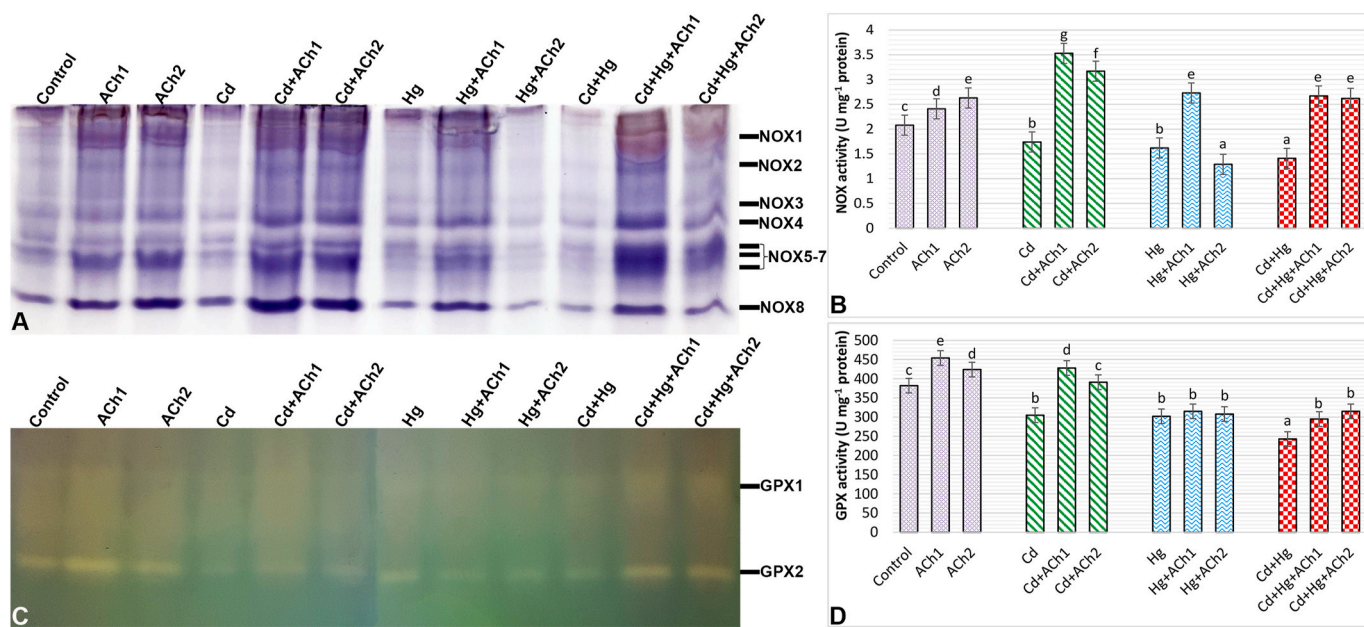


Fig. 7. Relative band intensity of NADPH oxidase isoenzymes (NOX, A) and NOX activity (B) and relative band intensity of different types of glutathione peroxidase isoforms (GPX, C) and GPX activity (D) in acetylcholine (ACh1, 10 μ M; ACh2, 100 μ M) treated wheat leaves under Hg and/or Cd stress (Hg, 50 μ M; Cd, 100 μ M).

on the dose and context, ACh might influence the balance between different stress responses, sometimes leading to a reduction in proline accumulation, as in the Hg + ACh2 and Cd + Hg + ACh1 groups in this study. Similarly, Qin et al. (2019) found that in tobacco plants under salt stress, different doses of ACh (1, 10, 50, and 100 μ M) led to varying levels of proline accumulation. Specifically, the 10 μ M dose resulted in the highest proline accumulation when applied to the roots, while the 50 μ M dose was most effective for foliar application. In contrast, the lower (1 μ M) and higher (100 μ M) doses resulted in reduced proline content.

Efficient photosynthetic yield requires an ample supply of water, light, and carbon dioxide (CO₂). Stomata regulate the exchange of CO₂

and water vapor between the interior of leaves and the surrounding atmosphere (Dusenge et al., 2019). In this study, we noted a reduction in carbon assimilation (A) rate in the Cd, Hg, and Cd + Hg groups. Heavy metals have the potential to induce a decrease in chlorophyll content and impair the activity of enzymes responsible for CO₂ fixation (Anjum et al., 2017). The decrease in A value coincided with a parallel decrease in E, g_s, and C_i, which could be attributed to stomatal abnormalities. Stomatal limitation (L_s) increased under stress conditions (Cd, Hg, Cd + Hg), which was consistent with other gas exchange parameters, providing evidence for this phenomenon. Similarly, Simonová et al. (2007) observed a notable reduction in plant biomass along with an elevation in stomatal resistance, subsequently leading to decreased

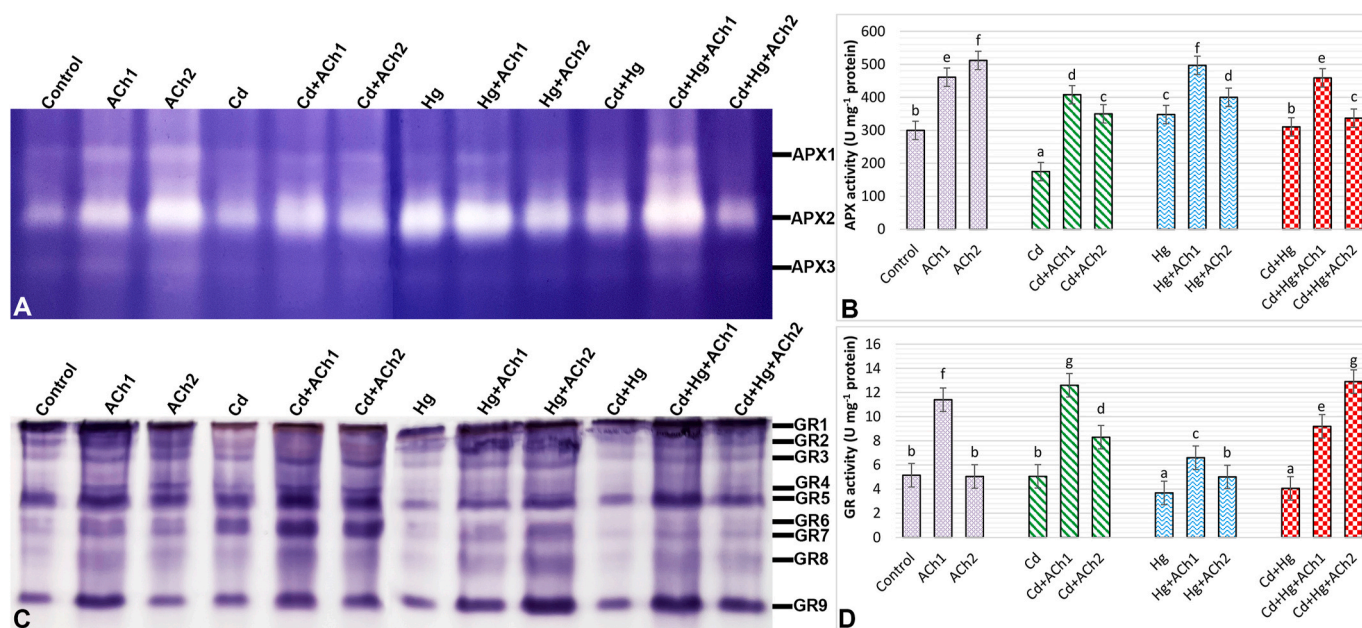


Fig. 8. Relative band intensity of ascorbate peroxidase (APX, A), APX activity (B), relative band intensity of glutathione reductase isoenzymes (GR, C) and GR activity (D) in acetylcholine (ACh1, 10 µM; ACh2, 100 µM) treated wheat leaves under Hg and/or Cd stress (Hg, 50 µM; Cd, 100 µM).

photosynthesis and transpiration in the leaves of *Brassica juncea* and *Vigna radiata* under Cd stress. Conversely, ACh1 and ACh2 treatments reversed these effects under stress conditions, increasing photosynthesis, transpiration, intercellular CO₂ concentration, and stomatal conductance. Su et al. (2020b), in line with our findings, reported that treatment with ACh effectively countered the reduction in chlorophyll content and mitigated the adverse effects on photosynthetic characteristics induced by Cd.

Heavy metals can disrupt both the light and dark reactions of photosynthesis. Additionally, among the non-stomatal factors, a significant limitation arises from the inactivation of enzymes engaged in carbon assimilation, leading to reduced CO₂ utilization by mesophyll cells. Furthermore, heavy metals contribute to a decrease in the photokinetic activities of photosystem II (PSII) and photosystem I (PSI). PSII and PSI are essential components sensitive to abiotic stress in photosynthetic light reactions (Huihui et al., 2020). The onset of stress typically results in a reduction, and occasionally mild deactivation, of both PSI and PSII reaction centers (Zhang et al., 2018). Heavy metal stress typically results in a decrease in the activity of both PSI and PSII (Baycu et al., 2017). The effects of Cd and Hg stress on PSII and PSI function in wheat leaves are indicated by parameters such as F_v/F_m and F_v/F_o (Israr et al., 2006). In this study, we observed a decrease in the F_v/F_o and F_v/F_m parameters under stress conditions induced by Cd, Hg, and Cd + Hg. This decrease in the F_v/F_m is consistent with the findings of Chen et al. (2022). The decrease in the F_v/F_o value indicates changes in the electron transport rate from PSII to primary electron acceptors, as well as a reduction in the number and size of reaction centers (Singh et al., 2022). The decrease in PSII efficiency or F_v/F_m caused by heavy metal-induced decrease leads to a decrease in the utilization of light energy. This, in turn, results in an increase in non-photochemical quenching (F_o/F_m) under Cd and Hg stress. According to these results, ACh may alleviate photosynthetic inhibition caused by Cd and Hg by regulating photosynthetic capacity and improving PSII activity (Xu and Zhou, 2008).

In this study, we investigated the impact of Cd and Hg stress on PSII photochemistry of wheat plants by employing the JIP test. This test is particularly valuable for elucidating how the photosynthetic system adapts to stress conditions (Mathur et al., 2013). The values of TR_o/RC, ABS/RC, and DI_o/RC increased under Cd and Hg stress, hindering the

energy flow to the reaction center of PSII. These results are consistent with the study by Zhuo et al. (2017). Two doses of ACh treatments to wheat plants caused a decrease in the effects of Cd and Hg stress on photosynthesis. This decrease led to an increase in energy flow to the reaction center. The DI_o/RC value increased in groups subjected to Cd and Hg stress, while a decrease was observed in groups treated with ACh. This decrease indicates stimulated and increased photon capture capacity of active reaction centers. The PI_{total} and PI_{ABS} performance indices are innovative parameters for analysing the photosynthetic reactions of plants under stress conditions. In this study, we found that ACh applications increased PI_{total} under Cd and Hg stress, which improved the overall performance of PSII's photokinetic activities in wheat leaves.

Heavy metals disrupt photosynthetic processes, leading to an elevation in ROS levels in plants, ultimately contributing directly or indirectly to oxidative damage (Sharma et al., 2020). ROS, including •OH, O₂^{•-}, and H₂O₂ are produced as byproducts during electron transfer in photosynthesis and respiration (Kadukova and Kavuličova, 2011). ROS play numerous signaling roles in plants and other organisms (Mittler, 2017). Under stress conditions, major sources of ROS include β-oxidation of fatty acids, disruptions in the electron transport chains of mitochondria and chloroplast, raised photorespiration, and NOX activity (Abdelgawad et al., 2015; Cassia et al., 2018). SOD activity modulates the relative quantities of O₂^{•-} and H₂O₂, the substrates of two Haber-Weiss responses, and reduces the threat of hydroxyl revolutionary conformation, which is largely reactive and can beget serious damage to membranes, proteins, and DNA (Zhang et al., 2007). In this study, no difference in SOD activity was observed under Cd and Hg stress compared to the control. Similarly, Zhang et al. (2015) reported no change in SOD activity in rice plants under abiotic stress.

NOX catalyzes the transfer of electrons from NADPH to oxygen in the cytoplasm, triggering a defense reply in plants when submitted to stress by generating O₂^{•-} and further serving as a source of H₂O₂ (Dumanovic et al., 2020). The reduction in NOX activity beneath Cd + Hg conditions is believed to be related to the accumulation of O₂^{•-} and H₂O₂ excited by Cd and Hg (Maksymiec and Krupa, 2006). ACh treatments successfully increased NOX activity under both normal and stress conditions, but this effect was not observed in the Hg + ACh2 group. The lower NOX activity in the Hg + ACh2 treatment compared to Hg stress alone could be due to

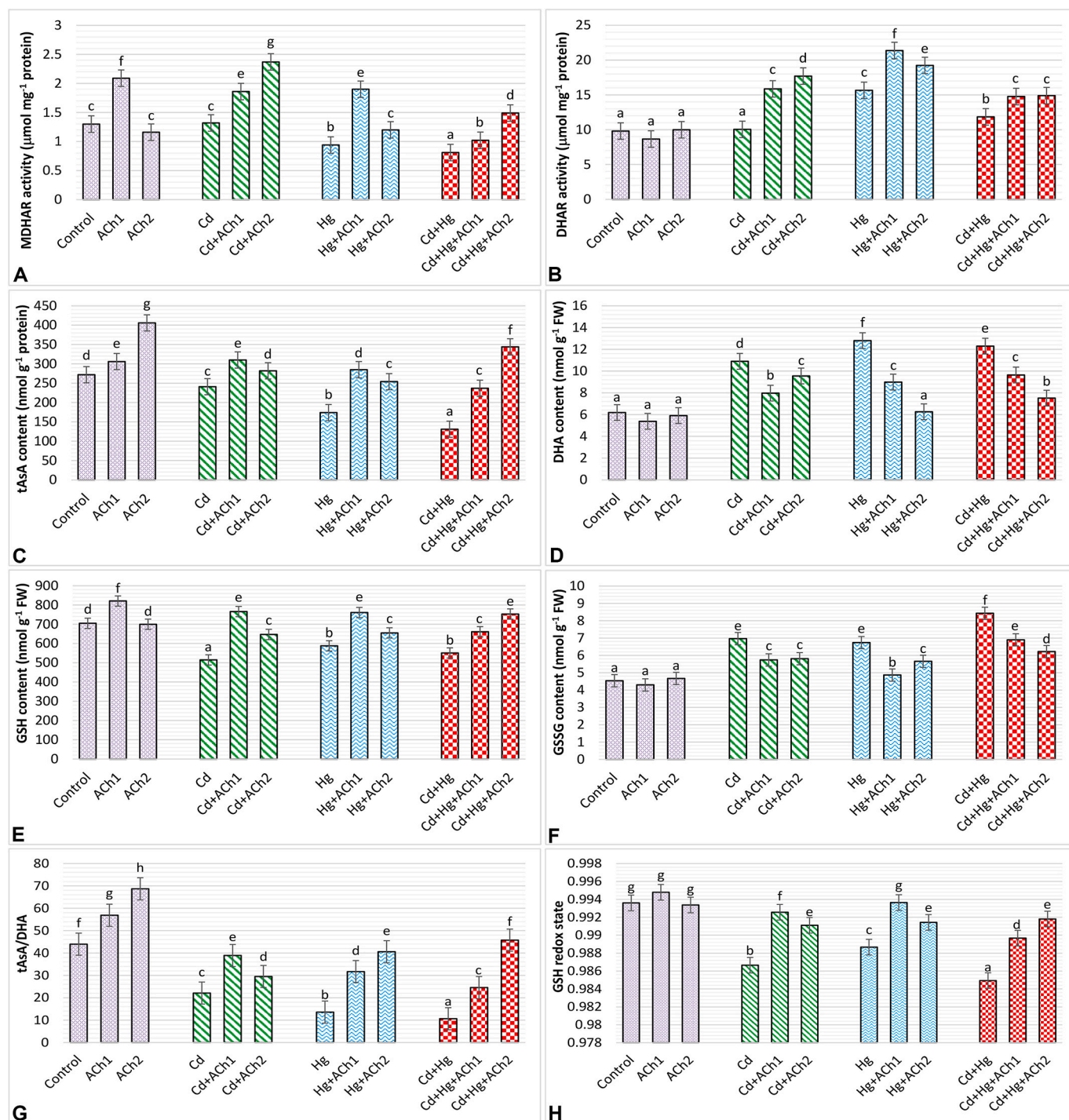


Fig. 9. The monodehydroascorbate reductase activity (MDHAR, **A**), dehydroascorbate reductase activity (DHAR, **B**), total ascorbate content (tAsA, **C**), dehydroascorbate content (DHA, **D**), glutathione content (GSH, **E**), oxidized glutathione content (GSSG, **F**), tAsA/DHA (**G**) and GSH redox state (**H**) in acetylcholine (ACh1, 10 μM ; ACh2, 100 μM) treated wheat leaves under Hg and/or Cd stress (Hg, 50 μM ; Cd, 100 μM).

the high concentration of ACh affecting NOX enzyme regulation. ACh may alter oxidative stress pathways differently in the presence of Hg, potentially leading to reduced NOX activity. Additionally, the fact that H_2O_2 levels were the lowest in this group suggests that the demand for NOX activity might have been diminished (Wei et al., 2020).

Even at low concentrations H_2O_2 , a potent photosynthetic inhibitor, can oxidize Calvin cycle enzymes, halving CO_2 fixation (Foyer and Shigeoka, 2011). Anjum et al. (2015) observed accumulation in H_2O_2 and MDA content in maize plants with Cd exposure. In this study, a boost in TBARS content was observed under Cd, Hg, and Cd + Hg conditions.

CAT, which is related to the major defense mechanism opposite the accumulation and toxicity of active oxygen species like H_2O_2 , plays a role in reducing the H_2O_2 content in plant cells. CAT enzyme directly converts H_2O_2 into water and oxygen. It is likely that CAT activity neutralizes excessive ROS production induced by heavy metal stress (Malar et al., 2016). However, in our study, a reduction in CAT activity was observed under Cd, Hg, and Cd + Hg conditions. This reduction is thought to be compensated by the increased activity of H_2O_2 -removing antioxidant enzymes (APX and POX). Although stress + ACh treatments successfully increased CAT enzyme activity under Cd and Cd + Hg

stress, CAT activity decreased with ACh applications under Hg stress. The reduction in CAT activity observed with the Hg + ACh1 treatment, in contrast to the elevated values under Cd and Cd + Hg stress, may be due to several factors. Different stressors can elicit distinct responses in antioxidant enzyme activities. Hg may affect CAT activity differently compared to Cd or the combined Cd + Hg stress. Specifically, Hg might have a more pronounced inhibitory effect on CAT activity or alter the ROS balance in a way that reduces the necessity for CAT activity in Hg + ACh1-treated plants. [Vighi et al. \(2016\)](#) have shown that CAT gene expression can vary depending on the type and duration of stress in *Oryza sativa*. Additionally, [Shim et al. \(2003\)](#) reported that increased oxidative stress leads to decreased CAT activity in rice, wheat, and cucumber seedlings. Therefore, the observed decrease in CAT activity under Hg stress reflects the complex relationship between stress and enzymatic response. Furthermore, the reduction in CAT activity in the Hg + ACh1 group may also be related to a compensatory increase in POX activity, as these enzymes often perform complementary roles in the antioxidant defense system.

In Cd and Cd + Hg conditions, POX activity increased, while GPX activity decreased. This increase in POX activity may serve as a defensive mechanism, thereby protecting cellular components from oxidation ([Kumar et al., 2023](#)). Stress + ACh treatments generally resulted in increased POX activity as well. This finding aligns with [Soares et al. \(2020\)](#), who reported elevated POX activity following brassinosteroid application. However, a significant decrease in POX activity was observed in the Hg + ACh2 group, which may be due to several factors. The interaction between heavy metals and ACh can influence POX activity. Hg might induce a stress response that overwhelms the POX system or interfere with ACh's typical activation of POX. [Dey et al. \(2007\)](#) also noted that different heavy metals affect POX activity in wheat leaves and roots in various ways. Additionally, high concentrations of ACh (such as under ACh2) might cause overcompensation or maladaptation in the antioxidant defense system, potentially leading to reduced POX activity.

In plants, antioxidants such as AsA and GSH play pivotal roles in mitigating oxidative stress induced by metal pollution. It has been suggested that both activities play a role in the removal of ROS and the maintenance of cellular redox buffering ([Kaya et al., 2020](#)). AsA prevents excessive H₂O₂ accumulation in plant cells, while GSH plays a momentous role as a ROS scavenger in plants ([Yan et al., 2018](#)). According to our results, AsA and GSH contents decreased below Cd and Hg stress conditions, which is supported by the results of [Nahar et al. \(2016\)](#) in lentil beans and [Ahmad et al. \(2018\)](#) in tomato plants. Plants can alleviate heavy metal-induced oxidative stress using antioxidant metabolites as electron acceptors with antioxidant and/or redox enzymes ([Hu et al., 2019](#); [Jung et al., 2020](#)). According to our results, under stress conditions, the ascorbate ingredient and the AsA/DHA ratio reduced, resulting in oxidative damage due to heavy metal uptake, consistent with increased ROS formation and lipid peroxidation as observed in several studies ([Foyer and Noctor, 2011](#); [Hasanuzzaman et al., 2017](#)). MDHAR and DHAR play a role in the renewal of AsA from dehydroascorbate (DHA), the oxidative form of AsA ([Hasanuzzaman et al., 2012](#)). However, under Cd, Hg, and Cd + Hg applications, the decrease in MDHAR, GR, tAsA/DHA, and GSH content, and the disturbance in GSH redox case, did not lead to the production of scavenging radicals through the AsA-GSH cycle. Moreover, the service of the DHAR enzyme increased, suggesting that the AsA produced by DHAR is used by other metabolic functions ([Fotopoulos et al., 2010](#)). Treatment with ACh enhanced the efficiency of certain antioxidant enzymes and also the elevated the levels of AsA and GSH.

These results have led to an increase in the potential to neutralize excessive ROS. Consistent with our findings, [Qin et al. \(2021\)](#) indicated the enhanced functionality of the antioxidant system due to ACh treatment under Cd and salinity stress. Exogenously applied ACh exhibits prominent features of the increased functionality of the antioxidant system and reduction in ROS accumulation in plants. The role of

neurotransmitters, including ACh, in alleviating oxidative damage in plants through enhanced antioxidant functionality is evident ([Saleh, 2024](#)). In our current study, it was observed that ACh treatment triggered tolerance to heavy metal stress in wheat leaves. This can be explained by the observations that, as previously suggested, ACh increases the cell wall's capacity to retain more heavy metals, sequestering heavy metals in vacuoles and subsequently helping them co-precipitate with the cytosol ([Su et al., 2020a](#)).

5. Conclusion

Exposure to heavy metal stress has resulted in a reduction in RGR and RWC of wheat seedlings. Cd and/or Hg conditions have led to stomatal inhibition, as evidenced by decreased A, g_s, E, C_i, and increased stomatal limitations. Treatment with ACh1 and ACh2 has positively mitigated the adverse effects on RGR and RWC observed in the stress groups. ACh treatment has countered the effects of stress conditions on gas exchange parameters. Moreover, our findings highlighted the accumulation of ROS and lipid peroxidation in wheat leaves under Cd and/or Hg stress. However, ACh treatment effectively bolstered the activities of antioxidant enzymes, thereby mitigating oxidative damage induced by heavy metals. In summary, our study underscores the potential of ACh treatment in conferring stress tolerance and mitigating the adverse effects of heavy metal toxicity in plants. The findings provide valuable insights into novel strategies for enhancing plant tolerance in the face of environmental stressors, contributing to sustainable agricultural practices and food security.

CRedit authorship contribution statement

Buket Koyukan: Writing – review & editing, Writing – original draft, Methodology, Investigation. **Busra Arıkan-Abdulveli:** Writing – review & editing, Writing – original draft, Methodology, Investigation. **Evren Yildiztugay:** Writing – review & editing, Writing – original draft, Methodology, Investigation. **Ceyda Ozfidan-Konakci:** Writing – review & editing, Writing – original draft, Methodology, Investigation.

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

The authors do not have permission to share data.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.envpol.2024.124978>.

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