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ORIGINAL PAPER

Stress-related physiological responses and ultrastructural changes in *Hypoxis hemerocallidea* leaves exposed to cadmium and aluminium

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Abstract

Hypoxis hemerocallidea is a medicinal plant containing hypoxoside (a pharmacologically active phytosterol diglucoside). This study evaluated the elemental composition in leaves of *H. hemerocallidea* treated with cadmium (Cd) and aluminium (Al) using scanning electron microscopy (SEM) combined with energy-dispersive X-ray spectroscopy (EDX). The impact of Cd and Al on photosynthetic pigments and performance, antioxidant activities and ultrastructure were also assessed. Corms of *H. hemerocallidea* were micropropagated, rooted and then exposed to varying concentrations of Cd, Al, and Cd + Al for six weeks. The SEM/EDX analysis indicated a two-fold increase in carbon content across all treated plants compared to the control. No/little Cd was detected in the leaves compared to a progressive increase in Al concentration with increasing Al treatment levels. This indicted that Al is more readily translocated to the shoots compared to Cd. Plants treated with Cd exhibited a significant decrease in total chlorophyll content accompanied by reduced photosynthetic performance and lower relative electron transport rates. Cd and Al exposure led to higher carotenoid, superoxide dismutase and malondialdehyde levels, indicating oxidative stress. Cd-treated plants displayed increased amylase activity and decreased carbohydrates content. Ultrastructural alterations occurred with exposure to Cd and Al, including abnormal swelling or disintegration of chloroplasts and thylakoid degeneration. An increase in starch grains and a decrease in plastoglobuli were also noted. In conclusion, this investigation provides evidence that both Cd and higher concentrations of Al exert detrimental effects on the ultrastructure, metabolism and photosynthetic performance of *H. hemerocallidea*, contributing to reduced growth and biological activity when stressed.

Keywords African potato · Antioxidant metabolites · Heavy metals · Medicinal properties · Photosynthesis

Abbreviations

Al	Aluminium
Cd	Cadmium
Chl a	Chlorophyll a
Chl a + b	Total chlorophyll
EDX	Energy-dispersive X-ray spectroscopy
Fo	Initial fluorescence
Fm	Maximum fluorescence
Fv	Variable fluorescence
MDA	Malondialdehyde
NBT	Nitroblue tetrazolium
NPQ	Non-photosynthetic quenching
PSII	Photosystem II
rETR	Relative electron transfer rate
ROS	Reactive oxygen species
SOD	Superoxide dismutase
SEM	Scanning electron microscopy
TBARS	2-thiobarbituric acid reactive substances
TEM	Transmission electron microscopy

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Introduction

The accumulation of harmful heavy metal contamination in soils from human activities has increased significantly over the past few decades (Priya et al. 2023; Yan et al. 2018, 2020; Zhong et al. 2018). The impact of heavy metal toxicity

on plants varies depending on the plant species. Exposure to heavy metals elicits a range of toxic responses including physiochemical and ultrastructural changes which hinders the plant's ability to carry out fundamental metabolic processes (Ghuge et al. 2023). Plants deploy several defence mechanisms such as the accumulation of specific stress-related metabolites and the activation or alteration of various enzymatic and non-enzymatic antioxidant systems to reduce the negative impact of metal toxicity (Emamverdian et al. 2015; Mashabela et al. 2023).

Most South African soils are acidic (South African Department of Agriculture 2007). The availability of aluminium (Al) increases in acidic soil, leading to Al toxicity. This is a major limiting factor for crop productivity as it blocks mechanisms essential for cell division (Panda et al. 2009). Cadmium (Cd) has considerable phytotoxic effects, even at low concentrations (Li et al. 2023). It interfers with enzymatic activity involved in carbon assimilation, ultimately leading to the suppression of chlorophyll biosynthesis and disruption of photosynthetic activities (Gratão et al. 2005; Liu et al. 2018). Analysis of chlorophyll fluorescence is a valuable tool in investigating how excitation energy is utilized within the photosynthetic apparatus (Kalaji et al. 2016) and provides insight into the mechanisms and regulation of photosynthesis in living plant systems. Heavy metal toxicity disrupts the ability of chlorophyll fluorescence by hindering the efficient transfer of electrons to photosystem II (PSII), resulting in oxidative stress and physiological impairments. For instance, in PSII, the chlorophyll molecules absorb light and then re-emit it as chlorophyll fluorescence. Measuring this fluorescence offers valuable insights into the efficiency of PSII (Genty et al. 1989; Roháček 2002). A deeper understanding of how plants cope with heavy metal toxicity and other stressors can be gained by measuring chlorophyll fluorescence, thereby contributing to the development of effective strategies for plant protection and environmental conservation (Joshi and Mohanty 2004).

Hypoxis hemerocallidea Fisch. & C.A. Mey, known as the African Potato and "miracle muthi", is one of the most utilized medicinal plants in South African traditional medicine owing to its numerous pharmacological properties (Ncube et al. 2013). A pharmacologically active phytosterol diglucoside named hypoxoside with antitumor properties and anti-HIV activities was identified in *H. hamerocallidea* (Bayley and van Staden 1990). Plants growing in their natural environment are often exposed to multiple toxic contaminants (Bvenura and Afolayan 2012). Wild harvested *H. hemerocallidea* purchased from a street market and "muthi shop" in KwaZulu-Natal had high levels of Al and iron in the corms. This poses a potential health hazard when consumed for medicinal purposes. Cd levels were within the World Health Organization permissible limits (Okem et al. 2014). However, there is limited information available concerning the combined effects of heavy metal toxicity on medicinal plants. In a greenhouse trial where *H. hemerocallidea* plants were treated with Cd and Al, increasing Cd concentrations resulted in a significant decrease in plant growth (shoot and root length) and biomass. Al did not affect these growth parameters (Okem et al. 2015). Both Cd and Al were taken up by the plant in a dose-dependent response. Al had a high translocation factor with high Al concentrations recorded in the shoots. Cd was poorly translocated with low Cd concentrations in the shoots (Okem et al. 2015). Antioxidant activity and secondary metabolites (phenolics and flavonoids) increased with exposure to Cd and Al as a defence mechanism against oxidative stress. However, these decreased at the highest Cd and Al concentrations, indicating the loss in the ability to synthesize these metabolites to combat stress (Okem et al. 2015). This negatively affected the medicinal efficacy of the plant with a decrease in hypoxoside concentration and decreasing antibacterial activity, indicating a reallocation of resources to sustain primary metabolic processes (Okem et al. 2015).

Wild populations of *H. hemerocallidea* are declining due to overharvesting (Katerere and Eloff 2008). Owing to the high demand, there is potential for large-scale commercial cultivation. Given the prevalence of heavy metal contamination in South African soils (Shabalala et al. 2022), a comprehensive understanding of the physiological responses, ultrastructural changes and stress-associated metabolites in medicinal plants subjected to heavy metal exposure is required before they can be grown commercially. Such knowledge is vital for developing effective cultivation strategies to ensure that high quality plants with the target bioactive compound(s) are produced. This study aimed to evaluate the elemental composition in leaves of *H. hemerocallidae* exposed to heavy metal stress and to investigate the interactive effects of Cd and Al on physiological (specifically photosynthetic) responses, biochemical alterations and anatomical changes in the leaves of *H. hemerocallidea*.

Materials and methods

Micropropagation and establishment of *H. hemerocallidea* plants

Corms of *H. hemerocallidea* were collected from the University of KwaZulu-Natal Botanical Garden. A voucher specimen (A. Okem 21 NU) was deposited in the Bews Herbarium, University of KwaZulu-Natal, Pietermaritzburg Campus. The corms were micropropagated and the resulting shoots were augmented through sub-culturing. Subsequent steps involving rooting and acclimatization of the in vitro-derived plants were carried out as detailed in Okem et al. (2015). Thereafter, the plantlets were potted into

acid-washed quartz sand and transferred to a greenhouse. Plants were watered with 50% Hoaglands solution for 7 months until well established (Okem et al. 2015).

Heavy metal treatments

Once acclimatized, healthy plants were selected for the heavy metal treatment. Hoagland's nutrient solution was spiked with different concentrations of $Cd(NO_3)_2$ (2, 5, and 10 mg L^{-1}), $Al(NO_3)_3$ (500, 1000, and 1500 mg L^{-1}) and a combination of both metals (Cd 2 + Al 500, Cd 5 + Al 1000, Cd 10 + Al 1500 mg L^{-1}). Hoagland's nutrient solution without Cd and Al was used as the control. The Cd and Al concentrations were selected based on previous field studies (Jonnalagadda et al. 2008). Each treatment was replicated ten times. The plants were treated with 100 mL per pot every two days. The experiment was terminated after 6 weeks. Growth parameters were recorded and are presented in conjunction with heavy metal distribution in the roots and shoots, hypoxoside content and antibacterial activity (Okem et al. 2015). Leaf material was collected for the physiological and photosynthetic analysis detailed in the present paper.

Elemental analysis

Elemental distribution on the abaxial leaf surface was visualized using a scanning electron microscope (SEM) coupled with energy-dispersive X-ray spectroscopy (EDX). Leaf samples were air-dried and mounted on aluminum stubs using double-sided sticky carbon tape. The material on the stubs was coated with a 20 nm conductive film of carbon using a Quorum Q150RS Carbon Coater. The EDX analysis was conducted on a Zeiss EVO LS15 VP SEM equipped with the Oxford X-Max EDX 80 mm SDD (silicon drift detector). A 60s scan time and approximately 1 µm scan depth were set to enable a detailed elemental analysis of the abaxial leaf surface to provide data on the spatial distribution of different elements in the plant samples. The EDX system could detect elements with a 0.1% detectability limit and heavy elements at a lower than 0.1% detectability limit. The analysis was carried out using the INCA v4.14 software (Oxford Instruments).

Chlorophyll and carotenoid content

Total chlorophyll (Chl a + b) of fresh leaf samples extracted in acetone was determined quantitatively following the method of Lichtenhaler (1987). The absorbance was measured at 644.8, 661.6 and 470.0 nm (UV–vis spectrophotometer, Varian Cary 50, Australia). Chlorophyll and carotenoid concentrations were expressed using the equations:

$$\text{Chl a} + \text{b} = 7.05A_{661.6} + 18.71A_{644.8}$$

$$\text{Carotenoids} = \left(1000A_{470} - 1.90\text{Chl a} - 63.14\text{Chl b}\right)/214$$

The pigment content expressed as mg g^{-1} FW. The pigment analysis was performed in triplicate.

Chlorophyll a (Chl a) fluorescence

The impact of Cd and Al on the photochemical activities of *H. hemerocallidea* was assessed using chlorophyll a (Chl a) fluorescence (FMS 2 modulated fluorometer, Hansatech Instruments, King's Lynn, U.K.). Fully expanded leaves from 10 plants in each treatment were securely clamped in standard Hansatech leaf clips. The leaves were adapted to the dark for 10 min to allow for the oxidation of the photosynthetic electron transport system and then the initial fluorescence (F_o) and maximum fluorescence (F_m) was measured. A 10 min adaptation time was chosen as it enabled the fast-relaxing non-photosynthetic quenching (NPQ) to diminish in unstressed plants (control plants). The steady-state level of fluorescence in the light is termed *F´*. The fluorescence intensity was measured by activating the actinic light with a saturation light pulse of 3000 µmol photons m^2 s^{-1} and F_m was measured after 5 min. The activation of a regular saturation pulse under actinic illumination transiently closed all the reaction centres and provides a value of maximal fluorescence in the light-adapted state, termed $F_{m´}$ which is less than the dark-adapted F_m. The variable fluorescence (F_v) was determined using the formula $F_v = F_m - F_o$. The maximum quantum efficiency of PSII photochemistry was calculated as $F_v/F_m = (F_m - F_o)/F_m$. The quantum efficiency of PSII electron transport in the light was calculated as $\Phi PSII = (F_{m´} - F)/ F_{m´}$. The relative electron transfer rate (rETR) was calculated as rETR= ΦPSII x 0.42 x photosynthetic photon flux density (Schreiber et al. 1995). NPQ was estimated as the Stern-Volmer quotient ($(F_m - F_{m´})/ F_{m´}$) and its components using the method described by Thiele et al. (1997).

Superoxide dismutase (SOD) activity

SOD activity was determined using the nitroblue tetrazolium (NBT) method (Sadasivam and Manickam 1996) with some modifications. Briefly, 1 g FW leaf samples were homogenized in 10 mL ice-cold potassium-phosphate buffer (pH 7.8) and the homogenate centrifuged at 10 000 x *g* for 10 min at 4 °C. The supernatant was used as the enzyme source. A reaction mixture was prepared with 50 mM potassium-phosphate buffer (pH 7.8), 13 mM methionine, 75 mM NBT, 0.1 mM ethylenediamine tetraacetic acid, 2 mM riboflavin and 50 µL enzyme extract and the volume adjusted to 3 mL with distilled water. The reaction was initiated by switching

on a fluorescent lamp (48 µmol photons m^2 s^{-1}). The reaction was terminated after 15 min by switching off the lamp. The absorbance of the samples was measured at 560 nm. One unit of SOD activity was defined as the amount of enzyme extract that caused a 50% inhibition of the SOD-inhibitable fraction of the NBT reduction in the presence of methionine. The enzyme activity was calculated as.

$$\text{SOD activity} = \left(\left(OB_b - OD_s\right)/50\%\ OD_b\right) \times \text{aliquot of sample}$$

where OD_b = optical density of the blank, OD_s = optical density of the sample. Enzyme activity was expressed as mg protein.

The experiment was conducted in triplicate.

Lipid peroxidation rate

The rate of oxidative damage to leaf lipids was assessed by measuring the total 2-thiobarbituric acid reactive substances (TBARS) expressed as malondialdehyde (MDA) equivalents based on the method of Cakmak and Horst (1991) with minor modifications. Briefly, fresh leaf samples (0.5 g FW) were homogenized in 5 mL 0.1% (w/v) trichloroacetic acid at 4 °C. After centrifugation at 12 000 x *g* for 5 min, 1 mL supernatant was mixed with 4 mL 0.5% (w/v) 2-thiobarbituric acid in 20% (w/v) trichloroacetic acid. The samples were incubated at 90 °C for 30 min, followed by the termination of the reaction by placing the samples in an ice bath. After centrifugation at 10 000 x *g* for 5 min, the absorbance of the supernatant was measured at 532 nm. To account for non-specific turbidity, the absorbance at 600 nm was subtracted from the reading. The resulting value was used to calculate the TBARS content using the formula:

$$\text{TBARS content(nmol g}^{-1}\text{FW)} = \left(\left(A_{532} - A_{600}\right) V \times 1000\right)/\varepsilon XW$$

where ε is the specific extinction coefficient (= 155 $mM.cm^{-1}$), V is the volume of crushing medium, W is the fresh weight of the leaf, A_{532} and A_{600} is the absorbance at 532 and 600 nm. The experiment was performed in triplicate.

Amylase activity

The activity of α- and β-amylase (starch-degrading enzymes) in leaf samples was determined using the method of Sadasivam and Manickam (1996) with slight modifications. Briefly, 1 g freshly cut leaf sample was homogenized in 10 mL ice-cold 10 mM CaCl solution and the resulting homogenate was centrifuged at 54 000 x *g* at 4 °C for 20 min. The supernatant was used as the enzyme source for amylase. In the assay, 1 mL enzyme-enriched supernatant and 1 mL 1% starch solution were incubated at 27 °C for 15 min. The reaction was terminated by adding 2 mL dinitrosalicylic acid reagent and heating the mixture in boiling water for 5 min. Immediately thereafter, 1 mL potassium sodium tartrate solution was added and the tubes cooled in running tap water. The volume was adjusted to 5 mL with distilled water and the absorbance measured at 560 nm. The activity of α- and β-amylase was expressed as mg maltose produced during 5 min incubation. The assay was performed in triplicate.

Total carbohydrates content

Total carbohydrates content was determined using the method of Dubois et al. (1951) outlined by Buysse and Merckx (1993) with slight modifications. Dried powdered shoot and root samples (25 mg DW) were pooled from 10 plants per treatment and extracted in 10 mL 80% ethanol where the mixture was heated at 95 °C for 60 min. The extract was centrifuged at 2000 x *g* for 15 min at room temperature. The supernatant was adjusted to 10 mL with distilled water. From this, 500 µL supernatant was mixed with 3 mL Anthrone reagent. A standard solution of glucose (100 µg mL^{-1}) was also prepared for calibration. The mixture was heated for 10 min in boiling water and the reaction stopped by cooling on ice. The absorbance was measured at 620 nm. The amount of glucose in the samples was calculated based on a glucose standard curve and expressed as µg mg^{-1} DW. The experiment was performed in triplicate.

Transmission electron microscopy (TEM)

Leave samples were fixed in 2.5% glutaraldehyde in 0.05 M potassium phosphate buffer (pH 7.1) for 8 h prior to the sample being prepared for TEM. Ultrathin sections, measuring 100 nm were obtained from each sample using an ultramicrotome (Leica UC7 RT) equipped with a diamond knife. These sections were placed on copper grids, air-dried and positively stained with aqueous uranyl acetate for 5 min, followed by rinsing with distilled water. The grids were air-dried and subsequently imaged using a JEOL JEM 1400 120 kV TEM.

Statistical analysis

Statistical analyses were carried out using SPSS for Windows by one-way ANOVA to test the different significance levels. Results are presented as mean ± SD.

Table 1 Energy-dispersive spectral analysis of essential element and heavy metal distribution in the abaxial leaf surface of *Hypoxis hemerocallidea* after six weeks of exposure to Cd and Al

Treatment (mg L^{-1})	Weight (%) of elemental content										
	C	O	Na	Mg	Si	P	S	K	Ca	Cd	Al
Control	30.54	43.67	0.18	0.24	0.68	2.05	9.09	2.07	11.48	–	
Cd 2	62.74	33.05	–	0.19	0.13	-	0.33	2.18	1.24	0.02	–
Cd 5	65.67	30.63	–	0.21	0.10	0.02	0.33	2.07	0.88	0.1	–
Cd 10	65.14	31.23	–	0.21	–	–	0.22	2.11	1.09	–	–
Al 500	62.93	30.00	0.10	0.19	2.72	–	0.17	2.48	0.58	–	0.75
Al 1000	64.20	26.43	–	0.28	1.81	0.48	1.22	0.96	2.40	–	2.00
Al 1500	68.86	25.59	–	0.27	1.45	0.18	0.25	1.15	0.49	–	1.37
Cd2:Al500	52.72	30.40	–	0.17	7.48	–	0.10	0.79	0.33	–	3.95
Cd5:Al1000	58.02	34.07	0.09	0.14	0.60	0.06	0.07	0.44	025	–	5.76
Cd10:Al10:1500	60.81	31.15	–	0.35	–	0.05	0.98	4.41	0.60	–	1.39

Results

EDX micrographs of elemental distributions in leaves of *H. hemerocallidea*

Nine essential elements (C, O, Na, Mg, P, S, K, Ca, and Si) were detected in the leaves of the control plants (Table 1). The C content increased two-fold in plants exposed to Cd or their combination. The amounts of the other elements in the leaves were reduced with no Na and low amounts of P detected. At the highest Cd concentration (Cd 10 mg L^{-1}), only six essential elements were detected (Table 1). Exposure to Al generally reduced the elemental content to a lesser extent compared to Cd (Table 1; Online Resource 1).

The uptake of Cd into the leaves was minimal with only trace amounts being detected in plants exposed to Cd 2 mg L^{-1} and Cd 5 mg L^{-1}. There was an increase in Al content across all plants subjected to Al treatments up to Al 1000 mg L^{-1}. Al content decreased with Al 1500 mg L^{-1} treatment, including the high level combined Cd and Al treatment. Cd was not detected in the leaves of plants subjected to the combined treatment (Table 1). The EDX analysis indicated that Cd showed poor distribution in the leaves compared to Al.

Effect of Cd and Al on photosynthetic pigments

Plants treated with heavy metals had significantly reduced levels of total chlorophyll (Fig. 1A). Cd exposure was the most detrimental with a dose-dependent decrease in Chl a + b with increasing Cd concentrations and prolonged exposure. By week 6, plants treated with Cd 10 mg L^{-1} had significantly lower chlorophyll content. Similarly, the highest concentrations of the combination treatments of Cd and Al significantly decreased the total chlorophyll content from week 4 to 6. Exposure to Al reduced the Chl a + b content from weeks 4–6 (Fig. 1A).

Exposure to heavy metals caused a dose- and time-dependent increase in the carotenoid content in *H. hemerocallidea*. The significantly highest carotenoid content was in plants exposed to the highest combination treatment for 6 weeks (Fig. 1B).

Effect of cC and Al on Chl a fluorescence

There was a progressive dose-dependent decrease in F_v/F_m in dark-adapted leaves of *H. hemerocallidea* with the lowest values in the combination treated plants (Fig. 1D). The exception was plants treated with Al 500 mg L^{-1} which had a slightly higher F_v/F_m ratio than the control (Fig. 1D). There was a comparable pattern for F_v/F_o activity (Fig. 1C).

There was a significant increase in NPQ activity in all Cd-treated plants (Fig. 2A). Plants exposed to low Al concentrations exhibited a lower NPQ activity compared to control plants but increasing Al concentrations significantly increased NPQ activity (Fig. 2B). The Cd and Al combination treatments significantly increased the NPQ activity at the higher concentrations (Fig. 2C). Increasing concentrations of Cd, Al and combination treatments significantly decreased the rETR (Fig. 3A). The exception was the lowest Al concentration where the rETR was similar to the control (Fig. 3B).

Effect of Cd and Al on SOD activity

SOD activity was higher in plants exposed to high levels of Cd and Al and the combination treatments compared to the control plants (Fig. 4A). SOD activity increased with increasing heavy metal concentrations with the highest activity in plants treated with Al 1000 mg L^{-1}.

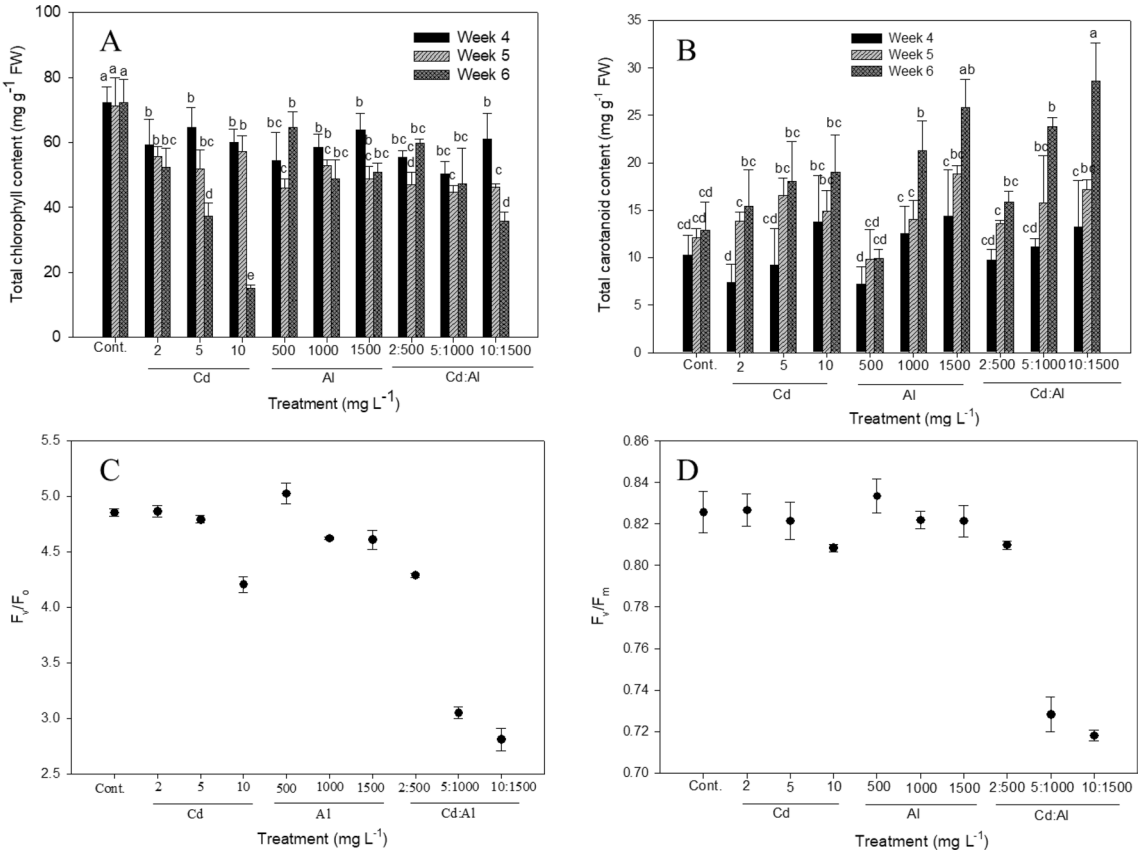

Fig. 1 Effect of Cd and Al exposure for 6 weeks on photosynthetic parameters in *Hypoxis hemerocallidea.* (A) total chlorophyll content; (B) carotenoid content; (C) ratio of variable fluorescence and initial fluorescence (F_v/F_o) and (D) ratio of variable fluorescence and maximum fluorescence (F_v/F_m). Results are presented as mean ± SD. Different letters on each bar (A and B) indicate significant differences ($p < 0.05$)

Effect of cC and Al on MDA content

There was a dose-dependent response in the accumulation of MDA with a significant increase especially in the combined treatment of Cd 10:Al 1500 mg L^{-1} (Fig. 4B).

Effect of heavy metal stress on amylase activity

The activity of hydrolytic enzymes (α- and β-amylases) was significantly higher in plants exposed to the lowest Cd concentrations (Fig. 4C). Control plants had comparable amylase activity to those treated with Cd 5 mg L^{-1} and Cd 10 mg L^{-1}. Increasing concentrations of Cd and Al caused a significant reduction in hydrolytic enzyme activity following a dose-dependent pattern. The lowest levels of amylases occurred in plants from the Cd 10:Al 1500 mg L^{-1} treatment (Fig. 4C).

Effect of Cd and Al on total carbohydrates content

There was a significant dose-dependent decrease in the carbohydrates content in leaves of plants treated with Cd (Fig. 4D). The lowest Al treatment (Al 500 mg L^{-1}) significantly enhanced and higher Al concentrations significantly decreased the carbohydrates content. Cd and Al combination treatments elicited a dose-dependent increase in the carbohydrates content (Fig. 4D).

Ultrastructure of leaf samples

H. hemerocallidea leaf samples from the control group had normal chloroplasts characterized by a bean-seed shape. There were numerous well-organized grana stacks and evenly spaced thylakoids (Fig. 5A). Electron-dense plastoglobuli (lipid droplets) were discernible between the thylakoids. Exposure to Cd modified the ultrastructure of the

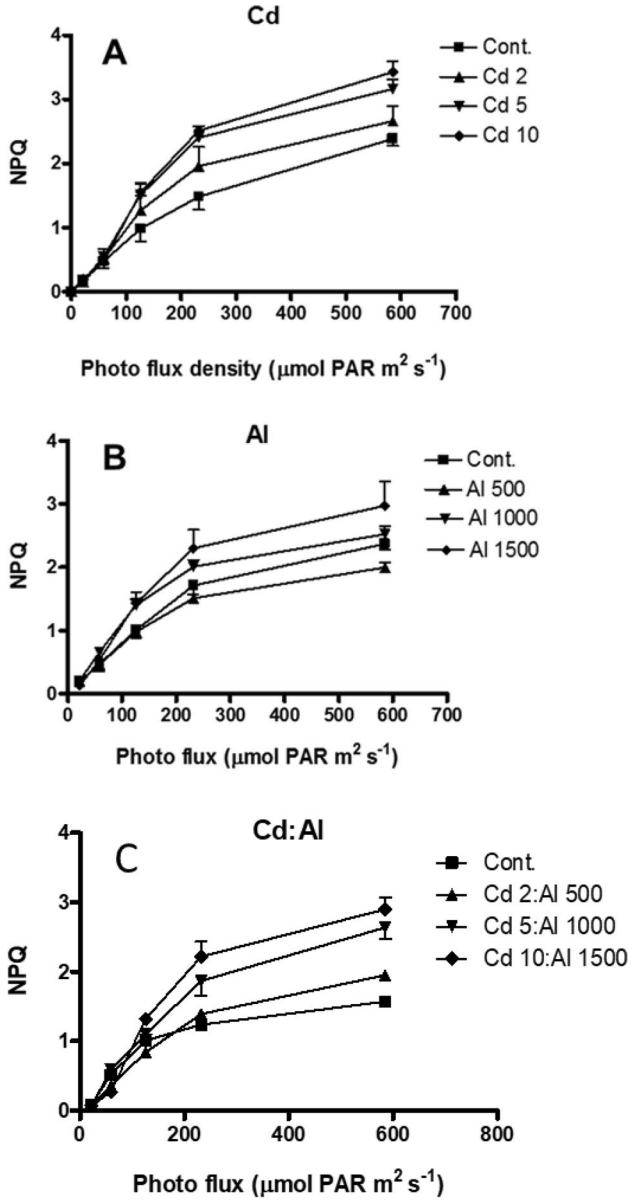

Fig. 2 Effect of Cd and Al on non-photosynthetic quenching (NPQ) activity in the leaves of *Hypoxis hemerocallidea* after exposure for 6 weeks to (A) Cd; (B) Al; and (C) combination of Cd and Al. Results are presented as mean ± SD ($n = 10$)

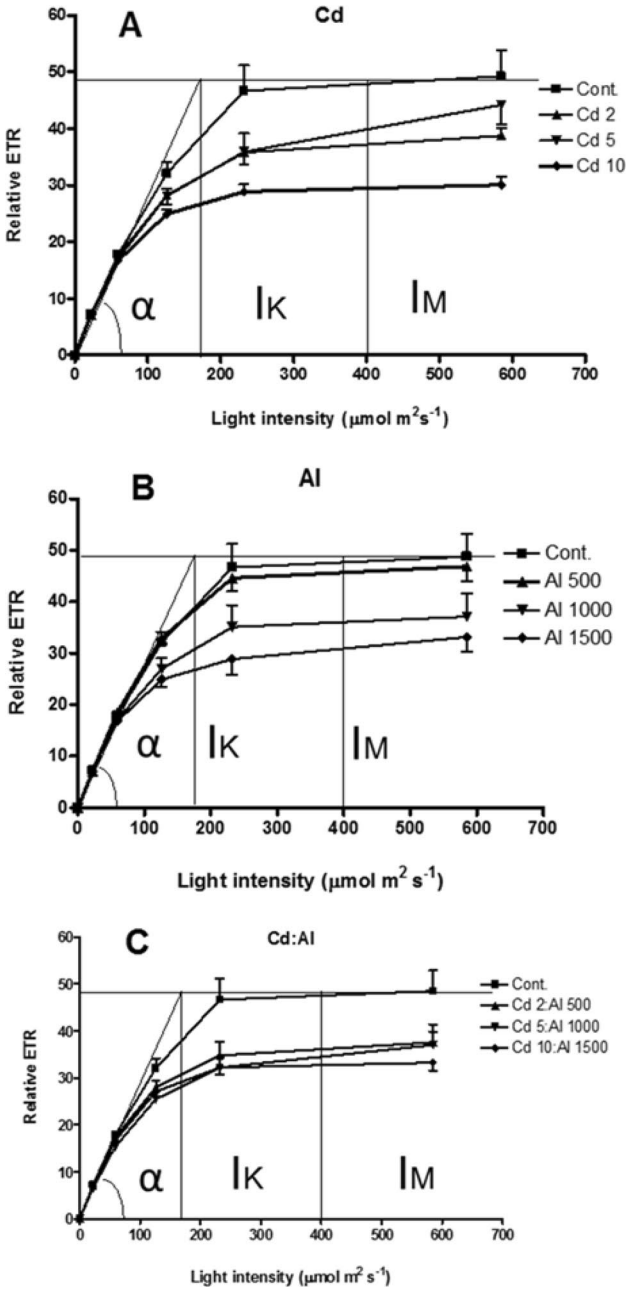

Fig. 3 Effect of Cd and Al on relative electron transfer rate (rETR) activity in the leaves of *Hypoxis hemerocallidea* after exposure for 6 weeks to (A) Cd; (B) Al; and (C) combination of Cd and Al. Results are presented as mean ± SD ($n = 10$)

chloroplasts and thylakoids Chloroplasts displayed anomalous swelling and disintegration and there was an increase in starch grains (Fig. 5B–D). Plants treated with the lowest Al concentration had similar ultrastructural features as the control plants (Fig. 5E). Higher Al concentrations induced the transformation of the chloroplast structure into a disc-like configuration (Fig. 5F, G). Plants subjected to the combination treatments displayed a decrease in plastoglobuli quantity and complete disintegration of the chloroplast (Fig. 5H).

Discussion

A substantial increase in carbon in leaves of *H. hemerocallidea* treated with heavy metals compared to the control plants indicated an imbalance in carbon partitioning (Table 1). This can be attributed to several interconnected physiological and biochemical responses. The changes signify the impact of Cd and Al toxicity on plant metabolism

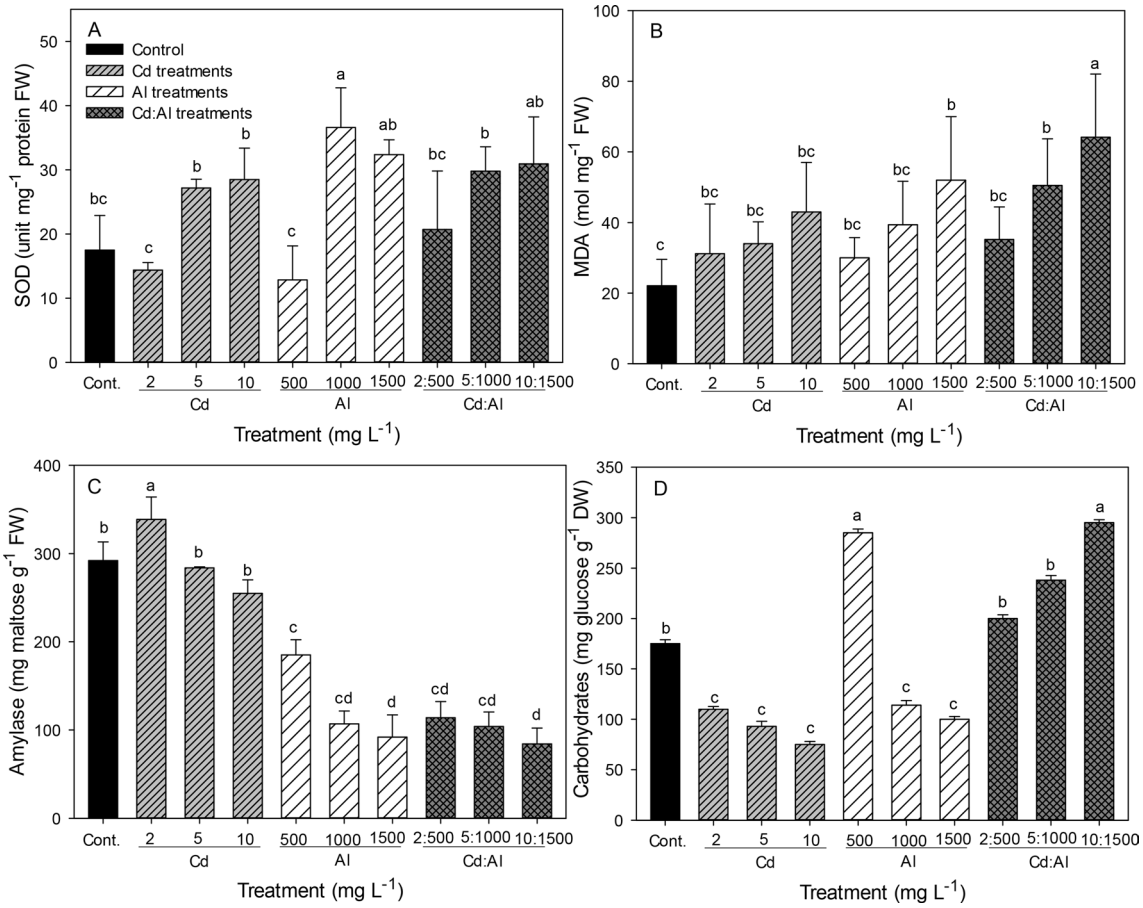

Fig. 4 Effect of Cd and Al treatment on (A) SOD activity; (B) MDA activity; (C) amylase activity; and (D) total carbohydrates content in leaves of *Hypoxis hemerocallidea* after exposure to Cd and Al for 6 weeks. Results are presented as mean ± SD ($n = 3$). Different letters on each bar indicate significant differences ($p < 0.05$)

and growth, leading to stress and disruption (Guo et al. 2023; Ofoe et al. 2023). The shift in carbon partitioning may also have contributed to the buildup of carbon-based compounds within the leaves as evidenced by the rise in starch grains observed in the TEM micrographs (Fig. 5). The decrease in oxygen levels might be due to the reduced photosynthetic activity, leading to less oxygen being produced (Zhao et al. 2021).

Al is a highly reactive element and can bind to various sites, including the cell wall, plasma membrane surface, cytoskeleton and the nucleus (Panda et al. 2009). This reactivity could be responsible for the uptake and translocation of Al into the shoot leading to the elevated levels of Al detected in the present study. In EDX, only the surface region of the leaf is analyzed. The likelihood that Cd was not uniformly distributed on the surface of the *H. hemerocallidae* leaf samples but was present in a patchy or localized manner in low levels would cause it to be undetected in the analysis and could be the reason for non-detection of Cd in most of the treatments (Table 1). These results confirm that while both Al and Cd are taken up by the roots, Al has a high translocation factor compared to Cd in *H. hemerocallidae* (Okem et al. 2015). The restriction of Cd to the root involves processes such as immobilization inside vacuoles, metal precipitation and binding of metal cations to cell walls (Akhter et al. 2014) which slows down Cd movement into the shoot.

Cd and Al induce oxidative stress in plants, leading to the degradation of chlorophyll molecules. Oxidative stress triggers the breakdown of chlorophyll pigments, resulting in a reduction of chlorophyll content. This decline in chlorophyll concentration in *H. hemerocallidea* could be an indicator of cellular damage and stress response (Fig. 1A) . Carotenoids are antioxidant pigments that play a critical role in protecting plants from oxidative damage caused by reactive oxygen species (ROS) which are produced as a result of heavy metal stress. In response to stress, *H. hemerocallidea* increased the synthesis of carotenoids as a defense mechanism (Fig. 1B). Carotenoids effectively neutralize ROS and protect cellular components from oxidative harm (Zuluaga et al. 2017).

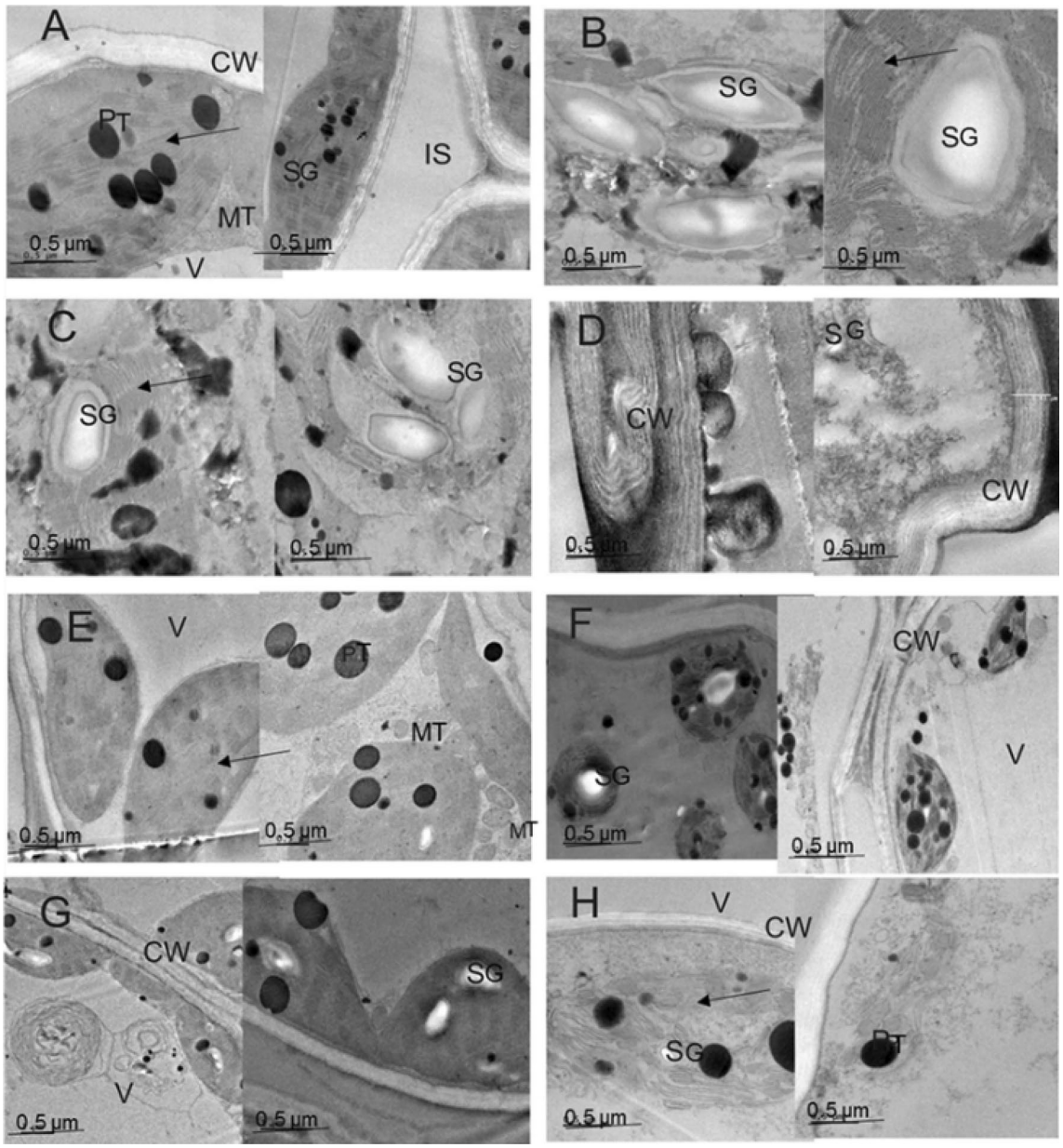

Fig. 5 Ultrastructural changes in the leaves of *Hypoxis hemerocallidea* grown in Cd and Al for 6 weeks in (A) Control; (B) Cd 2 mg L^{-1} (note the abnormally swollen chloroplast); (C) Cd 5 mg L^{-1}; (D) Cd 10 mg L^{-1}; (E) Al 500 mg L^{-1}; (F) Al 1000 mg L^{-1}; (G) Al 1500 mg L^{-1}; (H) Cd 5:Al 1000 mg L^{-1} (note the damaged, disintegrating chloroplast). Labels indicate CW = Cell wall; IS = Intracellular space; MT = Mitochondria; PT = Plastoglobuli; SG = Starch grain and V = Vacuole and the arrow shows the thylakoids

The analysis of fluorescence photochemistry in *H. hemerocallidea* revealed the toxic effects of Cd and Al on chlorophyll fluorescence capacity, especially at the highest concentrations of heavy metal treatment. This was evident from the significant decrease in maximum quantum efficiency of PSII photochemistry (Fig. 1C and D), indicating inhibition of photoactivation of PSII and disruption of electron transport. The more pronounced activity of F_v/F_o was an indication that F_v/F_o was more sensitive than F_v/F_m (Schreiber et al. 1995). Toxicity of heavy metals can impair thylakoidal and stromal membranes, resulting in a diminished capacity of plants to utilize absorbed light energy for photosynthesis (Santos et al. 2012).

In stressful conditions, an increase in NPQ is often accompanied by the photoinactivation of PSII reaction centres which dissipate excitation energy as heat instead of photochemistry (Malnoë 2018). This process, known as photoinactivation, can lead to oxidative damage and loss

of PSII reaction centres, resulting in an increase in F_o. In the present study, escalating heavy metal stress caused *H. hemerocallidea* to lose the ability to utilize absorbed light energy (Fig. 2). Thus, the changes induced by heavy metals disrupted the balance between electron transfer and excitation rates, ultimately reducing the state of PSII reaction centres and affecting the effective utilization of captured light (Aydin et al. 2016). With the increased concentration in heavy metal treatments, there was significant inhibition of photosynthetic electron transport through PSII in *H. hemerocallidea*. This was evident with gradual reduction in rETR (Fig. 3). A similar pattern occurred in barley and the microalga *Chlorella pyrenoidosa* where Cd significantly impaired photosynthetic electron transport through PSII (Vassilev et al. 2004; Wang et al. 2022).

Abiotic stress such as exposure to heavy metals, triggers the generation of free radicals and ROS. This disrupts normal metabolism through oxidative damage to cellular components (Cakmak and Horst 1991). Plants activate enzymatic and non-enzymatic antioxidant processes to scavenge ROS to counter these effects. An increase in heavy metal stress correlated with an increase in SOD in *H. hemerocallidea* (Fig. 4A). Lipid peroxidation, as indicated by the accumulation of MDA, is a general indicator of oxidative stress in plants. Exposure to increasing concentrations of heavy metals led to higher levels of lipid peroxidation in *H. hemerocallidea* (Fig. 4B). Al binds to phospholipids within the cell membrane (Jones and Kochian 1997), potentially explaining the high levels of lipid peroxidation observed in the Al-treated plants.

Exposure to Cd and Al had an impact on metabolic processes such as starch and sugar metabolism in *H. hemerocallidea*. Plants treated with Cd exhibited lower carbohydrates content and higher amylase activity (Fig. 4C and D). The higher amylase levels suggest enhanced enzymatic activity, leading to more rapid starch degradation. Consequently, the breakdown of starch into soluble sugars may have outpaced the plant's capacity to utilize these sugars for immediate energy needs or storage, thus resulting in a lower overall accumulation of carbohydrates even though the amylase activity was heightened. The lower amylase activity in Al-treated plants could be a result of *H. hemerocallidea* diverting its resources away from amylase production or experiencing a downregulation in amylase-related pathways due to the prevailing environmental stressors, resulting in an increase in carbohydrates content. Amylase plays a crucial role in converting starch reserves into usable sugars for energy production in plants. A decrease in amylase activity can lead to an imbalance of carbohydrates (Devi et al. 2007).

Alterations in the ultrastructure of chloroplasts and thylakoid systems indicated that the photosynthetic machinery of *H. hemerocallidea* was affected by exposure to Cd and Al stress. There was severe damage to the chloroplast membranes and structural integrity as seen by the anomalous swelling and disintegration of chloroplasts, especially at the highest Cd concentration. This disruption hindered the plant's ability to efficiently capture light energy and perform photosynthesis, ultimately affecting its overall growth, productivity and efficacy as a medicinal plant (Okem et al. 2015). Synthesis of secondary metabolites has a high energy demand (Gershenzon 1994) and reduced photosynthetic capacity due to exposure to heavy metals may be linked to decreased antibacterial activity and lower hypoxoside concentrations in *H. hemerocallidea* when exposed to Cd and Al stress (Okem et al. 2015).

In conclusion, exposure to Cd and Al caused ultrastructural changes in the leaves of *H. hemerocallidea* and had a detrimental effect on photosynthetic performance. This compromised other metabolic functions including sugar and starch metabolism and the reallocation of resources to combat oxidative stress e.g. increased SOD activity and MDA content. These results suggest that while *H. hemerocallidea* is tolerant to low levels of heavy metal stress, the photosynthetic performance is impaired. This impacts secondary metabolite synthesis, thus reducing the efficacy of this extensively utilized medicinal plant when grown in heavy metal contaminated soils. Future research needs to focus on ways (e.g. use of natural biostimulants) to enhance the tolerance of *H. hemerocallidea* to heavy metal stress. This will enable plants with good biological activity (e.g. high hypoxoside content) to be cultivated on a commercial scale. Monitoring will be essential to ensure that the Al and Cd content in the edible plant parts does not exceed the WHO permissible limits.

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Author contributions AO conceptualized the research idea, performed the experiment and prepared the manuscript. WAS, JFF and JvS supervised the project, provided technical advice and improved the narrative. All authors have read and approved the manuscript for submission.

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Data availability The data generated during this study is available from the corresponding author on reasonable request.

Declarations

Conflict of interest The authors declared no conflict of interest.

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