Photoinhibition of Photosynthesis in Leaves of Cowpea (*Vigna unguiculata* L. Walp. P152) in Response to Zinc and High Irradiance Stresses

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**Abstract:** Seedlings of cowpea (*Vigna unguiculata* L. Walp. P152) were fertilized every alternate day for 14 days with half strength Hoagland solution containing 0 ppm (deficient), 5 ppm (sufficient) and 50 ppm (excess) ZnSO$_4$.7H$_2$O. Photoinhibition of photosynthesis and photosynthetic recovery were studied in detached leaves under zinc (Zn) and high irradiance (HI) stresses. Changes in photosynthetic pigments, soluble proteins, soluble starch, photosystem (PSII) activities and thylakoid membrane proteins were investigated. The degree of photoinhibition was determined by the ratio of variable to maximum chlorophyll (Chl) fluorescence ($F_{v}/F_{m}$). Photosynthetic pigments, soluble proteins and soluble starch decreased significantly in Zn-deficient and Zn-excess leaves, the degree of decrease being greater in Zn-excess. Maximum quantum yield of primary photochemistry ($F_{v}/F_{m}$), maximum variable fluorescence ($F_{v}$) were significantly decreased to a greater extent in Zn-excess leaves than in Zn-deficient ones. The exogenous electron donors, diphenylcarbazide (DPC) and NH$_2$OH slightly restored the loss of PSII activity in Zn-deficient leaves when compared to Zn-excess leaves. The marked loss of PSII activity in Zn-deficient and Zn-excess leaves was evidently due to the loss of 33, 28-25, 23 and 17 kDa polypeptides under Zn and Zn + HI stress. Thus, the cowpea plants seem to be highly susceptible to excess Zn. To the best of our knowledge, this is the first report on the effect of Zn stress combined with HI stress in cowpea plant.

**Key words:** Chlorophyll fluorescence · Cowpea · High irradiance · Photoinhibition · PSII · Thylakoid membrane protein · Zinc

**INTRODUCTION**

Heavy metal pollution of soil and water is a worldwide problem of increasingly importance, because of the contamination of large areas due to the anthropogenic activity (e.g., industrialization, application of pesticides and fertilizers, mining, military applications, etc.) [1-3] and it has a strong impact on the environment and on the human health through the food chain. Zinc is an essential element for plant nutrition. It plays structural and/or catalytic roles in many enzymes such as Cu-Zn superoxide dismutase, alcohol dehydrogenase, RNA polymerase and DNA-binding proteins and is associated with the carbohydrate metabolism [4, 5]. However, when massively present in the environment, Zn can reach supraoptimal concentrations in all plant organs, thus inducing toxic effects and metabolic disorders. Zn inputs on soils are related with mining, industrial activities and agricultural practices [6]. Excessive Zn in plants can delay or diminish the growth [7] and root development [8] and causes leaf chlorosis [9].

Though Zn plays a vital in stability of biomembranes and proteins [10], Zn deficiency can affect the photochemical processes in the thylakoids and thus inhibits biophysical processes of photosynthesis. The flow of electrons through PSII is indicative of the overall rate of photosynthesis and is an estimation of photosynthetic performance. Chlorophyll fluorescence measurements could be used to estimate the operating quantum efficiency of electron transport through PSII in leaves [11]. Zinc deficiency can also cause a drastic decrease in chlorophyll content as well as a severe damage to the fine structure of chloroplasts [12]. Zinc at higher concentrations inhibits plant growth [13, 14], chlorophyll formation [15] and photosynthesis and transpiration rates [16].
Most plants during the daytime encounter light intensities that exceed their photosynthetic capacity and then a part of the excess light energy is used to produce ROS and/or other highly oxidizing species in the PS II. The presence of these active species results in oxidative stress and biological damage, both in animals and in plants [17, 18]. Among various stresses encountered by plants in tropical environments, high intensity of irradiance is the most significant that accounts for remarkable alterations in plant metabolism [19, 20]. Photoinhibition of photosynthesis will arise when the rate of transfer of excitation energy from the antennae to the photochemical reaction center exceeds the rate of electron transport [21, 22]. Photoinhibition of photosynthesis is caused by exposing plants to irradiance much higher than that used during growth [23, 24].

Cowpea is one of the important kharif pulses grown in India. The crop is grown from March to April and is harvested between June and July depending upon its end use. Cowpea is highly responsive to fertilizer application and the dose of fertilizer depends on the initial soil fertility and moisture availability [25]. So far, to our knowledge there is no report available on the combined effect of Zn and HI in cowpea plants. Therefore the objective of the present work is to study the effects of Zn and HI induced changes in chlorophyll content, soluble protein, soluble starch, Chl fluorescence, DCPIP photoreduction and thylakoid protein profile of cowpea plant.

MATERIALS AND METHODS

Plant Culture and Zinc Treatments: Cowpea seeds (Vigna unguiculata L. Walp. P152) were rinsed in distilled water and surface sterilized with 1% sodium hypochlorite for 20 min, rinsed again, imbibed overnight in distilled water and germinated on moistened filter paper in trays for 3 days in darkness at 23°C. After 3 days, uniformly germinated seedlings were transferred to plastic cups and grown hydroponically in half-strength Hoagland’s nutrient solution [26] for 7 days. From the 8th day onwards Zn treatments were given in the concentrations of 0 ppm (Zn-deficient), 5 ppm (Zn-sufficient) and 50 ppm (Zn-excess) as ZnSO₄·7H₂O. The growth solutions were adjusted to pH 5.6 ± 0.2 and were replaced every two days. Plants were grown in a growth chamber with the following conditions: day/night temperature, 22 ± 2°C/18 ± 2°C; relative humidity, 60-70%; 16-h light: 8-h dark photo-cycle; light intensity, 150 μmol m⁻² s⁻¹. Two weeks after germination, the plants were collected and analyzed.

Photoinhibition and Recovery under Controlled Conditions: Detached leaves which were already subjected to Zn stress were placed in a controlled environment chamber equipped with a 24 V/250 W metal-halide lamp. The upper leaf surface was exposed to a photosynthetic photon flux density (PPFD) of 1900 μmol m⁻² s⁻¹ for up to 60 min. Air temperature was 20 °C and relative humidity was 65%. After this period, some leaves exposed to HI were returned to normal condition (Recovery) by adapting dark recovery for 60 min before sampling and analyzed.

Determination of Photosynthetic Pigments: The chlorophyll (Chl) and carotenoid (Car) contents were assayed according to Arnon [27]. Fresh leaf (100 mg) was homogenized in 10 ml of pre chilled 80% acetone. The homogenate was centrifuged at 3,500 g for 15 min. The supernatant was collected and the centrifugation was repeated until the pellets became colourless. The supernatant collected was made up to 10 ml with 80% acetone and the absorbance was read at 480, 645 and 663 nm. The Chl content was calculated using the formula of Arnon and Car content was calculated based on the formula of Kirk and Allen [28] and were expressed in mgg⁻¹ FW.

\[
\begin{align*}
\text{Chl } a & = 12.7 \times A_{645} - 2.69 \times A_{663} \\
\text{Chl } b & = 22.9 \times A_{663} - 4.68 \times A_{645} \\
\text{Tot Chl } & = 20.2 \times A_{663} + 8.02 \times A_{645} \\
\text{Carotenoids} & = A_{663} (0.114 \times A_{645} - 0.638 \times A_{645})
\end{align*}
\]

Estimation of Total Soluble Protein and Soluble Starch Content: Total soluble protein was estimated according to the method of Lowry et al. [29]. The pellets obtained after chlorophyll estimation were dissolved in 2 ml of 0.1 N NaOH and were boiled for 15 min and centrifuged at 2,500 g for 3 min. The supernatant was collected and to 0.5 ml of the supernatant, 5 ml of Lowry reagent (1% CuSO₄·5H₂O + 1% sodium potassium tartarate + 2% Na₂CO₃ in 0.1 N NaOH) was added and allowed to stand for 5 min. Then 0.5 ml of Folin-phenol reagent was added and kept in darkness for 45 min and the absorbance was read at 730 nm. The total soluble protein content was calibrated with the help of a standard graph and expressed in mgg⁻¹ FW. Bovine serum albumin was used as standard.

Soluble starch was determined following the method of McCreary et al. [30]. Fresh leaf (100 mg) was ground in 2 ml of 50% alcohol and the homogenate was centrifuged. The supernatant was discarded and to the pellets, 1 ml of 1 N H₂SO₄ and 1 ml of distilled water was added.
The contents were boiled for 30 min, cooled, centrifuged and the supernatant was collected. To 0.5 ml of the supernatant, 0.5 ml of distilled water and 4 ml of anthrone reagent (0.2% anthrone in Conc. H₂SO₄) were added and the contents were heated for 8 min, cooled and the absorbance was read at 630 nm. The soluble starch content was calibrated with the help of a standard graph and expressed in mg g⁻¹ FW.

**Chlorophyll Fluorescence:** Measurements of Chl fluorescence was performed with OS-30 P pulse modulated chlorophyll fluorimeter (Opti-sciences, Hudson, USA.). Before each measurement, leaves were dark-adapted for 30 min with leaf-clips. To determine the minimal fluorescence (F₀), the weak measuring light was turned on and F₀ was recorded. The leaves were then exposed to 0.1 s saturated flash of approximately 6000 µmolm⁻²s⁻¹ to obtain the maximal fluorescence yield (Fₘ). The ratio of variable to maximal fluorescence (Fᵥ/Fₘ) was calculated automatically according to Fᵥ and Fₘ measured \[ Fᵥ/Fₘ = (Fₘ-F₀)/Fₘ \].

**Isolation of Thylakoid Membranes:** Thylakoid membranes were isolated at 4 °C as described by Berthold et al. [31]. Leaf samples were homogenized in an ice-cold grinding medium containing 25 mM Tris-HCl pH 7.8, 10 mM NaCl, 5 mM MgCl₂ and 330 mM sucrose. The homogenate was filtered rapidly through four layers of miracloth and chloroplasts were collected by centrifugation at 8,000 g for 5 min. The chloroplast pellets were suspended with a low osmotic medium containing 25 mM Tris-HCl pH 7.8, 10 mM NaCl, 5 mM MgCl₂ and 100 mM sucrose for washing and then the suspension was centrifuged again at 9,000 g for 10 min. The thylakoid membrane pellets were resuspended in a small volume of the low osmotic medium and stored at -20 °C for later use.

**DCPIP Photoreduction:** The rate of 2,6-dichlorophenol indophenol (DCPIP) photoreduction was determined following the decrease in absorbance at 590 nm. The reaction mixture contained 20 mM Tris-HCl pH 7.5, 5 mM MgCl₂, 10 mM NaCl, 100 µM sucrose, 100 µM DCPIP and thylakoid membranes equivalent to 20 µg of Chl. Electron donation to the oxidizing side of PSII was measured in the presence of 5 mM MnCl₂, 0.5 mM DPC and 5 mM NH₄OH as electron donors [32].

**SDS-PAGE:** Thylakoid membrane proteins were separated using the polyacrylamide gel electrophoresis system of Laemmli [33], with the following modifications.

The contents were boiled for 30 min, cooled, centrifuged and the supernatant was collected. To 0.5 ml of the supernatant, 0.5 ml of distilled water and 4 ml of anthrone reagent (0.2% anthrone in Conc. H₂SO₄) were added and the contents were heated for 8 min, cooled and the absorbance was read at 630 nm. The soluble starch content was calibrated with the help of a standard graph and expressed in mg g⁻¹ FW.

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**Isolation of Thylakoid Membranes:** Thylakoid membranes were isolated at 4 °C as described by Berthold et al. [31]. Leaf samples were homogenized in an ice-cold grinding medium containing 25 mM Tris-HCl pH 7.8, 10 mM NaCl, 5 mM MgCl₂ and 330 mM sucrose. The homogenate was filtered rapidly through four layers of miracloth and chloroplasts were collected by centrifugation at 8,000 g for 5 min. The chloroplast pellets were suspended with a low osmotic medium containing 25 mM Tris-HCl pH 7.8, 10 mM NaCl, 5 mM MgCl₂ and 100 mM sucrose for washing and then the suspension was centrifuged again at 9,000 g for 10 min. The thylakoid membrane pellets were resuspended in a small volume of the low osmotic medium and stored at -20 °C for later use.

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**Statistical Analysis:** The data compiled were submitted to one-way analysis of variance (ANOVA) by using SigmaPlot 11.0. Each data point was the mean of five replicates (n = 5) and comparisons with P-values < 0.01 were considered significantly different by Tukey’s test.

**RESULTS**

**Photosynthetic Pigments:** Under Zn, Zn + HI stress and recovery, Chl a, Chl b, Tot Chl, Chl a/b ratio and Car contents were significantly decreased in Zn-deficient and Zn-excess leaves when compared to Zn-sufficient leaves, the percent of decline being much higher in Zn-excess leaves (Fig. 1A-C and Fig. 2A and B). Though there was a decrease in photosynthetic pigments in Zn-stressed plants, there was a 92% of recovery from the imposed HI stress whereas in Zn-deficient and Zn-excess leaves, recovery rate was low.

**Total Soluble Protein and Soluble Starch Content:** The total soluble protein content was significantly reduced in Zn-deficient and Zn-excess leaves compared to Zn-sufficient leaves (Fig. 2C) under Zn, Zn + HI stress and recovery. In Zn-excess leaves, the decline was by 40% and 54% under Zn and Zn + HI stress respectively when compared to Zn-sufficient leaves. Upon recovery, the Zn-sufficient leaves showed a maximum recovery of 91% whereas the rate of recovery was very low in Zn-deficient and Zn-excess leaves. The soluble starch content was found to be significantly decreased in Zn-deficient and Zn-excess leaves when compared to Zn-sufficient leaves (Fig. 2D). Though the starch content decreased under Zn + HI stress in all the Zn stressed plants, the degree of decrease was much higher in Zn-excess leaves (44%).
Fig. 1: Changes in Chl $a$ (A), Chl $b$ (B) and total Chl (C) in the leaves of *Vigna unguiculata* L. Walp. P152 under Zn, Zn + HI stress and recovery. Values are Mean ± SE ($n = 5$). The different letters indicate significant differences compared.
Chlorophyll Fluorescence: There was no significant increase or decrease in the F₀ value in the cowpea leaves in all Zn treatments under Zn, Zn + HI stress and recovery (Fig. 3A). The F₀ and F₀/Fm_ratio declined significantly in Zn-deficient and Zn-excess leaves when compared to Zn-sufficient leaves under Zn, HI stress and recovery, the percent of decrease being higher under Zn-excess condition (Fig. 3B and C). Though there was a significant decline in F₀ value and F₀/Fm ratio in Zn-sufficient leaves under Zn + HI stress, the percent decline was much lower than Zn-deficient and Zn-excess leaves. Upon recovery from HI stress, the Zn-sufficient leaves showed a maximum recovery of 86% and 95% in F₀ and F₀/Fm ratio respectively.

DCPIP Photoreduction: DCPIP photoreduction was carried out to find the possible site(s) of inhibition in the PSII reaction by providing various exogenous electron donors used in thylakoids isolated from Zn and Zn + HI stressed leaves. We used MnCl₂, DPC and NH₂OH as electron donors [34] which could donate electrons to the PSII reaction. Fig. 4A and B shows the electron transport activity of PSII in the presence and absence of H₂O₂, MnCl₂, NH₂OH and DPC in cowpea plants under Zn and Zn + HI stress. PSII activity was reduced to about 73% and 89% in Zn-excess leaves under Zn and Zn + HI stress, when H₂O served as electron donor. Similar results were obtained for MnCl₂. In contrast, under Zn and Zn + HI stress, a significant restoration of PSII mediated DCPIP reduction was observed when NH₂OH and DPC were used as electron donors in Zn-deficient than in Zn-excess leaves. The results showed that the PSII damage was much pronounced in Zn-excess leaves than the Zn-deficient ones.

SDS-PAGE: Thylakoid Membrane Proteins: Fig. 5 shows the changes in the thylakoid membrane proteins in the cowpea leaves under Zn, Zn + HI stress and Rec. Under Zn stress, a slight degradation in 47, 33, 23 and 17 kDa proteins were observed in the Zn-deficient and Zn-excess leaves when compared to the Zn-sufficient leaves. Upon further HI stress, the 47, 33, 23 and 17 kDa proteins were drastically degraded in the Zn-deficiency Zn-excess thylakoids. Upon recovery from HI stress, the Zn-deficient and Zn-excess thylakoids couldn’t restore the 33 and 17 kDa polypeptides and couldn’t withstand the HI stress imposed on them.
Fig. 4: Effect of various exogenous electron donors on PS II activity in the thylakoids isolated from Vigna unguiculata L. Walp. P152 leaves under Zn and Zn + HI stress. Values are Mean ± SE (n = 5). The different letters indicate significant differences compared to the sufficient level (5 ppm Zn) at P = 0.01 as determined by Tukey’s test.

Fig. 5: SDS-PAGE polypeptide profiles of thylakoids isolated from Zn, Zn + HI treated and recovered leaves of Vigna unguiculata L. Walp. P152. Gel lanes were loaded with equal amounts of thylakoid proteins (100 µg). Lane A, Zn stress; lane B, Zn + HI stress; lane C, Zn + HI + recovery.

DISCUSSIONS

Heavy metals have been found to decrease the Chl content and the Chl a/b ratio, in many terrestrial plants [35, 36]. The decrease in Chl a, Chl b and Chl a/b ratio under Zn deficiency and Zn toxicity has been recently reported by Cherif et al. [37] in tomato plants which is in good agreement with our results. The decreased concentration of Chl under deficiency [38] and excess of Zn [39] suggested a possible indirect effect of Zn on Chl biosynthesis. Moreover, similar to our results, a significant decrease in chlorophyll contents has been reported in different cultivars of maize under salinity stress [40] and Zn stress [41]. The highest pigment loss in the Zn + HI stressed cowpea leaves suggests that when the Zn-deficient and Zn-excess leaves experience two different stresses simultaneously, because of the additive action, the effect was more compared to the plants exposed to single stress. The HI stress might have resulted in greater damage to the...
photosynthetic apparatus and the generation of higher concentrations of ROS. This might have lead to the rapid photo-bleaching and hence the loss of photosynthetic pigments under Zn-deficient and Zn-excess conditions [42].

The total soluble protein content was significantly reduced in the cowpea plants under Zn-deficiency and Zn-excess conditions as has been reported earlier by Tandon and Gupta [43] and Singh et al. [44], at increased doses of heavy metals. Decrease in soluble proteins in Zn-deficient and Zn-excess leaves under HI could be due to rapid degradation of proteins as well as overall inhibition of protein synthesis [20]. The higher rate of protein loss in the Zn-deficient and Zn-excess leaves under Zn + HI stress could be due to additive as well as synergetic effect of both the stresses.

Nutrient deficiencies and heavy metal toxicities are known to produce starch accumulation within leaves [45]. On the contrary, in our results no significant increase of starch accumulation in the Zn-deficient and Zn-excess leaves occurred, which indicates that Zn had no effect on vein loading or, more probably, Zn-deficiency and Zn-excess inhibited photosynthesis more intensively than the translocation of photo-assimilates [46]. The decreased starch concentration under Zn deficiency could be a result of reduced starch synthetase activity [47]. It was previously suggested that the accumulation of starch could play an important role in regulating the non-osmotic volume of cells, thus facilitating osmotic adjustment [48]. The accumulation of starch in the Zn-sufficient plants in our experiments has no effect on the photosynthetic rate as this hypothesis has been reported earlier by Claussen and Biller [49].

Cowpea plants grown under Zn-sufficient condition had Fv/Fm values of approx. 0.82, close to the 0.83 value typical for non-photo-inhibited vascular plants [50]. However, in the Zn-deficient and Zn-excess cowpea leaves, a steep decrease in Fv/Fm was observed, indicating a strong inhibition of photosynthetic efficiency. Fv/Fm remained depressed even after further HI stress and did not fully recovered from HI stress after dark incubation for 1 hr. This rapid decrease in Fv/Fm suggests a significant transient loss of PS II function followed by a slow and possibly incomplete recovery [42] in the Zn-deficient and Zn-excess cowpea leaves. Fv/Fm ratio was clearly affected in the Zn-excess by Zn + HI stress, indicating that excess Zn enhanced the photoinhibition induced by light stress. Similar results were obtained by Vaillant et al. [51] in Datura metel.

Significant decreases in the rate of DCPIP photoreduction in thylakoid membranes after the Zn and Zn + HI stress treatments might be due to changes in the proper alignment of thylakoid membranes and also dissociation of some extrinsic proteins. Significant restoration of PSII activity in Zn-deficient and Zn-excess leaves by the artificial electron donors suggest that the damage might be located on the donor side of PSII, in the oxygen evolving system which is similar to the results obtained by Bertamini et al. [52]. Hence Zn-deficient and Zn-excess leaves were affected on the donor side of PSII by photoinhibition. The HI stress has further caused a deleterious effect on the PSII activity of Zn-deficient and Zn-excess leaves. In agreement to our results Pagliano et al. [53] has reported a loss in the capability to reduce DCPIP, which is partially restored if DPC is used as an electron donor, indicating that the water splitting activity is affected in rice plants under cadmium stress. Accordingly, when the activity of PSII was measured in thylakoids isolated from Zn and HI treated cowpea leaves, a loss in the capability to reduce DCPIP was observed in Zn-deficient and Zn-excess leaves. Therefore, when both Zn and HI are applied simultaneously, the photochemical activity was further reduced due to the additive and a possible synergetic action of both the stresses in the Zn-deficient and Zn-excess leaves.

Induction of marked loss of 47, 33, 23 and 17 kDa proteins in the Zn-deficiency and Zn-excess thylakoid membranes under Zn and Zn + HI stress might be due to greater disruption of the PS II complex. This could be the reason for the observed marked loss of PS II activity under both stress conditions. No significant changes were found in the number of protein bands under Zn and Zn + HI stress treatment. Our result supports the findings of Maslenkova et al. [54] reported a marked depletion of all PSII polypeptides such as 47 and 43 kDa polypeptides, 33, 23 and 17 kDa polypeptides of OEC. Our results are in good agreement with those of Pandey and Yeo [55] who observed similar results in barley leaves under HI stress. ROS generated inside PS II under HI might have damaged the OEC subunits in isolated thylakoid and PS II preparations under Zn-deficient and Zn-excess conditions [56].

**CONCLUSION**

In conclusion, Zn deficiency and Zn excess conditions together with HI treatment caused a range of effects in the cowpea plants. These include decrease in photosynthetic pigments, soluble proteins, soluble starch,
F<sub>o</sub> and F<sub>n</sub>/F<sub>o</sub> ratio, DCPIP photoreduction and changes in the extrinsic polypeptides of the PSII complex along with a marked loss of 47, 33, 23 and 17 kDa. Our results show that Zn deficient leaves were less photoinhibited than the Zn excess leaves. Moreover, a sufficient level of Zn favored the better growth of the cowpea plant. Thus, the cowpea plants seem to be very sensitive to excess Zn and Zn + HI stress. Further studies, especially dealing with production of antioxidants in quenching reactive oxygen species under excess Zn and HI stress needs to be conducted in order to better understand the nature of the cowpea plants towards these stresses.

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