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ORIGINAL ARTICLE

Combined Effects of Zinc and High Irradiance Stresses on Photoinhibition of Photosynthesis in Bean

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Seedlings of bean plants (*Phaseolus vulgaris* L.Sel 9) were grown for 2 weeks with half strength Hoagland solution containing 0 ppm (deficient), 5 ppm (sufficient) and 50 ppm (excess) $ZnSO_4.7H_2O$. 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. Though the 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 the restoration was excellent. The exogenous electron donors, diphenylcarbazide (DPC) and NH_2OH restored the loss of PSII activity in Zn-deficient and Zn-excess leaves. A slight degradation 33, 28-25, 23 and 17 kDa polypeptides were observed in the Zn-deficient and Zn-excess leaves. Upon recovery from HI stress, the Zn-excess leaves restored the 47, 33, and 28-25 kDa polypeptides.

Key words: Bean; Chlorophyll fluorescence; High irradiance; PSII; Zinc

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Environment contamination and plant exposure to heavy metals is a growing problem throughout the world. Toxic levels of heavy metals were reported to affect a variety of processes in plants (Toppi et al., 2001). Zinc plays multiple important roles in the various physiological and metabolic processes of plants and may limit plant growth. Zinc deficiency is the most widespread micronutrient deficiency worldwide (Graham et al., 1992). It is estimated that about 50% of soils used for cereal production in the world have low levels of plant-available Zn (Graham and Welch, 1996). However, when massively present in the environment, Zn can reach supraoptimal concentrations in all plant organs, thus inducing toxic effects and metabolic disorders. In recent years, the concentration of Zn in soils and the area polluted by it have continually increased due to wastes of nonferrous metallurgy,

the chemical industry, and the introduction of high doses of phosphate fertilizers (Zakrutkin and Shishkina, 1997). Zn inputs on soils are related with mining, industrial activities and agricultural practices (Bi et al., 2006). Excessive Zn in plants can delay or diminish the growth (Andrade et al., 2009) and causes leaf chlorosis (Wang et al., 2009).

Though Zn plays a vital in stability of bio-membranes and proteins (Cakmak 2000), Zn deficiency can affect the photochemical processes in the thylakoids, and thus inhibits biophysical processes of photosynthesis. The measurement of Chl fluorescence is a useful tool for quantification of the effect of stress on photosynthesis (Schreiber et al., 1994). The ratio F_v/F_m is one of the fluorescence parameters most widely used to estimate the degree of photoinhibition (Solhaug and Haugen, 1998). Zinc deficiency causes a drastic decrease in chlorophyll content as well as a severe damage to the fine structure of chloroplasts (Chen et al., 2007). Zinc at higher concentrations inhibits plant growth (Sharma et al., 2008), chlorophyll formation (Kaya et al., 2000) and photosynthesis and transpiration rates (Van Assche et al., 1979).

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 (Finkel and Holbrook, 2000). 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 (Dubey, 1999). Photoinhibition of photosynthesis is caused by exposing plants to irradiance much higher than that used during growth (Powles, 1984).

The common bean, *Phaseolus vulgaris* L., is a herbaceous annual plant grown worldwide as an edible bean, popular for both its dry seeds and green beans. The leaf is occasionally used as a leafy vegetable, and the straw is used for fodder. Common bean (*Phaseolus vulgaris* L.) is a predominantly self-pollinated crop originated mainly in Latin America. From Latin America, it spread to other parts of world and now it is widely cultivated in the tropics and subtropics as well as in temperate regions of the world (Zeven et al., 1999). In India, common bean is known by the names like 'Rajmash' and 'Frash bean' (green bean) and grows in certain parts of the country. Since India being a tropical country the incidence of high light is inevitable and there is no report available on the combined effect of Zn and HI on bean plants which made us to carry out the present investigation.

MATERIALS AND METHODS

Plant culture and zinc treatments:

Bean seedlings (*Phaseolus vulgaris* L.Sel 9) 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 (Hoagland and Arnon, 1950) for 7 days. From the 8^{th} day onwards Zn treatments were given in the concentrations of 0 ppm (Zn-deficient), 5 ppm (Zn-sufficient) and 50 ppm (Zn-excess) as $ZnSO_4.7H_2O$. 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 (Rec-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 (1949). 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 (1965) and were expressed in mg·g^{-1} FW.

Chl *a*=12.7 x A_{663} – 2.69 x A_{645}

Chl *b*=22.9 x A_{645} – 4.68 x A_{663}

Tot Chl= 20.2 x A_{645} + 8.02 x A_{663}

Carotenoids=A_{480} + (0.114 x A_{663} – 0.638 x A_{645})

Estimation of total soluble protein and soluble starch content:

Total soluble protein was estimated according to the method of Lowry et al. (1951). 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% $CuSO_4.5H_2O$ + 1% sodium potassium tartarate + 2% Na_2CO_3 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 mg·g^{-1} FW. Bovine serum albumin was used as standard.

Soluble starch was determined following the method of McCready et al. (1950). 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 H_2SO_4 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. H_2SO_4) 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 (F_o), the weak measuring light was turned on and F_o was recorded. The leaves were then exposed to 0.1 s saturated flash of approximately 6000 $\mu mol \cdot m^{-2} \cdot s^{-1}$ to obtain the maximal fluorescence yield (F_m). The ratio of variable to maximal fluorescence (F_v/F_m) was calculated automatically according to F_o and F_m measured [$F_v/F_m = (F_m-F_o)/F_m$].

Isolation of thylakoid membranes:

Thylakoid membranes were isolated at 4 °C as described by Berthhold et al. (1981). Leaf samples were homogenized in an ice-cold grinding medium containing 25 mM Tris–HCl pH 7.8, 10 mM NaCl, 5 mM $MgCl_2$ 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 $MgCl_2$ 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 $MgCl_2$, 10 mM NaCl, 100 mM 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 $MnCl_2$, 0.5 mM DPC and 5 mM NH_2OH as electron donors (Prasad et al., 2001).

SDS-PAGE:

Thylakoid membrane proteins were separated using the polyacrylamide gel electrophoresis system of Laemmli (1970) with the following modifications. Gels consisted of 8-15% gradient of polyacrylamide. Samples were solubilized at 20 °C for 5 min in 2% (w/v) SDS, 60 mM DTT and 8% sucrose using SDS-Chl ratio of 20:1. The wells were loaded with equal amount (100 µg) of thylakoid protein. Electrophoresis was performed at 20 °C with a constant current of 5 mA. Gels were stained in methanol/acetic acid/water (4:1:5, v/v/v) containing 0.1% (w/v) coomassie brilliant blue R250 and destained in methanol/acetic acid/water (4:1:5, v/v/v). Thylakoid membrane proteins were estimated according to the method of Lowry et al. (1951).

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

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 24% and 29% under Zn and Zn + HI stress respectively when compared to Zn-sufficient leaves. Upon recovery, the Zn-sufficient leaves showed a maximum recovery of 94%. 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 (34%).

Chlorophyll fluorescence:

There was no significant increase or decrease in the F_o value in the bean leaves in all Zn treatments under Zn, Zn + HI stress and recovery (Fig.3A). The F_v and F_v/F_m 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-C). Though there was a significant decline in F_v value and F_v/F_m 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 94% and 98% in F_v and F_v/F_m 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 $MnCl_2$, DPC, and NH_2OH as electron donors (Wydrzynski and Govindjee, 1975) which could donate electrons to the PSII reaction. Fig.4A-B shows the electron transport activity of PSII in the presence and absence of H_2O, $MnCl_2$, NH_2OH, and DPC in bean plants under Zn and Zn + HI stress. PSII activity was reduced to about 63% and 74% in Zn-excess leaves under Zn and Zn + HI stress, when H_2O served as electron donor. Similar results were obtained for $MnCl_2$. In contrast, A significant restoration of PS II mediated DCPIP reduction was observed when NH_2OH and DPC were used as electron donors by about 57 % and 61 % and by 48 % and 50 % in the Zn-deficient and Zn-excess leaves respectively.

SDS-PAGE: Thylakoid membrane proteins:

Fig.5 shows the changes in the thylakoid membrane proteins in the bean leaves under Zn, Zn + HI stress and Rec. Under Zn stress, a slight degradation 33, 28-25, 23 and 17 kDa polypeptides were observed in the Zn-deficient and Zn-excess leaves when compared to the Zn-sufficient leaves. Under HI stress, there was a slight decline in 33 and 23 kDa polypeptides under Zn-deficiency condition and a diminished polypeptide changes in 33, 28-25, 23 and 17 kDa in Zn-excess conditions. Upon recovery from HI stress, the Zn-excess leaves restored the 47, 33, and 28-25 kDa polypeptides.

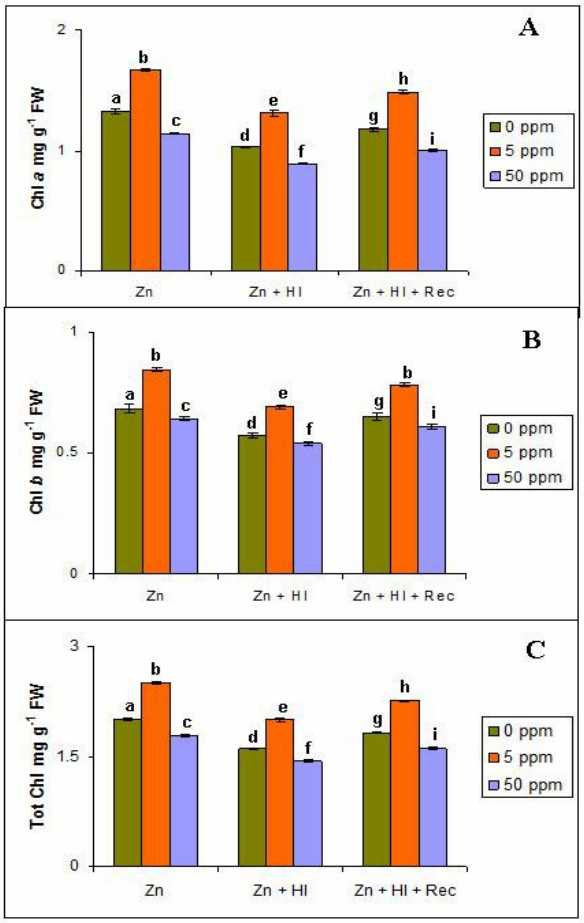

Figure 1. Changes in Chl *a* (A), Chl *b* (B), and total Chl (C) in the leaves of *Phaseolus vulgaris* L. Sel 9 under Zn stress, Zn + HI stress and recovery. Values are means ± SE (n = 5). The different letters indicate significant differences at P ≤ 0.01 as determined by Tukey's test.

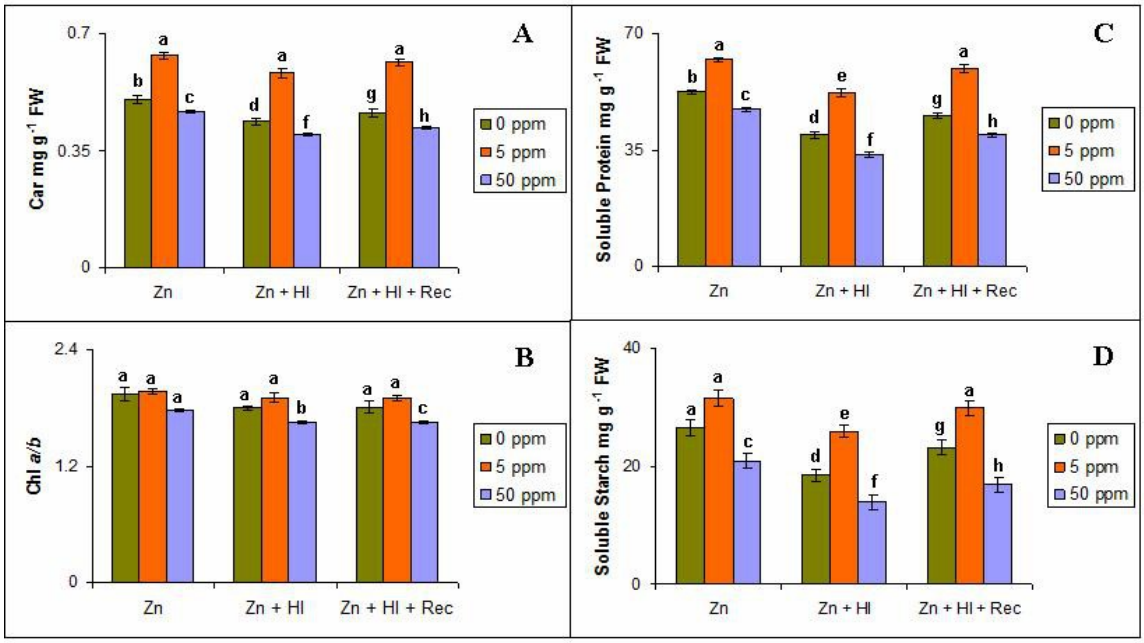

Figure 2. Changes in Car (A), Chl *a/b* (B), soluble protein (C) and soluble starch (D) in the leaves of *Phaseolus vulgaris* L. Sel 9 under Zn stress, Zn + HI stress and recovery. Values are means ± SE (n = 5). The different letters indicate significant differences at P ≤ 0.01 as determined by Tukey's test.

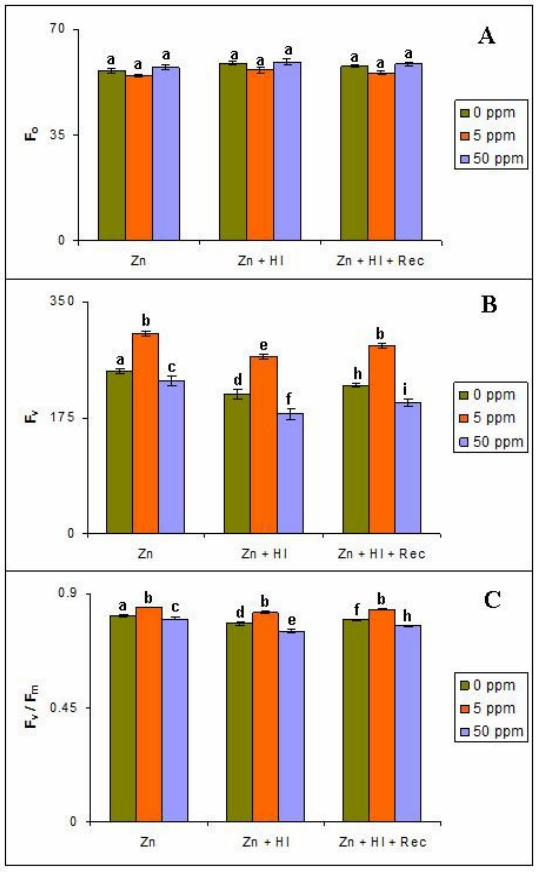

Figure 3. Alterations in initial fluorescence (F_o) (A), variable fluorescence (F_v) (B), and variable to maximum fluorescence ratio (F_v/F_m) (C) in the leaves of *Phaseolus vulgaris* L. Sel 9 under Zn stress, Zn + HI stress and recovery. Values are means ± SE (n = 5). The different letters indicate significant differences at P ≤ 0.01 as determined by Tukey's test.

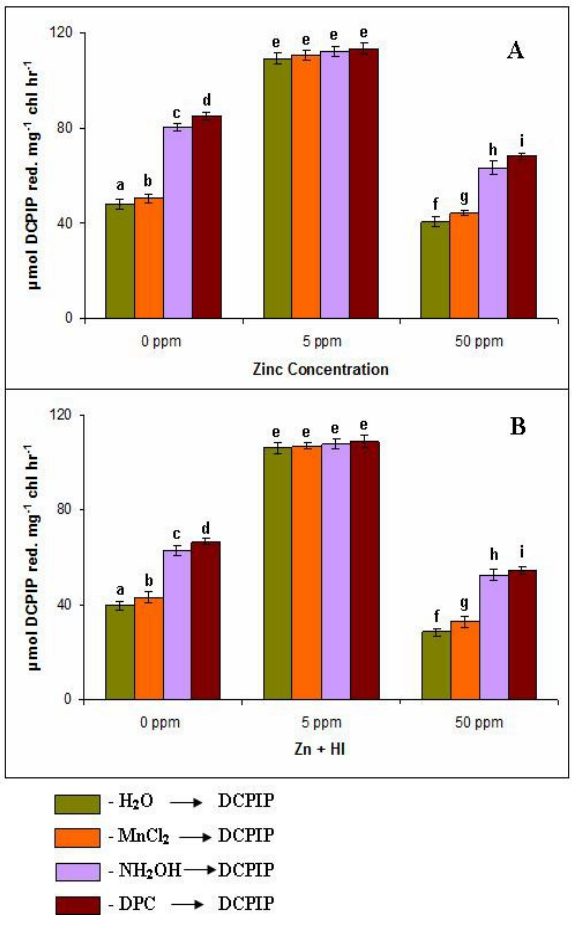

Figure 4. Effect of various exogenous electron donors on PS II activity in thylakoids isolated from *Phaseolus vulgaris* L. Sel 9 leaves under Zn stress and Zn + HI stress. Values are means ± SE (n = 5). The different letters indicate significant differences at P ≤ 0.01 as determined by Tukey's test.

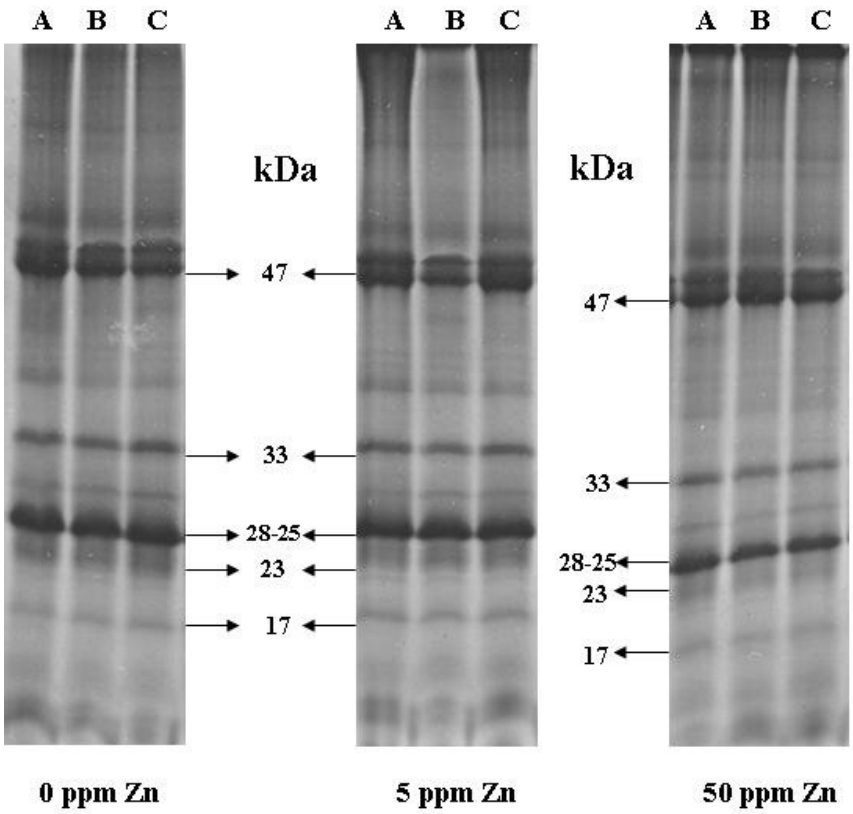

Figure 5. SDS-PAGE polypeptide profiles of thylakoids isolated from Zn stress, Zn + HI treated and recovered leaves of *Phaseolus vulgaris* L. Sel 9. Gel lanes were loaded with equal amounts of thylakoid proteins (100 µg). Lane A, Zn stress; lane B, Zn + HI stress; lane C, Zn +HI + recovery.

DISCUSSION

Zinc deficiency depresses plant leaf's photosynthetic capacity. The reduction in Chl level might have led to a decrease in photosynthesis in Zn-deficient plants. Zinc deficiency might have also affected some photosynthetic enzymes (Ohki, 1976). The decreased concentration of Chl under deficiency (Sharma et al., 1994) and excess of Zn (Kaya, 2002) suggested a possible indirect effect of Zn on Chl biosynthesis. Decrease in pigment in Zn-excess plants suggests that the Chl synthesizing system and chlorophyllase activity were affected at higher exposure concentrations of Zn (Van Assche and Clijsters, 1990). Carotenoids in general, efficient quenchers of the triplet excitation state of Chl and of singlet oxygen (Asada and Takahashi, 1987). The higher Car content in the Zn-sufficient leaves might have protected the photosynthetic machinery exposed to HI stress and acted as a protective mechanism against the ROS generation. Moreover, the Zn-deficient and Zn-excess leaves had a higher percent recovery in Car content thus implicating that the Zn-deficient and Zn-excess bean plants had the capability to withstand or recover from the HI stress imposed on them.

The protein content normally declines under heavy metal stress (Bhattacharya and Choudhuri, 1994). The decrease in soluble protein content under Zn-deficiency and Zn-toxicity has been recently reported by Tavallali et al. (2009) in pisatachio seedling which is similar to our results. The lower content of soluble proteins in the Zn-deficient and Zn-excess leaves under HI stress could be due to high rate of breakdown caused by photon stimulation of protease activity (Paulsen, 1997). The decreased starch concentration under Zn deficiency

could be a result of reduced starch synthatase activity (Jyung et al., 1975). 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 (Ackerson and Hebert, 1981). The accumulation of starch in the Zn-sufficient plants in our experiments has no effect on the photosynthetic rate as has been reported earlier by Potter (1980).

Under HI stress, photoinhibition, or a decrease in quantum efficiency of PS II, can be significant (Kitao et al., 2000). F_v/F_m is used frequently as an expression of photoinhibition (Schansker and Van Rensen, 1999). Hernandez et al. (2006) reported an insignificant increase in F_o and a significant decline in F_v and F_v/F_m ratio in pea plants under HI which was similar to our results. In agreement to our results, a decrease in the F_v/F_m ratio has already been reported in rice grown under cadmium stress (Pagliano et al., 2006) and in bean plants under copper stress (Patsikka et al., 1998). A slight decline in F_v/F_m ratio under Zn-deficiency and Zn-excess conditions in our study indicated that the intrinsic quantum efficiency of the PS II unit was little damaged.

The effect of DCPIP, a Q_B site of electron acceptor, which is generally used for the measurement of PS II activity and oxygen evolution, is well known (Henmi et al., 2004). Significant restoration of PS II activity in the Zn-deficient and Zn-excess leaves by the artificial electron donors suggests that the damage might be located on the donor side of PS II, in the OEC which is similar to the results obtained by Bertamini et al. (2002). Hence the Zn -deficient and Zn -excess leaves were affected on the donor side of PS II by photoinhibition. Since both Zn-deficient and Zn-excess leaves had the capability to restore the PS II activity which is shown by the addition of DPC, the bean plant though underwent some photodamage had the capability to restore from the photodamage caused by Zn and HI stress.

Although the SDS-PAGE of thylakoids revealed that the intensity of the many polypeptide bands were slightly decreased in Zn and Zn + HI stressed thylakoid membranes as compared to Zn-sufficient, the highest decrease was observed during HI-treatment. Similarly, marginal reduction in the amounts of various membrane polypeptides under HI-treatment compared with exogenously applied Zn stress indicated that HI-induced endogenously generated ROS caused deleterious effects not only in the decrease of PS II mediated electron transfer rate, but also in the reduction of contents of other thylakoid membrane polypeptides. Our result supports the findings of Suzuki et al. (2004) of significant reduction in the amounts and completes release of OEC subunits (33, 23, and 17 kDa) from the PS II complexes, which plays a major role in oxygen evolution. Similarly, a loss of 33 kDa polypeptide of OEC was reported by Nanda and Biswal (2008) in papaya leaves. Moreover, there was no change in the number of polypeptide bands. Thus the bean leaves underwent a slight depletion in the thylakoid polypeptides under extreme Zn toxicity when compared to the Zn-sufficient condition which has been overcome after recovery from HI stress.

CONCLUSION

In conclusion, Zn deficiency and Zn excess conditions together with HI treatment caused a range of effects in the bean plants. These include decrease in photosynthetic pigments, soluble proteins, soluble starch, F_v and F_v/F_m ratio, DCPIP

photoreduction and changes in the extrinsic polypeptides of the PSII complex. 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 bean plant. Though there was a slight decline in the F_v/F_m ratio, and a lower inhibition of PS II activity, and slight degradation of 33, 28-25, 23 and 17 kDa polypeptides under Zn-excess condition, the recovery rate was well enough which suggested that the bean plants might have possessed the capability to overcome the imposed Zn and HI stress which might be due to the antioxidant enzyme activities.

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