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Waterlogging induced oxidative stress and antioxidant enzyme activities in pigeon pea

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Abstract

An experiment was conducted with two contrasting pigeon pea (*Cajanus cajan* L.) genotypes, ICPL 84023 (tolerant) and ICP 7035 (susceptible), to study the physiological and molecular basis of waterlogging tolerance in relation to oxidative stress and antioxidant enzyme activities. Waterlogging resulted in visible yellowing and premature senescence of leaves, and greater decline in relative water content, chlorophyll content, and membrane stability index in ICP 7035 than in ICPL 84023. Superoxide radical and hydrogen peroxide contents increased at day 4 and 6 of waterlogging probably due to activation of NADPH-oxidase. $O_2^{\bullet -}$ production was inhibited, by diphenylene iodonium chloride, a specific inhibitor of NADPH oxidase and expression of *NADPH oxidase*-mRNA was increased under waterlogging condition in ICPL 84023. ICP 7035 showed higher contents of ROS in control condition and after recovery, however, during waterlogging the $O_2^{\bullet -}$ production was higher in ICPL 84023. Activities of antioxidant enzymes superoxide dismutase (SOD), ascorbate peroxidase (APX), glutathione reductase and catalase increased under waterlogging more in ICPL 84023 than in ICP 7035. *Cu/Zn-SOD* and *APX*-mRNA expression in 24-h waterlogged plants showed enhanced expression in ICPL 84023 compared to ICP 7035. The cloning and sequencing of *APX* gene of tolerant and susceptible genotypes yielded cDNAs of 622 and 623 bp, having 95 % homology with each other and 92 % with the corresponding sequences of *Vigna unguiculate APX*-gene.

Additional key words: anoxia, ascorbate peroxidase, *Cajanus cajan*, catalase, gene expression, glutathione reductase, hydrogen peroxide, hypoxia, superoxide radical, superoxide dismutase.

Introduction

Lack of oxygen or anoxia is a common environmental challenge, which plants have to face throughout their life. Seed imbibitions, flood irrigation, floods and excess of rainfall are examples of natural conditions leading to root hypoxia or anoxia. A decrease in adenylate energy charge, cytoplasmic acidification, anaerobic fermentation, elevation in cytosolic Ca^{2+} concentration, changes in the redox state [NAD(P)H/NAD(P)] and a decrease in the membrane barrier function, are the main features caused by lack of oxygen (Richard *et al.* 1994, Crawford and Braendle 1996, Drew 1997, Tadege *et al.* 1999). Regulation of anoxic/hypoxic metabolism is complex and not all the features are well elucidated.

Excessive generation of reactive oxygen species (ROS), or oxidative stress, is an integral part of many stress situations, including hypoxia. Hydrogen peroxide accumulation under hypoxic conditions have been shown in the roots and leaves of barley (Kalashnikov *et al*. 1994) and in wheat roots (Biemelt *et al*. 2000). The presence of H_2O_2 in the apoplast and in association with the plasma membrane has been visualized by transmission electron microscopy under hypoxic conditions in four plant

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Abbreviations: APX - ascorbate peroxidase; CAT - catalase; Chl - chlorophyll; DEDC - diethyldithiocarbamate; DPI - diphenyleneiodonium chloride; DMSO - dimethylsulfoxide; DTNB - 5,5-dithiobis-2-nitrobenzoic acid; EB - ethidium bromide; GR - glutathione reductase; GSSG - glutathione disulfide (oxidized glutathione); NBT - nitroblue tetrazolium chloride; ROS - reactive oxygen species; RT-PCR - reverse transcriptase - polymerase chain reaction; SOD - superoxide dismutase; TBARS - thiobarbituric acid relative substances; TBE - Tris-borate-EDTA.

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species (Blokhina *et al.* 2001). Indirect evidence of ROS formation such as thiobarbituric acid relative substances (TBARS; lipid peroxidation products) under low oxygen has also been detected (Chirkova *et al.* 1998, Blokhina *et al*. 1999). To control the level of ROS and to protect cells under stress conditions, plant tissues contain several ROS scavenging enzymes such as superoxide dismutase (SOD), ascorbate peroxidase (APX), glutathione reductase (GR) and catalase (CAT), and a network of low molecular mass antioxidants (ascorbate, glutathione, phenolic compounds and α-tocopherol). An increase in the activity of antioxidant enzymes in response to various environmental stresses have been reported (*e.g.* Elstner 1986, Bowler *et al*. 1992, Sairam *et al*. 2000, 2001, 2002). A 14-fold increase in SOD activity under waterlogged condition has been reported in *Iris pseudacorus* by Monk *et al*. (1989). An increase in total SOD activity has been reported in wheat roots under anoxia, and the degree of increase was positively correlated with duration of anoxia (Van Toai and Bolles 1991). Induction of enzymes involved in the ascorbate-glutathione cycle (APX, MDHAR, DHAR and GR) has been shown for anaerobically germinated rice seedlings and roots of wheat (*Triticum aestivum*) seedlings (Ushimaru *et al.* 1997, Albrecht and Wiedenroth 1994).

Pigeon pea is an important pulse crop, cultivated in Africa, Asia and Australia, and being a summer-rainy season crop, is exposed to waterlogging condition during germination and early vegetative growth phases. In spite of a large data pool on the activity of antioxidant enzymes during and post hypoxia stress, the origin of ROS during waterlogging is still not clear. The objective of this study is, therefore, to characterize the molecular basis of ROS production during waterlogging and to clone the gene of APX, an important enzyme involved in the scavenging of ROS in pigeon pea.

Materials and methods

Plants and growth conditions: A preliminary experiment was conducted with 13 pigeon pea genotypes procured from *ICRISAT*, Hyderabad, India and Division of Genetics, Indian Agricultural Research Institute, New Delhi, India. Based on relative water content, membrane stability index of leaf and root tissues, leaf chlorophyll contents, loss in leaf area and dry matter per plant, the genotypes were grouped into tolerant and susceptible to waterlogging stress. The present study has been conducted with a highly tolerant genotype ICPL 84023 and a susceptible genotype ICP 7035. Seeds were treated with the required *Rhizobium* culture and seedlings were grown in pots filled with clay-loam soil and farm yard manure in 3:1, supplied with basal dose of phosphorus and potassium. Waterlogging treatment was given by placing pots with 25-d-old plants in plastic troughs filled with water to a height just 1 - 2 cm below the soil level in pots. Treatments consisted of control, 2, 4 and 6 d of waterlogging, and recovery 5 and 10 d. Some plants of susceptible genotype ICP 7035 survived up to 6 d of waterlogging, but about 92 % of them died during recovery, therefore, recovery was uniformly studied in the both genotypes for 4 d waterlogged plants only. Observations were recorded on relative water content (RWC), membrane stability index (MSI), chlorophyll (Chl) content, superoxide radicals and hydrogen peroxide contents, and activity of SOD, APX, GR and CAT. Gene expression of *NADPH oxidase, Cu/Zn-SOD* and *APX* was studied by RT-PCR. The design of the experiment was complete randomized block design and data was analyzed by factorial randomized block design (RBD).

Physiological parameters: Leaf relative water content (RWC) was estimated by recording the mass of water saturated leaf samples by keeping in water for 4 h, followed by drying in hot air oven till constant mass and calculated according to Weatherley (1950).

For estimation of membrane stability index 100 mg leaf or root material was taken in test tubes containing 10 cm^3 of double distilled water (Sairam *et al.* 1997). One set was heated at 40 °C for 30 min in a water bath, and the electrical conductivity of the solution was recorded (C_1). Second set was boiled at 100 °C on a boiling water bath for 10 min, and its conductivity was measured (C_2). Membrane stability index (MSI) = $[1 - (C_1/C_2)] \times 100$

Chlorophyll content was estimated by extracting 0.05 g of the leaf material in 10 cm^3 dimethylsulfoxide (Hiscox and Israelstam 1979). Samples were heated in an incubator at 65 °C for 4 h and after cooling to room temperature the absorbance of extracts were recorded at 663 and 645 nm, and their chlorophyll content was calculated.

Assay of oxidative stress: Superoxide radical content was quantified by its capacity to reduce nitroblue tetrazolium chloride (NBT) and the absorption of end product measured at 540 nm. Root tissue (1 g) was homogenized in 10 cm^3 of pre-cooled phosphate buffer (0.2 M, pH 7.2) containing 1 mM diethyldithiocarbamate (DEDC; to inhibit SOD activity) and 10 μM diphenyl-eneiodonium chloride (DPI; optional, to inhibit NADPH oxidase dependent $O_2^{\bullet-}$ generation). The homogenate was centrifuged in refrigerated centrifuge (model *3K 30*, *Sigma*, Osterode, Germany) at 9 400 g for 10 min and supernatant was immediately used for the estimation of $O_2^{\bullet-}$ (Chaitanya and Naithani 1994). The reaction mixture contained 0.25 cm^3 tissue extract, 0.075 mM NBT, 25 mM Na_2CO_3, 0.1 mM EDTA, 13.33 mM L-methionine and water to make volume 3 cm^3. Reaction mixture was incubated at 30 °C for 10 min and absorbance was recorded at 540 nm (coefficient of absorbance 12.8 mM^{-1} cm^{-1}). NADPH oxidase independent $O_2^{\bullet-}$ production (DPI-insensitive) was

obtained by addition of DPI (10 μM) in the extraction buffer, which inhibited NADPH oxidase activity. Difference of total and DPI-insensitive $O_2^{\bullet-}$ production gave the NADPH oxidase dependent (DPI-sensitive) $O_2^{\bullet-}$ production.

H_2O_2 was estimated by forming titanium-hydro-peroxide complex (Rao *et al.* 1997). Root material (1 g) was ground with liquid nitrogen and the fine powdered material was mixed with 10 cm^3 cooled acetone in a cold room (10 °C). Mixture was filtered with *Whatman No.1* filter paper followed by the addition of 4 cm^3 titanium reagent and 5 cm^3 ammonium solution to precipitate the titanium-hydro peroxide complex. Reaction mixture was centrifuged at 9 400 g for 10 min. Precipitate was dissolved in 10 cm^3 2 M H_2SO_4 and then recentrifuged. Supernatant was read at 415 nm against blank in UV-visible spectrophotometer (model *Specord Bio-200*, *AnalytikJena*, Jena, Germany). H_2O_2 contents were calculated by comparing with a standard curve.

Antioxidant enzymes: Enzyme extract was prepared by freezing 1 g root samples in liquid nitrogen, followed by grinding with 10 cm^3 extraction buffer consisting of 0.1 M phosphate buffer, pH 7.5, containing 0.5 mM EDTA in case of SOD, GR and CAT, or 0.1 M phosphate buffer, pH 7.5, 0.5 mM EDTA, 1 mM ascorbic acid in case of APX. Brie was passed through 4 layers of cheesecloth and filtrate was centrifuged for 20 min at 4 °C and 21 100 g, and the supernatant was used as enzyme extract.

SOD activity was assayed by following the decrease in absorbance of formazone formed by superoxide anion with itroblue tetrazolium (NBT; Dhindsa *et al.* 1981). The 3 cm^3 of reaction mixture contained 13.33 mM methionine, 25 mM NBT, 0.1 mM EDTA, 50 mM potassium phosphate buffer (pH 7.8), 50 mM sodium carbonate and 0.1 cm^3 enzyme. Reaction was started by adding 2 μM riboflavin and placing the tubes under two 15 W fluorescent lamps for 15 min. To distinguish SOD isoforms the sensitivity of Cu/Zn-SOD to KCN (3 mM), and Cu/Zn-SOD and Fe-SOD to H_2O_2 (5 mM) were used, whereas Mn-SOD is unaffected (Yu and Rengel 1999). A complete reaction mixture without enzyme served as a blank. Reaction was stopped by switching off the light and the absorbance was recorded at 560 nm. One unit of enzyme activity was taken as that amount of enzyme, which reduced the absorbance reading to 50 % in comparison with tubes lacking enzyme.

APX activity was assayed by recording the decrease in absorbance due to enzyme induced oxidation of ascorbic acid at 290 nm (Nakano and Asada 1981). The 3 cm^3 reaction mixture contained 50 mM potassium phosphate buffer (pH 7.0), 0.5 mM ascorbic acid, 0.1 mM EDTA, 1.5 mM H_2O_2 and 0.1 cm^3 enzyme. The reaction was started with the addition of H_2O_2. Absorbance was measured at 290 nm.

GR activity was assayed as per the method of Smith *et al.* (1988). The reaction mixture contained, 66.67 mM potassium phosphate buffer (pH 7.5) and 0.33 mM EDTA, 0.5 mM DTNB (in 0.01 M potassium phosphate buffer, pH 7.5), 66.67 μM NADPH, 666.67μM oxidized glutathione (GSSG), 0.1 cm^3 enzyme extract. Reaction was started by adding GSSG. The increase in absorbance at 412 nm was recorded. The activity was expressed as changes in absorbance (ΔA_{412}) per mg protein per min.

CAT activity was assayed by monitoring the decrease in absorbance due to decomposition of H_2O_2 at 240 nm (Aebi 1984). The reaction mixture consisted of potassium phosphate buffer 50 mM, 12.5 mM H_2O_2, enzyme extract 0.05 cm^3, and water to make up the volume to 3.0 cm^3. Adding H_2O_2 started reaction and decrease in absorbance was recorded for 1 min. Enzyme activity was computed by calculating the amount of H_2O_2 decomposed by referring to a standard curve.

Soluble protein content was estimated as per dye binding method of Bradford (1976).

RT-PCR analyses were done in 25-d-old plants subjected to waterlogging for 24 h, and RNA from root tissue was extracted using *Trizol* reagent (*GibcoBRL*, USA) according to the manu-facturer's recommendations. DNA contamination was removed from the RNA samples using DNase I (*Qiagen Science*, Maryland, USA). RNA (1 μg) was reverse transcribed using *Qiagen* one step RT-PCR kit. PCR conditions were standardized using gene-specific primers for tubulin content. Linear amplification for semi-quantitative RT-PCR was obtained with 35 cycles. Reactions were conducted using *My Genie 32* thermal block PCR (*Bioneer*, Daejeon, South Korea) under the following conditions: initial activation for 15 min at 95 °C, reverse transcription for 30 min at 50 °C, denaturation for 1 min at 94 °C, annealing for 1 min at 60 °C, extension for 1 min at 72 °C, final extension for 10 min at 72 °C. The amplification products were electrophoresed on 1.2 % agarose gel at 120 V in TBE buffer (0.4 M Tris-borate, 0.001 M EDTA, pH 8.0) using known concen-tration DNA ladders. Gels were stained with ethidium bromide and visualized on *Uvi Pro* gel documentation system (*UviTec*, Cambridge, UK).

The desired cDNA fragment were excised from the agarose gel and three volumes of QC buffer (containing guanidine thiocyanate) was added and incubated at 50 °C for 10 min. After complete dissolution of gel slice, one volume of isopropanol was added. The contents were placed in *QIAquick* spin column with the 2 cm^3 collection tube and centrifuged for 1 min at 10000g. The flow through was discarded. The column was given a wash with 0.75 cm^3 PE buffer and centrifuged again at 10 000 *g* for 1 min. The flow through was again discarded and column was placed in another clean micro-centrifuge tube and cDNAs were eluted with 0.05 cm^3 EB buffer. The eluted DNA was sequenced by following dideoxychain termination method (Sanger *et al*. 1977.

Table 1. Primer sequences for various genes.

Gene	Source		Primers
NADPH oxidase	*Vigna radiata*	forward	ATG TAA GCG TTC ACA TTC GGA C
		reverse	CCN TGA AAC AAT ATC CAC ACC AC
Cu/Zn-SOD	*Cicer arietinum*	forward	ATG GTK AAG GCT GTG GCA GTT C
		reverse	ACC TTT CCC AAG ATC ATC AGG ATC
APX	*Pisum sativum*	forward F_1	TCT ACG AAA ATT GAA TAC ATT GTA C
		forward F_2	CTC TGT CAT AAC TCA TCA CGC AAC
		reverse	CGC CAA ACG GAG AAT TAG AGG AG
Tubulin	*Vigna radiata*:	forward	CTT GAC TGC ATC TGC TAT GTT CAG
		reverse	CTT GAC TGC ATC TGC TAT GTT CAG

Results

Waterlogging treatment of 2, 4 and 6 d resulted in significant decline in RWC in both the pigeon pea genotypes as compared to control (Table 2). However, ICPL 84023 maintained higher RWC even after 6 d of waterlogging. ICP 7035 showed a sharp decline in RWC, and the 6 d of waterlogging caused RWC to decline beyond the level of recovery. The recovery recorded after 5 and 10 d of 4-d waterlogged plants showed that ICPL 84023 recovered faster and the RWC was comparable to that of control plants even at day 5 after recovery.

Membrane stability index (MSI) in the roots and leaves (Table 2) decreased in both the pigeon pea genotypes under waterlogging. The rate of decline corresponded with the duration of waterlogging stress, with decline being greater in ICP 7035, while ICPL 84023 managed to maintain higher MSI even after 6 d of waterlogging. ICP 7035 showed slow recovery, while ICPL 84023 recorded a faster recovery.

Total chlorophyll content decreased after 6 d of water logging in both genotypes but more in ICP 7035 than in ICPL 84023 (Table 2). During recovery, recorded after 5 and 10 d of 4-d waterlogged plants, none of genotypes reach the initial content even after 10 d of recovery.

Two days after waterlogging total superoxide radical ($O_2^{\bullet-}$) content declined over control plants in both the genotypes (Fig. 1*A*). However, continuous waterlogging upto 6 d resulted in significant increase in $O_2^{\bullet-}$ production over 2-d waterlogged plants in both the genotypes, though remaining less than the control plants. The increase was greater in ICPL 84023 than in ICP 7035. Recovery recorded after 5 d resulted in a drastic increase in $O_2^{\bullet-}$ content over that observed in water-logged and control plants of the two genotypes, however, ICP 7035 showed significantly higher $O_2^{\bullet-}$ content than the ICPL 84023. In ICPL 84023 the $O_2^{\bullet-}$ content after 10 d of recovery decreased below the control level. The content of $O_2^{\bullet-}$ in the presence of DPI decreased under waterlogging, recording only a marginal variation in the two genotypes (Fig. 1*B*). However, the DPI-insensitive $O_2^{\bullet-}$ production increased after 5 and 10 d of recovery, and ICP 7035 recorded higher DPI-insensitive $O_2^{\bullet-}$ production. In case of ICPL 84023 the DPI-sensitive (NADPH oxidase dependent) $O_2^{\bullet-}$ generation was lowest in control plants, and continuously increased under waterlogging stress, reaching a peak on day 6, while in case of ICP 7035 it decreased on day 2 of waterlogging, and showed only a marginal increase after 4 and 6 d (Fig. 1*C*). NADPH oxidase dependent $O_2^{\bullet-}$ generation

Table 2. Effect of waterlogging treatment on leaf relative water content (RWC), chlorophyll (Chl) content and membrane stability index (MSI) of leaf and root tissues in susceptible and tolerant *Cajanus cajan* genotypes. Recovery was studied for 4-d waterlogged plants for both the genotypes, as 6-d waterlogged plants of ICP 7035 failed to survive during recovery. Means ± SE, $n = 8$.

Parameter	Genotype	Waterlogging [d]				Recovery [d]	
		0	2	4	6	5	10
RWC [%]	ICP 7035	79.77 ± 5.05	71.7 ± 4.13	58.57 ± 3.55	46.03 ± 2.42	68.50 ± 2.69	74.83 ± 7.07
	ICPL 84023	83.37 ± 2.25	77.90 ± 1.68	72.57 ± 2.88	64.93 ± 3.98	80.40 ± 2.83	82.53 ± 2.46
Chlorophyll	ICP 7035	2.97 ± 0.02	2.47 ± 0.04	1.84 ± 0.03	1.23 ± 0.03	1.93 ± 0.07	2.35 ± 0.06
[mg g^{-1} (f.m.)]	ICPL 84023	3.18 ± 0.04	3.16 ± 0.02	2.92 ± 0.06	2.43 ± 0.03	2.88 ± 0.06	3.04 ± 0.01
MSI (leaves)	ICP 7035	78.74 ± 4.16	57.45 ± 2.66	46.48 ± 3.73	33.28 ± 0.92	40.72 ± 1.09	60.76 ± 1.07
[%]	ICPL 84023	82.93 ± 1.69	74.23 ± 2.87	68.73 ± 1.82	56.23 ± 1.51	72.32 ± 3.90	82.65 ± 2.74
MSI (roots)	ICP 7035	72.76 ± 4.37	52.39 ± 2.98	45.79 ± 2.53	31.44 ± 2.30	39.26 ± 2.80	56.04 ± 5.26
[%]	ICPL 84023	80.69 ± 2.42	72.42 ± 1.82	64.10 ± 2.90	54.16 ± 3.24	68.18 ± 2.19	78.80 ± 1.09

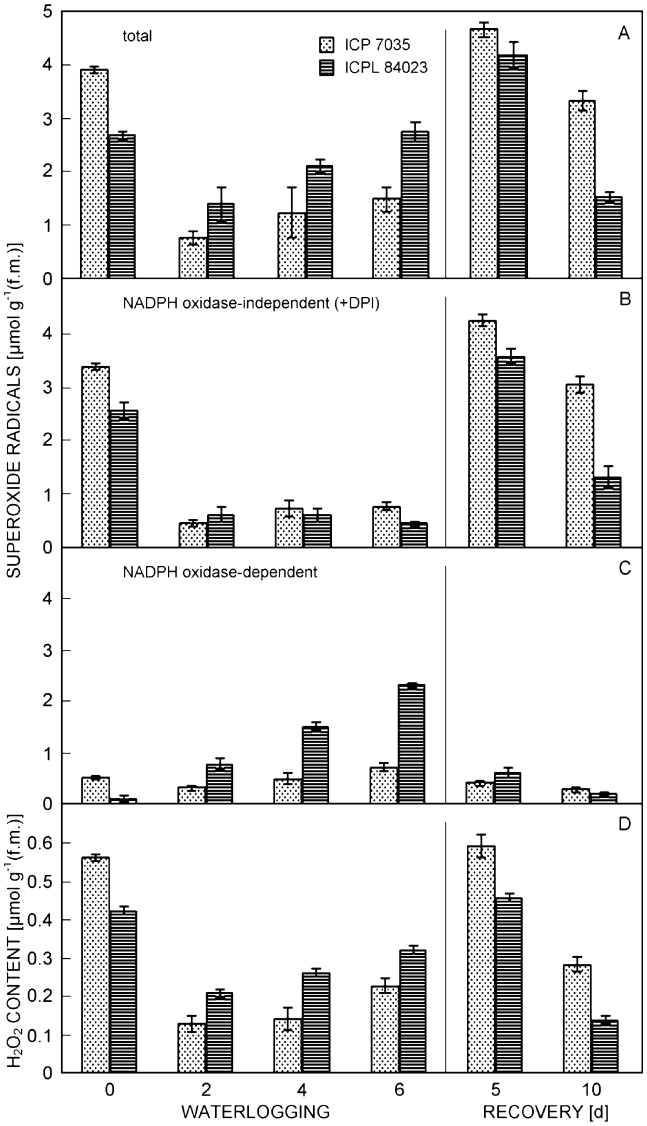

Fig. 1. Effect of waterlogging on contents of total superoxide radical (*A*), superoxide radical in the presence of DPI (*B*), DPI-sensitive (NADPH oxidase dependent) superoxide radical (*C*) and hydrogen peroxide (*D*) in the root tissue of tolerant and susceptible *Cajanus cajan* genotypes. *Vertical bars* show ± SE, $n = 8$.

was significantly greater in ICPL 84023. With the onset of recovery the DPI-sensitive $O_2^{\bullet-}$ content declined in both the genotypes.

H_2O_2 content showed pattern similar to $O_2^{\bullet-}$ content. In control and recovered plants H_2O_2 content was higher in ICP 7035 than in ICPL 84023 (Fig. 1*D*). Two days of waterlogging resulted in significant decline in hydrogen peroxide content in both the genotypes compared to that observed in control plants. However, waterlogging upto 6 d resulted in significant increase in H_2O_2 content compared to 2-d waterlogged plants, but remained lower than control plants in both the genotypes. During the waterlogging phase ICPL 84023 showed significantly greater H_2O_2 content than observed in ICP 7035. Recovery resulted in a drastic increase in H_2O_2content over that observed on 4th day, the day recovery was started, as well as in control plants of the two genotypes, and ICP 7035 showed significantly higher H_2O_2 content than the ICPL 84023.

Waterlogging caused significant increase in the activity of SOD and its isozymes in ICPL 84023 upto 6 d of stress, while in ICP 7035 a slight increase was observed only on day 2 of waterlogging and thereafter the activity decreased with duration of stress, going down to almost 20 % of its maximum (Fig. 2*A*). During recovery the SOD activity decreased in ICPL 84023 and slightly increased in ICP 7035, however the activity in ICPL 84023 was 80 % higher than that observed in ICP 7035. Waterlogging induced increase in total SOD activity in ICPL 84023 was mainly due to the increase in Cu/Zn-SOD, which contributed to about 47, 46 and 45 % at 2, 4 and 6 d of waterlogging treatment, respectively. Mn-SOD

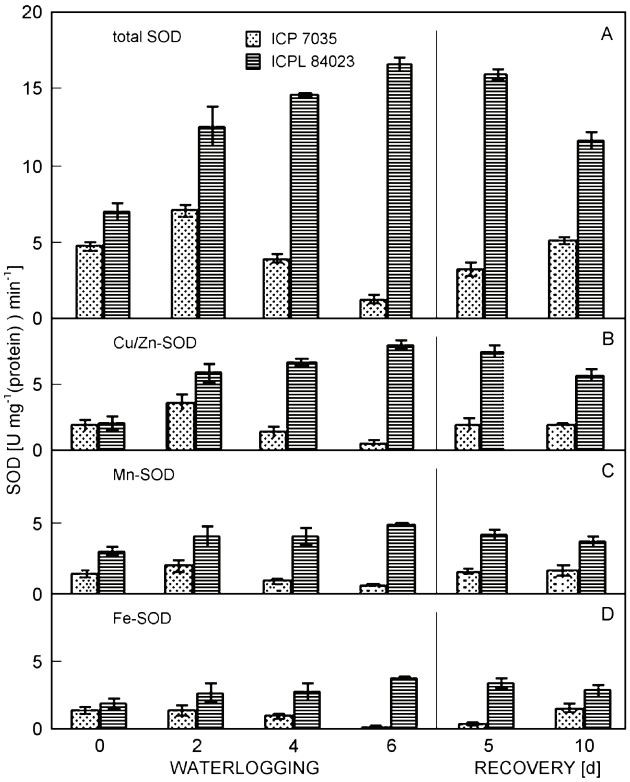

Fig. 2. Effect of waterlogging on the activity of total superoxide dismutase (*A*), Cu/Zn-SOD (*B*), Mn-SOD (*C*) and Fe-SOD (*D*) in the root tissue of tolerant and susceptible *Cajanus cajan* genotypes. *Vertical bars* show ± SE, $n = 8$.

accounted for about 32, 32 and 29 % at 2, 4 and 6-d of waterlogging, respectively, in tolerant genotype. Fe-SOD had lesser role with its contribution of only 21, 19 and 23 % of the total SOD activity at 2, 4 and 6-d of waterlogging, respectively. All the isoforms of SOD were greatly reduced at 4 and 6 d of waterlogging stress in ICP 7035 (Fig. 2*B-D*). There was significant increase in the activities of APX, GR and CAT at 2 d of waterlogging in both the genotypes (Fig. 3). Further waterlogging of 4 and 6 d resulted in decrease in APX, GR and CAT activity in ICP 7035, while ICPL 84023 recorded continuous increase upto 6th day of waterlogging. During recovery, ICP 7035 showed slight increase in the activity of APX, GR and CAT, but the values were still lower than control plants. Though ICPL 84023 showed decline in APX, GR and CAT activities during recovery over the values observed on 4th and 6th day of waterlogging, the values were significantly greater than those in control plants and those observed in ICP 7035.

Gene expression studies for *NADPH oxidase, Cu/Zn-SOD* and *APX* by RT-PCR elicited bands of about 700 kbp. *NADPH oxidase gene* showed constitutive as well as induced expression in the case of ICPL 84023. However, the induced expression was much higher than constitutive expression. There was very little constitutive as well as induced expression of *NADPH oxidase* gene in the case of ICP 7035 (Fig. 4*A*). There was little constitutive expression of *Cu/Zn-SOD* gene in ICPL 84023 and non at all in ICP 7035. However, waterlogging treatment of 24 h induced significant *Cu/Zn-SOD* gene expression only in ICPL 84023 and very little in ICP 7035 (Fig. 4*B*). *APX*-gene showed significant expression in ICPL 84023 under 24 h waterlogging. In case of ICP 7035 very little *APX* gene expression was observed both in control and treated plants (Fig. 5*A*). The tubulin expression was almost constant in both the genotypes, and did not change under control and waterlogged conditions (Fig. 5*B*).

Sequencing of partial *APX* cDNAs obtained in this study yielded amplicons of 622 and 623 bp in the case of ICPL 84023 and ICP 7035, respectively in comparison to 753 bp of *Vigna unguiculata* (U 61379) complete *APX*-gene (Fig. 6). The deduced amino acid sequences of APX-proteins of the pigeon pea genotypes were having 207 (ICPL 84023) and 206 (ICP 7035) amino acid residues (Fig. 7) in comparison to 250 amino acid residues in the corresponding regions of the complete cytosolic APX-protein of *Vigna unguiculata*.

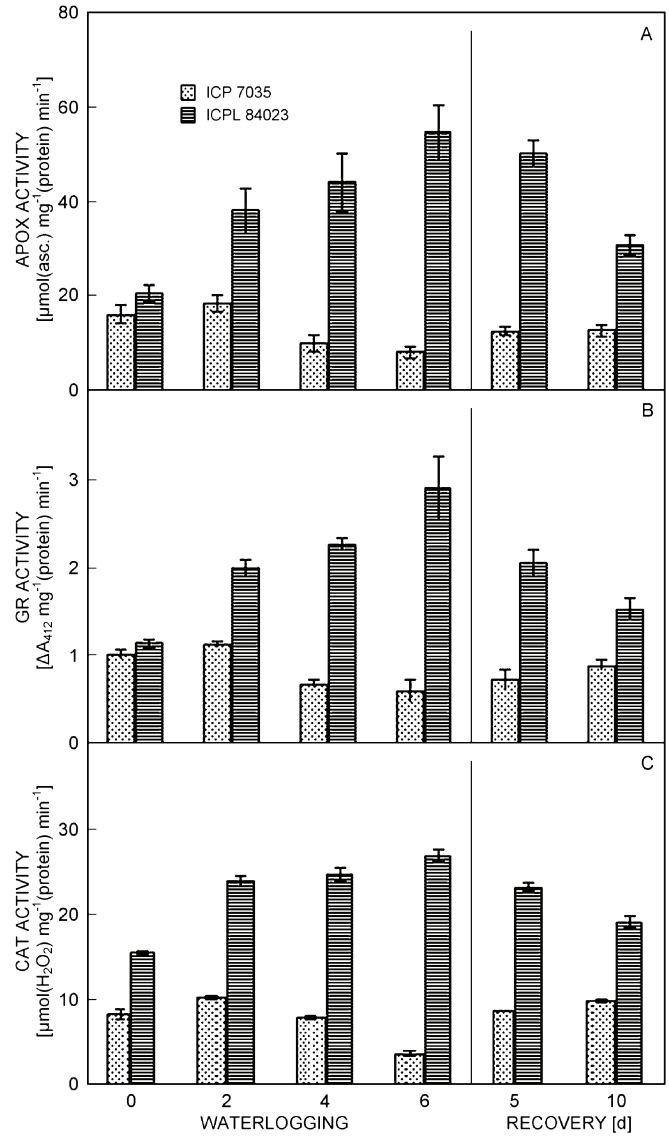

Fig. 3. Effect of waterlogging on the activity of ascorbate peroxidase (*A*), glutathione reductase (*B*) and catalase (*C*) in the root tissue of tolerant and susceptible *Cajanus cajan* genotypes. *Vertical bars* show ± SE, $n = 8$.

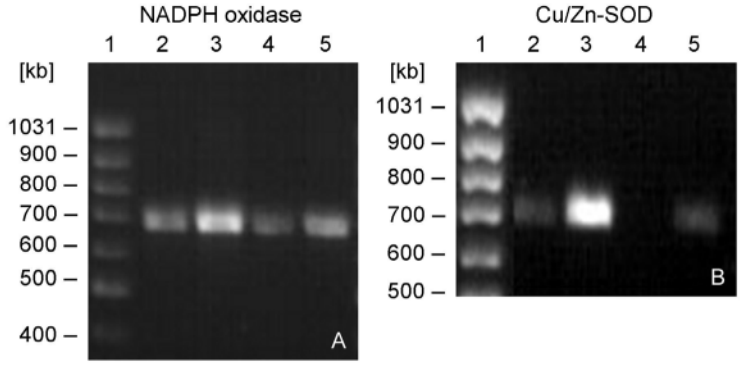

Fig 4. RT-PCR gene expression of *NADPH oxidase* (*A*) and *Cu/Zn-SOD* (*B*) as affected by 24 h waterlogging (WL) treatment in tolerant and susceptible *Cajanus cajan* genotypes (1 - ladder, 2 - control ICPL 84023, 3 - WL ICPL 84023, 4 - control ICP 7035, 5 - WL ICP 7035).

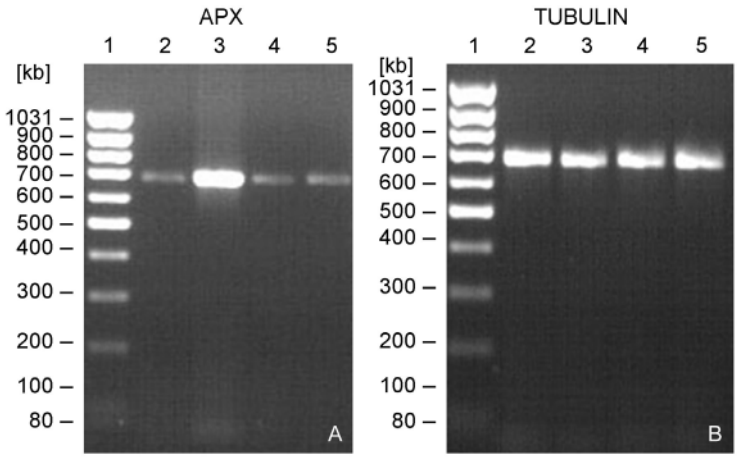

Fig. 5. RT-PCR gene expression of *APX* (*A*) and *tubulin* (*B*) as affected by 24 h waterlogging treatment in tolerant and susceptible *Cajanus cajan* genotypes (1 - ladder, 2 - control ICPL 84023, 3 - WL ICPL 84023, 4 - control ICP 7035, 5 -WL ICP 7035).

Discussion

As a result of 6 d of waterlogging pigeon pea genotypes sustained a higher decrease in RWC, chlorophyll contents, and higher membrane injury, both in roots and leaves, in ICP 7035 compared to ICPL 84023. Min and Bartholomew (2005) reported decrease in RWC during flooding, which further declined with the duration of flooding stress. Various workers have also reported waterlogging induced decrease in leaf water potential (Else *et al*. 1995, Naidoo 1983), and membrane damage (Oberson *et al.* 1999, Rawyler *et al*. 2002). Jackson *et al.* (1982) reported waterlogging induced membrane damage in pea plants resulting in more than 40 times increase in solute leakage. Waterlogging induced decline in Chl contents have been reported in wheat (Collaku and Harrison 2002), *Vigna sinensis* (Younis *et al.* 2003) and soybean (Sorte *et al.* 1996). Pigeon pea plants visually wilted within 4 d of imposing of flooding stress due to the higher resistance to mass flow of water through the roots (Jackson and Drew 1984). ICPL 84023 presumably has better survival mechanism, as it maintained greater RWC, MSI and Chl content under waterlogging stress.

A very striking observation under waterlogging is the production of various ROS, especially $O_2^{\bullet-}$ and H_2O_2 (Yan *et al.* 1996). Compared to control plants, 2 d waterlogged plants showed a decline in $O_2^{\bullet-}$ and H_2O_2 contents. However, after 4 and 6 d there was significant increase in the contents of ROS, though it remained lower than that of control or recovered plants. Further during the waterlogging tolerant genotype ICPL 84023 showed higher $O_2^{\bullet-}$ and H_2O_2 contents compared to ICP 7035 (susceptible genotype). The reduction in ROS production on 2nd day of waterlogging could be attributed to a shift from aerobic respiration to fermentation and consequently a blockage in the mitochondrial site of ROS production. The results obtained in this study clearly show that increase in ROS during waterlogging is primarily due to an increase in DPI-sensitive (NADPH oxidase dependent) $O_2^{\bullet-}$ production. The NADPH oxidase-dependent $O_2^{\bullet-}$ generation, which was minimum under aerobic condition increased significantly under waterlogging stress, but again declined during recovery. In contrast NADPH oxidase-independent (DPI-insensitive) $O_2^{\bullet-}$ generation declined under waterlogging, and increased significantly during the recovery phase, more in susceptible genotype ICP 7035, probably due to restoration of aerobic mitochondrial activity. Blokhina *et al.* (2001) reported H_2O_2 formation during anoxia as evidenced by electron dense insoluble precipitate of cerium perhydroxide, visualized through electron microscope. The enzymatic origin of ROS production during waterlogging was further confirmed by the waterlogging induced increase in expression of *NADPH oxidase* gene, which was significantly greater in tolerant genotype ICPL 84023. Waterlogging induced ROS production and expression of *NADPH oxidase* gene in tolerant genotype ICPL 84023 could also have a role in signaling anaerobiosis tolerance mechanism. H_2O_2 signaling has been reported in the induction of *ADH* gene (Fukao and Bailey-Serres 2004) and SOD and APX (Agarwal *et al.* 2005). The little amount of DPI-insensitive $O_2^{\bullet-}$ production during waterlogging could be due to the extremely reductive conditions in the rhizosphere, which might facilitate the reduction of dissolved oxygen to superoxide radical (Ponnamperuma 1972, Sairam *et al.* 2009).

Results observed on various antioxidative enzymes like SOD, APX, GR and CAT under waterlogged condition in tolerant and susceptible pigeon pea genotypes reveal a continuous increase in all the enzymes upto 6 d of waterlogging in tolerant genotype (ICPL 84023), while in case of susceptible genotype (ICP 7035) the increase in antioxidative enzyme activity was noticed only in 2 d waterlogged plants and at subsequent stages there was decline in activity of all the antioxidant

enzymes as compared to control and 2 d waterlogged plants. The increase in the activity of various antioxidant enzymes under waterlogging is due to the gene activation and mRNA synthesis, as is evident from waterlogging induced expressions of *Cu/Zn-SOD* and *APX* genes, which were significantly higher in tolerant genotype ICPL 84023. It has been suggested by various workers that increase in antioxidative enzymes activity during waterlogging is primarily to minimize the post hypoxia oxidative stress. Monk *et al.* (1987) were one of the first workers who observed a continuous increase in SOD activity in rhizomes of *Iris pseudocorus* under waterlogging stress. The results obtained from this study suggest that the increase in antioxidative enzymes during waterlogging may also be to scavenge build up of oxidative stress during waterlogging. However, plants experinced greater oxidative stress (ROS production) when they were shifted to aerobic condition and this explains overall higher antioxidant enzymes activity in tolerant genotype ICPL 84023 during waterlogging and

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CLUSTAL W (1.83) Multiple sequence alignment
APAX4-7035      ----ACTCTGCTGGTACCTTTGACGTTAGCACGAAGACCGGTGGTCCCTTCGGAACCATC 56
APAX3-84023     -----CTCTGCTGGTACCTTTGATGTTAGCACGAAGACCGGTGGTCCCTTCGGAACCATC 55
U61379          TGGCACTCTGCTGGTACCTTTGACGTCAGCACGAAGACCGGTGGTCCTTTTGGAACCATC 180
                     ****************** ** ******************** ** *********

APAX4-7035      AAGCACCCTGCGGAACTCGCTCACGGTGCCAACAACGGTCTCGACATAGCTGTTAGGCTT 116
APAX3-84023     AAGCACCCTGCGGAACTCGCTCACGGTGCCAACAACGGTCTCGATATCGCTGTTAGGCTT 115
U61379          AAGCACCCTGCCGAACTCGCTCACGGTGCCAACAACGGTCTTGATATCGCTGTTAGGCTA 240
                ********** ****************************** ** ** ***********

APAX4-7035      TTGGAGCCAATCAAAGCAGAGTTTCCTATCTTGAGCTACGCAGATTTCTACCAGCTGGCT 176
APAX3-84023     TTGGAGCCAATCAAAGCGGAGTTTCCTATCTTGAGCTACGCATATTTCTACCAGTTGGCT 175
U61379          TTGGAGCCAATTAAAGCGGAGTTTCCTATCTTGAGCTACGCAGATTTCTACCAGTTGGCT 300
                ********** ***** ********************** ********** *****

APAX4-7035      GGCGTTGTTGCAGTTGAGATAACTGGTGGACCCGAAGTACCTTTTCACCCGGGCAGAGAG 236
APAX3-84023     GGGGTTGTCGCAGTTGAGATAACTGGTGGACCCGAAGTACCTTTTCACCCGGGCAGAGAG 235
U61379          GGCGTTGTCGCAGTTGAGGTCACTGGTGGACCTGAAGTTCCTTTCCACCCGGGCAGAGAG 360
                ** ***** ********* * ********** ***** ***** ***************

APAX4-7035      GACAAACCAGAACCACCTCCAGAGGGTCGCTTGCCTGATGCAACCAAGGGGTCTGATCAT 296
APAX3-84023     GACAAGCCAGAACCACCTCCAGAGGGTCGCTTGCCTGATGCAACCAAGGGGTCTGATCAC 295
U61379          GACAAGCCAGAACCACCTCCAGAGGGTCGCTTGCCCGATGCAACCAAGGGGTCTGATCAC 420
                ***** ***************************** ***********************

APAX4-7035      CTTAGGGATGTGTTCGGCAAGGCTATGGGGCTTAGTGATCAGGATATTGTTGCTCTATCT 356
APAX3-84023     CTTAGGGATGTGTTCGGCAAGGCTATGGGGCTTAGTGATCAGGATATTGTTGCTCTATCT 355
U61379          CTAAGGGATGTGTTCGGCAAGGCTATGGGACTTAGTGATCAGGATATTGTTGCTCTATCT 480
                ** ************************* ******************************

APAX4-7035      GGTGGTCACACCATCGGAGCGGCACACAAGGAGCGTTCAGGATTTGAGGGTCCGTGGACC 416
APAX3-84023     GGTGGTCACACCATCGGAGCGGCACACAAGGAGCGTTCAGGATTTGAGGGTCCGTGGACC 415
U61379          GGTGGCCACACCATTGGTGCGGCACACAAGGAGCGTTCAGGATTTGAGGGCCCATGGACC 540
                ***** ******** ** ********************************** ** ******

APAX4-7035      TCAGACCCTCTTATATTTGACAACTCACACTTTAAGGAGTTGTTGAGTGGTGAAAAGGAA 476
APAX3-84023     TCAGACCCTCTTATATTTGACAACTCACACATTAAGGAGTTGTTGAGTGGTGAAAAGGAA 475
U61379          TCAAACCCTCTTATTTTTGACAACTCATACTTTAAGGAGTTGTTGAGTGGTGAAAAGGAA 600
                *** ********** ************ ** ******************************

APAX4-7035      GGCCTCCTTCAGCTGCCTTCTGACAAGGCACTTCTGTCAGATCCCGTATTCCGCCTTCTT 536
APAX3-84023     GGTCTCCTTCAGCTGCCTTCTGACAAGGCACTTTTGTCAGATACCGTATTCCGTCCTCTT 535
U61379          GGCCTCCTTCAGTTGCCTTCTGACAAGGCACTTTTGTCTGATCCTGTATTCCGCCCTCTT 660
                ** ********* ******************** **** *** * ******** * ****

APAX4-7035      GTTGAAAAATATGCAGCGGACGAAGATGCCTTCTTTGCTGAT-ATGCAGTTGCTCACCAT 595
APAX3-84023     GTTGAAAAATATGCAGCGGACGAAGATGCCATCTTTGCTGATTACGCAGTTGCTCACCAT 595
U61379          GTTGAAAAATATGCAGCGGACGAAGATGCCTTCTTCGCTGATTACGCTGTTGCTCACCAA 720
                ****************************** **** ****** * ** ***********

APAX4-7035      ATGCTCTCCGAGCTGGGTTTTGGCTTGA----- 623
APAX3-84023     AAGCTCTCCCAGCTAGGGTT-GGCAGGA----- 622
U61379          AAGCTTTCCGAGCTTGGGTTTGCTGATGCGTAA 753
```

Fig. 6. Comparison of nucleotide sequences of coding regions of *Cajanus cajan* genotypes (ICPL 84023 and ICP 7035) *APX* gene with *Vigna unguiculata* (Acc. No. U 61379) complete cytosolic *APX*-mRNA by *CLUSTAL W* (*1.83*) multiple sequence alignment.

Fig. 7. Comparison of deduced amino acid sequences of *Cajanus cajan* genotypes (ICPL 84023 and ICP 7035) APX-proteins with *Vigna unguiculata* (Acc. No. U 61379) complete cytosolic ascorbate peroxidase protein by *CLUSTAL W* (*1.83*) multiple sequence alignment.

recovery as compared to susceptible genotype ICPL 84023. The increase in activity of various antioxidative enzymes under waterlogging/ flooding have also been reported, *e.g*. SOD (Biemelt *et al.* 2000), APX (Biemelt *et al*. 1998), GR and CAT (Ushimaru *et al.* 1997).

The *Basic Local Alignment Search Tool* (*BLAST*; Altschul *et al*. 1997) for the gene coding region of ICPL 84023 and ICP 7035 revealed that pigeon pea *APX* gene has high homology to cowpea *APX* (Mittler and Zilinskas 1992; GenBank acc. No. U 61379). The gene coding regions of ICPL 84023 and ICP 7035 were of 622 and 623 bp. The *CLUSTAL W* (1.83) multiple sequence alignment analysis of the cDNAs (Thompson *et al.* 1994, Higgins *et al.* 1996) of ICPL 84023 (tolerant) and ICP 7035 (susceptible), revealed that both cDNAs have 92 % homology with the corresponding sequences of cowpea cytosolic *APX* (753 bp) and 95 % with each other. The deduced amino acid sequences of APX-proteins of the two pigeon pea genotypes were having 207 (ICPL 84023) and 206 (ICP 7035) amino acid residues, were highly homologous with each other (90 %) and also with the cowpea complete cytosolic *APX* (94 and 91 %), which has 250 amino acid residues in the corresponding region. Since the partial gene coding sequences obtained for tolerant and susceptible pigeon pea genotypes were almost 83 % of the complete cowpea *APX*-gene and also have 95 % homology with each other (and 92 % with cowpea), it would seem that the greater activity and gene expression level in case of APX of ICPL 84023 could primarily be due to some deficiency in the promoter region of the susceptible genotype (ICP 7035) or due to post trans-criptional modification of its gene.

From the results obtained it is clear that in spite of apparent oxygen deficiency during waterlogging pigeon pea plants suffer oxidative stress with increase in duration of exposure, though less than the control and recovered plants, and this oxidative stress was primarily due to increase in the gene expression and activity of DPI-sensitive NADPH oxidase. Tolerant genotype ICPL 84023 has better ROS scavenging system in terms of greater activity of SOD, APX, GR, and CAT, while the susceptibility of ICP 7035 can be attributed to its failure to augment the activity of important antioxidative enzymes during waterlogging stress. Secondly the greater ROS production during waterlogging treatment in tolerant genotype ICPL 84023, might have resulted in greater induction of antioxidant enzymes, which are required to scavenge not only the post hypoxic ROS build up, but also to detoxify ROS produced during hypoxia itself.

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