Waterlogging induced oxidative stress and antioxidant activity in pigeonpea genotypes

D. KUMUTHA, K. EZHILMATHI, R.K. SAIRAM*, G.C. SRIVASTAVA, P.S. DESHMUKH and R.C. MEENA

Division of Plant Physiology, Indian Agricultural Research Institute, New Delhi-110012, India

Abstract

The objective of this study was to examine the role of antioxidant enzymes in waterlogging tolerance of pigeonpea (*Cajanus cajan* L. Halls) genotypes ICP 301 (tolerant) and Pusa 207 (susceptible). Waterlogging resulted in visible yellowing and senescence of leaves, decrease in leaf area, dry matter, relative water content and chlorophyll content in leaves, and membrane stability index in roots and leaves. The decline in all parameters was greater in Pusa 207 than ICP 301. Oxidative stress in the form of superoxide radical, hydrogen peroxide and thiobarbituric acid reactive substances (TBARS) contents initially decreased, however at 4 and 6 d of waterlogging it increased over control plants, probably due to activation of DPI-sensitive NADPH-oxidase. Antioxidant enzymes such as superoxide dismutase, ascorbate peroxidase, glutathione reductase and catalase also increased under waterlogging. The comparatively greater antioxidant enzyme activities resulting in less oxidative stress in ICP 301 could be one of the factor determining its higher tolerance to flooding as compared to Pusa 207. This study is the first to conclusively prove that waterlogging induced increase in ROS is *via* NADPH oxidase.

Additional key words: anoxia, ascorbate peroxidase, *Cajanus cajan*, catalase, glutathione reductase, hydrogen peroxide, hypoxia, oxidative stress, 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, floods and excess of rainfall are examples of natural conditions leading to root hypoxia or anoxia. Low oxygen concentration can also be a normal attribute of a plants' natural environment. A decrease in adenylate energy charge, cytoplasmic acidification, anaerobic fermentation, elevation in cytosolic Ca^{2+} concentration, changes in the redox state 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, Vartapetian and Jackson 1997, Tadege *et al.* 1999). Regulation of anoxic metabolism is complex and not all the features are well established. For example De Carvalho *et al*. (2008) reported the significance of aerenchyma and adventitious roots formation.

Excessive generation of reactive oxygen species (ROS), or oxidative stress, is an integral part of many stress situations, including hypoxia. Post-hypoxic hydrogen peroxide accumulation has been shown in the roots and leaves of *Hordeum vulgare* (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 species (Blokhina *et al.* 2001). Indirect evidence of ROS formation such as TBARS contents (*i.e.* lipid peroxidation products) under low oxygen have been detected (Yan *et al.* 1996, Chirkova *et al.* 1998, Blokhina *et al.* 1999). Flooding treatment in *Zea mays* resulted in a significant increase in TBARS content, production of superoxide radical and hydrogen peroxide, and membrane permeability in the leaves (Yan *et al*. 1996). An excessive accumulation of superoxide due to the reduced activity of SOD under flooding stress has also been reported (Yan *et al.* 1996). To control the level of ROS and to protect cells under stress conditions, plant tissues contain several ROS scavenging enzymes, *e.g.*, superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidases (APX) and glutathione reductase (GR), enzymes detoxifying lipid peroxidation products (glutathione S-transferases,

Received 28 December 2006, *accepted* 18 August 2007.

Abbreviations: APX - ascorbate peroxidase; CAT - catalase; Chl - chlorophyll; DAA - days after anthesis; DPI - diphenylene iodonium chloride; GR - glutathione reductase, MSI - membrane stability index; ROS - reactive oxygen species; RWC - relative water content; SOD - superoxide dismutase; TBARS - thiobarbituric acid reactive substances.

* Corresponding author, fax: (+91) 11 25846420, e-mail: rks_ppl@yahoo.co.uk

phospholipid-hydroperoxide-glutathione peroxidase and ascorbate peroxidase), and a network of low molecular mass antioxidants (ascorbate, glutathione, phenolic compounds and α-tocopherol). Increase in the activity of antioxidant enzymes in response to various environmental stresses has also been reported in various studies (Elstner 1986, Bowler *et al.* 1992, Sairam and Srivastava 2001, Sairam *et al.* 2000, 2002). In *Iris pseudacorus* a 14-fold increase in SOD was observed during a reoxygenation period (Monk *et al.* 1989). Van Toai and Bolles (1991) demonstrated that high SOD activity may contribute to flooding tolerance by improving detoxification of superoxide upon re-admission of oxygen. Induction of enzymes involved in the ascorbate-dependent antioxidative system (APX, monodehydroascorbate reductase, MDHAR, dehydroascorbate reductase, DHAR and GR) has been shown for anaerobically germinated rice seedlings and roots of wheat (*Triticum aestivum*) seedlings after transfer to air (Ushimaru *et al.* 1997, Albrecht and Wiedenroth 1994). Sairam *et al*. (2008) have reviewed the significance of antioxidants in waterlogging tolerance in crop plants.

Pigeonpea is an important pulse crop, which is cultivated in Africa, Asia and Australia. Being a summer-rainy season crop (*kharif*), pigeonpea is exposed to waterlogging condition during germination and early vegetative growth phases. This is the crucial period, which determine the crop stand and ultimately crop growth and productivity. Singh *et al*. (1986) reported that seedling and early vegetative stages of pigeonpea are more sensitive to waterlogging stress than flowering stage. Our preliminary trials, done to screen tolerant and susceptible genotypes, indicated greater loss in chlorophyll, membrane stability index, relative water content (RWC), leaf area, dry matter and mortality when waterlogging treatment was given to 20 to 30-d-old crop than at flowering stage (un-published data).

The area of generation of oxidative stress and antioxidant enzymes activity during waterlogging/ hypoxia is still hazy. The present investigation, therefore, has been planned to study the waterlogging induced oxidative stress and antioxidant enzymes activity in tolerant and susceptible pigeonpea genotypes at early vegetative stage.

Materials and methods

A preliminary experiment was conducted with 13 pigeonpea (*Cajanus cajan* L. Halls) genotypes procured from *ICRISAT*, Hyderabad, India, and Division of Genetics, Indian Agricultural Research Institute, New Delhi, India, and based on relative water content, membrane stability index of leaf and root tissues and leaf chlorophyll contents, grouped into tolerant and susceptible to waterlogging stress. Subsequent studies were conducted with comparatively tolerant (ICP 301) and susceptible (Pusa 207) genotypes. Sowing was done in earthen pots of 30 × 30 cm, filled with clay-loam soil and farm yard manure in 3:1 ratio during the summer season. Pots were supplied with basal dose of 1 and 3.5 g of muriate of potash and single super phosphate, respectively per pot (containing about 10 kg air dried soil). Before sowing seeds were treated with the required *Rhizobium* culture. Initially four plants were sown in each pot, which were thinned to 2 plants per pot after 20 d. Waterlogging treatment was given by placing pots with 25-d-old plants in plastic troughs measuring 100 × 70 × 35 cm, filled with water to a height just 1 - 2 cm below the soil level in pots. Treatments consisted of control (S_0), 2 (S_2), 4 (S_4) and 6 (S_6) days of waterlogging, and recovery of 4-d waterlogged plants after 5 (R_5) and 10 (R_{10}) days of termination of treatment. Though some plants of susceptible genotype Pusa 207 survived up to 6 d of waterlogging, almost all of them died during recovery, therefore, recovery was uniformly studied in the 2 genotypes for 4 d waterlogged plants only. At each stage samples were collected in quadruplicate from 4 pots. Observations were recorded on the leaf area and dry matter per plant, relative water content (RWC), membrane stability index (MSI), chlorophyll (Chl) content, superoxide radical, hydrogen peroxide, lipid peroxidation as thiobarbituric acid reactive substances (TBARS) contents, superoxide dismutase activity and isozyme by native-PAGE, ascorbate peroxidase, glutathione reductase and catalase. In case of assay of ROS and antioxidant enzymes each sample was assayed twice (n = 8), while for leaf area, total dry matter production (TDMP), mortality, RWC, MSI and Chl content the number of observations were 4 only (n = 4). Except for RWC, Chl and leaf MSI all other observations were recorded in root tissues. The design of the experiment was complete randomized block design and data was analyzed by factorial RBD.

Leaf area was estimated by measuring the area of green leaves with the help of leaf area meter, model *Li 3100* (*LiCOR*, Lincoln, Nebraska, USA). For dry matter estimation, the above ground whole plant samples were dried at 65 °C in a hot air oven till constant masses were obtained. Leaf relative water content (RWC) was estimated by recording the water saturated mass of 0.5 g fresh leaf samples by keeping in water for 4 h, followed by drying in hot air oven till constant mass is achieved (Weatherley 1950).

For estimation of membrane stability index 100 mg leaf material, in two sets, was taken in test tubes containing 10 cm^3 of double distilled water (Sairam 1994). One set was heated at 40 °C for 30 min in a water bath, and the electrical conductivity of the solution was recorded on a conductivity bridge (C_1). Second set was boiled at 100 °C on a boiling water bath for 10 min, and its conductivity was measured on a conductivity bridge (C_2). Membrane stability index (MSI) is calculated as: $MSI = [1 - (C_1/C_2)] \times 100$.

Chlorophyll content was estimated by extracting 0.05 g of the leaf material in 10 cm^3 dimethylsulfoxide (DMSO) (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 were calculated (Arnon 1949).

Superoxide radical content was measured by its capacity to reduce nitro-blue tetrazolium and the absorption of end product was measured at 540 nm. Root tissue (1 g) was homogenized in pre-cooled phosphate buffer (0.2 M, pH 7.2) containing 1 mM diethyldithiocarbamate (to inhibit SOD) and with or without 10 μM diphenylene iodonium chloride (DPI, a specific inhibitor of NADPH oxidase was used to inhibit NADPH oxidase dependent $O_2^{\bullet-}$ generation). The homogenate was centrifuged at 6000 *g* for 5 min and supernatant was immediately used for the estimation of superoxide radical (Chaitanya and Naithani 1994). The reaction mixture contained 0.25 cm^3 leaf extract, 0.1 cm^3 NBT (2.25 mM), 0.05 cm^3 Na_2CO_3 (1.5 M), 0.1 cm^3 EDTA (3.0 mM), 0.2 cm^3 L-methionine (200 mM) 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. NADPH oxidase independent O_2^- production (DPI-insensitive) was obtained by addition of DPI (10 μM) in the extraction buffer. Difference of total and DPI-insensitive $O_2^{\bullet-}$ production gave the NADPH oxidase dependent (DPI-sensitive) $O_2^{\bullet-}$ production. Superoxide radical contents were calculated according to coefficient of absorbance 12.8 mM^{-1} cm^{-1}.

Hydrogen peroxide was estimated by forming titanium-hydroperoxide 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 10 000 *g* for 10 min in the *Beckman* centrifuge (Geneva, Switzerland) model *J2-21*. 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 (*Specord Bio-200*, *AnalytikJena*, Jena, Germany). Hydrogen peroxide contents were calculated by comparing with a calibration curve.

Level of lipid peroxidation was measured in terms of thiobarbituric acid reactive substances (TBARS) content (Heath and Packer 1968). Root sample (0.5 g) was homogenized in 10 cm^3 of 0.1 % trichloroacetic acid (TCA). The homogenate was centrifuged at 15 000 *g* for 15 min. To 1.0 cm^3 aliquot of the supernatant 4.0 cm^3 of 0.5 % thiobarbituric acid (TBA) in 20 % TCA was added. The mixture was heated at 95 °C for 30 min and then cooled in an ice bath. After centrifugation at 10 000 *g* for 10 min the absorbance of the supernatant was recorded at 532 nm. The TBARS content was calculated according to coefficient of absorbance 155 mM^{-1} cm^{-1}. The values for non-specific absorbance at 600 nm were subtracted.

Enzyme extract for superoxide dismutase, ascorbate peroxidase, glutathione reductase and catalase was prepared by first freezing root samples (1 g) in liquid nitrogen to prevent proteolytic activity 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 15 000 *g* and the supernatant was used.

Superoxide dismutase activity was estimated by following the enzyme mediated decrease in absorbance due to the formation of formazone by superoxide anion with nitro-blue tetrazolium chloride at 560 nm (Dhindsa *et al*. 1981). Three cm^3 of the reaction mixture contained 13 mM methionine, 25 mM nitro-blue tetrazolium chloride (NBT), 0.1 mM EDTA, 50 mM phosphate buffer (pH 7.8), 50 mM sodium carbonate and 0.1 cm^3 enzyme. Reaction was started by adding 2 μM (01. cm^3 of 60 μM) riboflavin and placing the tubes under two 15 W fluorescent lamps for 15 min. A complete reaction mixture with out enzyme, which gave the maximum colour, served as control. Reaction was stopped by switching off the light and putting the tubes into dark. A non-irradiated complete reaction mixture served as a blank. To distinguish SOD isoforms viz., Cu/Zn-SOD, Fe-SOD and Mn-SOD, the sensitivity of Cu/Zn-SOD to cyanide (3 mM), and Cu/Zn-SOD and Fe-SOD to hydrogen peroxide (5 mM) were used, whereas Mn-SOD is unaffected (Yu and Rengel 1999). Separate controls (lacking enzymes) were used for total SOD and inhibitor studies. The absorbance was recorded at 560 nm, and 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.

Ascorbate peroxidase was assayed by recording the decrease in absorbance due to ascorbic acid oxidation 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 hydrogen peroxide. Absorbance was measured at 290 nm.

Glutathione reductase (GR) 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, 666.67 μM GSSG, 0.1 cm^3 of enzyme extract. Reaction was started by adding 20.0 mM GSSG (oxidized glutathione). The increase in absorbance at 412 nm is recorded spectrophotometrically.

Catalase (CAT) was assayed by monitoring the decrease in absorbance due to hydrogen peroxide at 240 nm (Aebi 1984). The 3.0 cm^3 reaction mixture consisted of potassium phosphate buffer 50 mM,

hydrogen peroxide 12.5 mM, enzyme 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 of known concentrations of hydrogen peroxide.

SOD isozyme separation was done on native-PAGE. Samples containing equal amount of protein (200 μg) were subjected to non-denaturating PAGE, but without SDS (Laemmli 1970). For SOD samples were run at 4 °C in a 7.5 % resolving gel and 6 % stacking gel for 100 min at 300 V. Activity staining following electrophoresis was done with nitro-blue tetrazolium chloride (Beauchamp and Fridovich 1971 and Sandalio *et al.* 1987).

Results

Waterlogging resulted in yellowing and ultimately drying of leaves and death of shoots/branches, more in Pusa 207 than ICP 301. After 6 d plants of Pusa 207 were almost dead, while ICP 301 still showed green leaves (Fig. 1). Leaf area decreased under waterlogging in the two genotypes, and the greatest reduction was recorded on the 6th day of treatment (Table 1). Greater decline in leaf area was observed in Pusa 207 than ICP 301. The decline on 2nd, 4th and 6th day was 26.03, 52.75, 66.48 % and 3.64, 14.37, 20.18 % in Pusa 207 and ICP 301, respectively. Recovery studied for 4 d waterlogged plants was better in ICP 301 than Pusa 207, as decline over control at 5 and 10 d after recovery was 20.18, 8.0 % and 67.72, 52.15 % in ICP 301 and Pusa 207, respectively.

Total dry matter recorded in control and waterlogged plants also decreased at different durations of waterlogging, and greater reduction over control was recorded in Pusa 207 than ICP 301 (Table 1). The decline

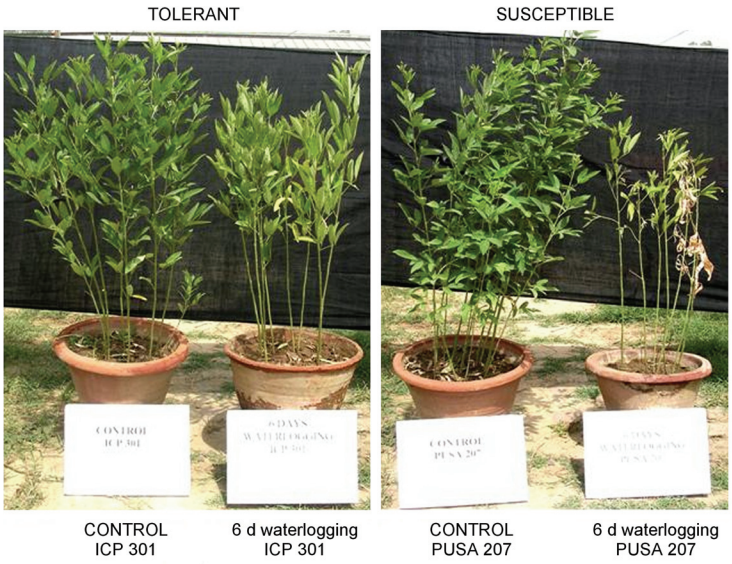

Fig. 1. Effect of waterlogging (6 d) on the growth of pigeonpea genotypes.

Table 1. Effect of waterlogging on leaf area and dry mass at various stages of waterlogging (2, 4 and 6 d) and recovery (5 and 10 d) in pigeonpea genotypes. Crop recovery was studied in 4-d waterlogged plants as susceptible genotypes failed to recover after 6 d of flooding. Means ± SE, $n = 4$. C - control, T - treated.

Parameter	Genotype		S2	S4	S6	R5	R10
Leaf area [cm^2 $plant^{-1}$]	Pusa 207	C	166.7 ± 5.74	189.0 ± 6.75	210.4 ± 4.89	284.2 ± 5.99	311.8 ± 9.46
		T	123.3 ± 8.99	89.3 ± 7.14	70.5 ± 4.17	91.7 ± 3.61	149.2 ± 5.37
	ICP 301	C	209.5 ± 7.70	237.8 ± 9.89	258.5 ± 10.69	336.0 ± 8.56	356.2 ± 8.92
		T	201.9 ± 9.21	203.6 ± 9.97	182.7 ± 9.09	268.2 ± 7.87	327.7 ± 6.20
Dry mass [g $plant^{-1}$]	Pusa 207	C	2.75 ± 0.066	2.94 ± 0.042	3.17 ± 0.108	3.77 ± 0.086	4.55 ± 0.091
		T	1.63 ± 0.042	1.60 ± 0.032	1.49 ± 0.039	2.32 ± 0.039	3.43 ± 0.042
	ICP 301	C	2.96 ± 0.075	3.18 ± 0.045	3.43 ± 0.037	4.05 ± 0.061	5.08 ± 0.096
		T	2.93 ± 0.038	2.91 ± 0.036	2.78 ± 0.109	3.97 ± 0.036	4.89 ± 0.048

on 2nd, 4th and 6th day was 40.73, 45.58, 53.00 % and 1.01, 8.49, 18.94 % in Pusa 207 and ICP 301, respectively. Recovery was better in ICP 301 than Pusa 207, as decline over control at 5 and 10 d after recovery was 1.97, 3.74 % and 38.46, 24.61 % in ICP 301 and Pusa 207, respectively.

ICP 301 did not show any mortality (%) during recovery even after 6 d of waterlogging, while in case of Pusa 207 mortality during recovery was 29.17 and 95.83 % in 4 and 6 d waterlogged plants.

There was significant decline in RWC in both the pigeonpea genotypes under the waterlogging treatment as compared to control (Fig. 2*A*). However, ICP 301 maintained higher RWC even after 6 d of waterlogging. Pusa 207 suffered a sharp decline in RWC, and the 6 d of waterlogging caused RWC to decline beyond the level of recovery. ICP 301 recovered faster and the RWC was comparable to that of control at 5 d after recovery.

Membrane stability index (MSI) in the roots (Fig. 2*B*) and leaves (Fig. 2*C*) decreased under waterlogging with greater decline in Pusa 207, while ICP 301 managed to maintain higher MSI even after 6 d of waterlogging. Pusa 207 showed slower recovery than ICP 301.

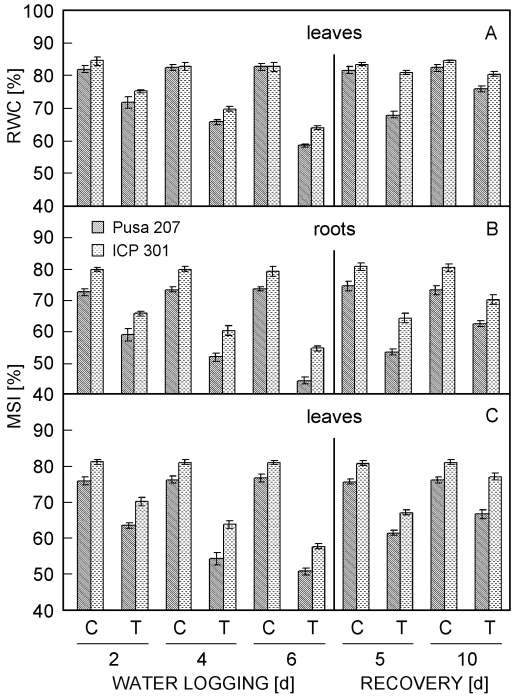

Fig. 2. Effect of waterlogging on leaf relative water content (*A*), membrane stability index of root tissue (*B*) and membrane stability index of leaf tissue (*C*) in pigeonpea genotypes. Means ± SE, *n* = 4. C - control, T - treatment.

Total chlorophyll (Chl) content drastically decreased after 6 d of waterlogging, recording a decrease of 56 % in Pusa 207 and 49 % in ICP 301 (Fig. 3*A*). ICP 301 showed better and quicker recovery than Pusa 207. There was gradual increase in the Chl *a*/*b* ratio during waterlogging in both the genotypes (Fig. 3*B*). The increase in chlorophyll *a*/*b* ratio was 39 % in Pusa 207and 21 % in ICP 301. Chl *a*/*b* ratio reached control level during recovery in ICP 301, while not in Pusa 207 even after 10 d of recovery.

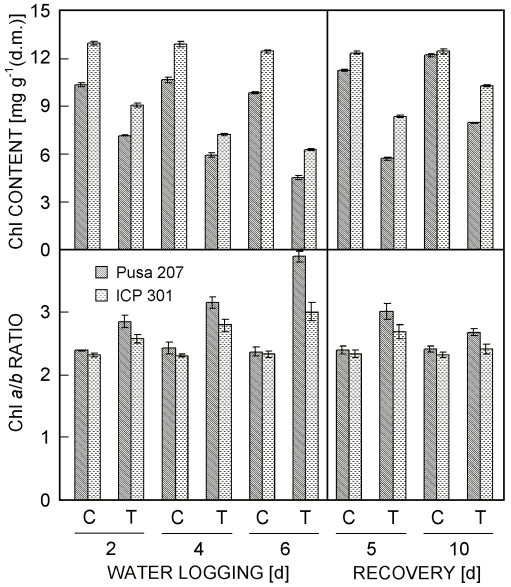

Fig. 3 Effect of waterlogging on chlorophyll content (*A*) and chlorophyll *a*/*b* ratio (*B*) in leaf tissue in pigeonpea genotypes.. Means ± SE, *n* = 4. C - control, T - treatment.

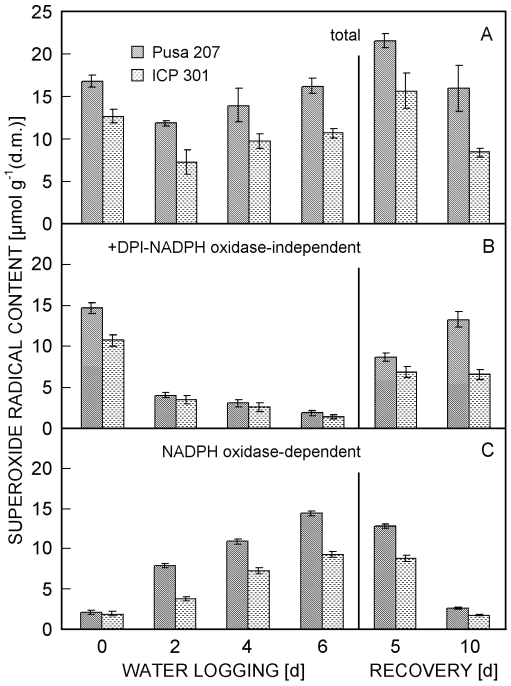

Fig. 4. Effect of waterlogging on total superoxide radical (*A*), superoxide radical in the presence of DPI (*B*) and DPI-inhibitable NADPH oxidase dependent superoxide radical (*C*) generation in the root tissue in pigeonpea genotypes. Means ± SE, *n* = 8.

Two days after waterlogging total superoxide radical production declined over control plants in both the genotypes (Fig. 4*A*). However, continuous waterlogging upto 6 d resulted in significantly higher total $O_2^{\bullet-}$ production in Pusa 207, where the contents were 53 % higher than control level, and more than double than observed on 2nd day of waterlogging. In ICP 301 the $O_2^{\bullet-}$ production on 6th day of waterlogging was just equal to that observed in control plants, and 60 % higher than observed on 2nd day of waterlogging. Recovery resulted in further increase in $O_2^{\bullet-}$ production and Pusa 207 maintained higher $O_2^{\bullet-}$ production, while it decreased in ICP 301 on 10th day of recovery to the control level. Superoxide radical content in the presence of DPI decreased with waterlogging, reaching a minimum value on 6th day (Fig. 4*B*) and increased after the recovery. The DPI-sensitive $O_2^{\bullet-}$ generation was lowest in control plants and increased under waterlogging, reaching a peak on 6th day (Fig. 4*C*). With the onset of recovery the DPI-sensitive $O_2^{\bullet-}$ content declined. DPI-insensitive and DPI-sensitive $O_2^{\bullet-}$ content were greater in Pusa 207 than in ICP 301. Hydrogen peroxide and TBARS content (Fig. 5) followed a pattern similar to total superoxide content.

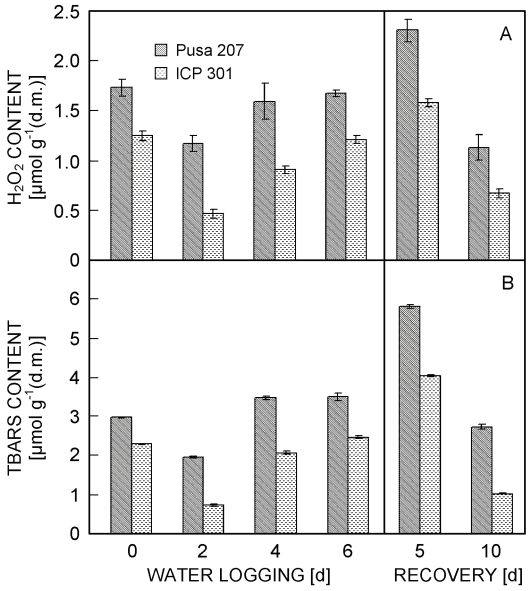

Fig. 5. Effect of waterlogging on hydrogen peroxide contents (*A*) and lipid peroxidation (TBARS contents; *B*) in the root tissue in pigeonpea genotypes. Means ± SE, $n = 8$.

Waterlogging caused increase in the activity of SOD and its isozymes in ICP 301 upto 6th day of stress, while in Pusa 207 a slight increase was observed only on 2nd day of waterlogging and thereafter the activity decreased with duration of stress, going down to almost 20 % of its maximum value recorded at 2 d waterlogging (Fig. 6*A*). During recovery the SOD activity decreased in ICP 301 and increased in Pusa 207, however, the activity in ICP 301 was 80 % higher than that of Pusa 207. Waterlogging induced increase in total SOD activity in ICP 301 was mainly due to the increase in Cu/Zn-SOD, which contributed to about 49 % at 2 d of waterlogging and 45 % at 6 d of waterlogging. Mn-SOD accounted for about 30 % at 2 d and 28 % at 6 d of waterlogging. Fe-SOD had lesser role with its contribution of only 20 % to the total SOD activity. In Pusa 207, all the isoforms of SOD were greatly reduced at 4 and 6 d of waterlogging (Fig. 6*B-D*).

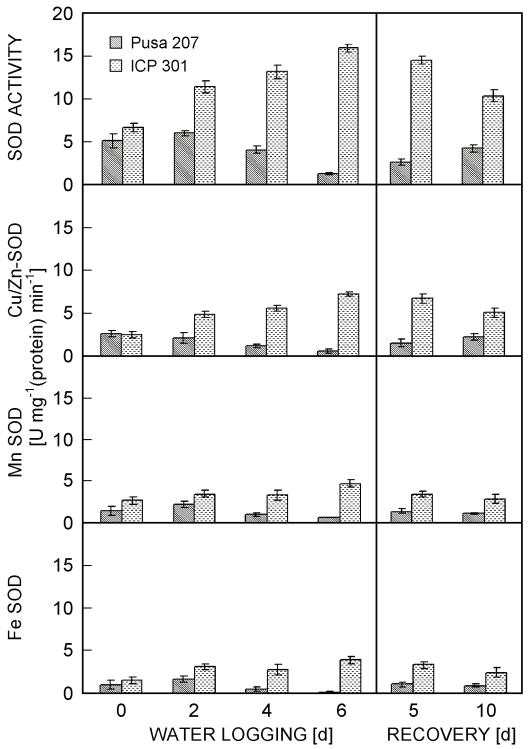

Fig. 6. Effect of waterlogging on the activity of total superoxide dismutase (SOD, *A*), Cu/Zn-SOD (*B*), Mn-SOD (*C*) and Fe-SOD (*D*) in the root tissue in pigeonpea genotypes. Means ± SE, $n = 8$.

The zymogram of SOD (Fig. 7) clearly showed 5 rows of bands, named SOD 1, 2, 3, 4 and 5. The upper row is most probably the high molecular mass isoform Mn-SOD. This band was present both in control as well as treated plants of the both the genotypes, but was more prominent in treated plants than control. Control plants of Pusa 207 showed prominently SOD 1, SOD 3, SOD 5 and a light band of SOD 4. In the 6-day waterlogged plants of Pusa 207 most of the bands disappeared, except for SOD 1 and very light SOD 2. In case of ICP 301 SOD 3, 4 and 5 were prominently expressed in control plants, while under waterlogged conditions all the bands (SOD 1, 2, 3, 4, 5) were highly expressed.

There was significant increase in APX activity at 2 d of waterlogging in all the genotypes (Fig. 8*A*), however, the increase was much higher in ICP 301. Further stress *viz*., 4 and 6 d of waterlogging resulted in decline in APX activity in Pusa 207, while increase in ICP 301. During

recovery APX activity increased in Pusa 207, however, the activity was significantly lower than in control plants. ICP 301 registered an increase in APX activity after 5-d recovery, which decreased after 10 d, though the values were higher than in Pusa 207.

Glutathione reductase (GR) and catalase (CAT) activities increased upto 2 d of waterlogging in both the genotypes (Fig. 8*B*,*C*). Further waterlogging resulted in decrease in GR and CAT activity in Pusa 207, while ICP 301 recorded continuous increase upto 6th day of waterlogging. During recovery, activities of GR and CAT increased in Pusa 207, but the values were still lower than in control plants. Though ICP 301 showed decline in GR and CAT activity during recovery, the values were greater than in control plants.

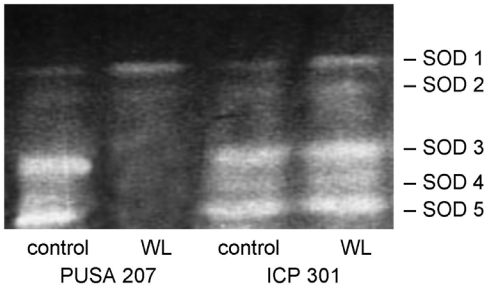

Fig. 7. Native PAGE expression of SOD isozymes in control and 4 d waterlogged (WL) susceptible (Pusa 207) and tolerant (ICP 301) pigeonpea genotypes.

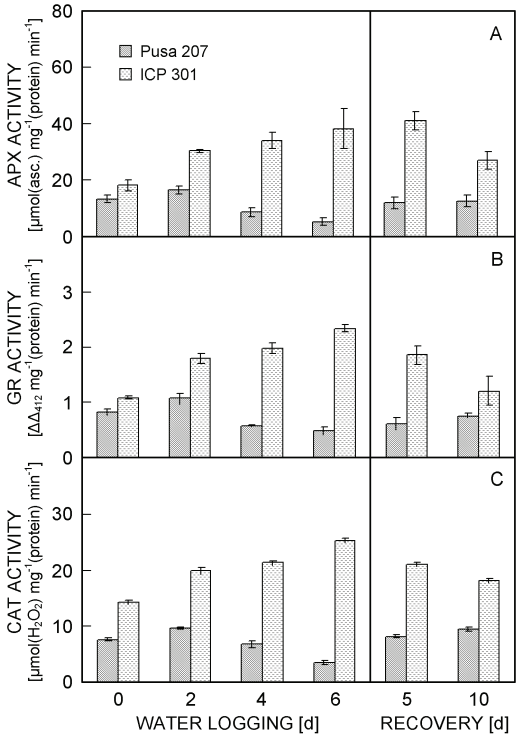

Fig. 8. Effect of waterlogging on the activity of (A) ascorbate peroxidase, (B) glutathione reductase and (C) catalase in the root tissue in pigeonpea genotypes. Means ± SE, n = 8.

Discussion

In tropical and subtropical regions, severe crop losses are caused by prolonged seasonal rainfall. Excess water produces anoxic soil condition within a few hours (Gambrell and Patrick 1978). Plant roots, consequently, suffer hypoxia or anoxia. As a result of 6 d of waterlogging pigeonpea genotypes suffered a sever loss in leaf area and dry matter per plant in both the genotypes, though the loss was more in susceptible genotype Pusa 207, which also suffered 96 % mortality during recovery. At physiological level 6 d of waterlogging caused a more severe decrease in RWC, MSI (higher membrane injury), both in roots and leaves and Chl contents in Pusa 207 compared to ICP 301. Min and Barthalomew (2005) reported gradual decrease in RWC during flooding. Plants visually wilt sometimes within a few hours of imposing a flooding stress (Jackson and Drew 1984) due to the lower root hydraulic conductance (Kramer and Jackson 1954). Various workers 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 more than 40 times increase in solute leakage from 4 d waterlogged pea plants. Collaku and Harrison (2002) also reported a decrease in Chl content in waterlogged wheat plants. Sorte *et al.* (1996) reported that 4 and 8 d of waterlogging in soybean caused reduction in Chl content. The increase in Chl content during recovery showed restoration of photosynthetic machinery once the 4-d waterlogged plants were provided normal aerobic environment. ICP 301 presumably has better survival mechanism, as it maintained greater RWC, MSI and Chl content under waterlogging stress. Increase in Chl *a*/*b* ratio, under declining total Chl, points to greater loss of Chl *b* under waterlogging condition, and more so in Pusa 207.

A very striking observation under waterlogging is the production of various ROS, especially superoxide radical and hydrogen peroxide, leading to an increase in lipid peroxidation. Though compared to control or untreated plants, 2 d waterlogged plants showed a decrease in production of $O_2^{\bullet -}$, H_2O_2 and TBARS content, on 4th and 6th day there was significant increase in the contents of ROS and TBARS. Pusa 207 showed a higher oxidative stress as compared to ICP 301, where the contents of the two ROS were less than 50 % of the Pusa 207 on the 6th day of treatment. The reduction in ROS production on 2nd day of waterlogging could be due to a shift from aerobic respiration to fermentation, as in non-green tissues mitochondrial electron transfer chain is the main 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. In contrast NADPH oxidase-independent (DPI-insensitive) $O_2^{\bullet-}$ generation declined under waterlogging. The NADPH oxidase-dependent $O_2^{\bullet-}$ generation, which was minimum under aerobic condition increased significantly under waterlogging stress. Blokhina *et al.* (2001) reported H_2O_2 formation during anoxia by an enzymatic process, as evidenced by electron dense insoluble precipitate of cerium perhydroxide. 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 have facilitated reduction of dissolved oxygen to superoxide and lower activity of superoxide scavenging enzyme, *i.e*., SOD, as observed in susceptible genotype, where ROS production was greater. Further the production of ROS under waterlogging can be confirmed by the enhanced lipid peroxidation in terms of TBARS content (Crawford *et al.* 1994, Chirkova *et al.* 1998).

Results observed on various antioxidative enzymes like SOD, APX, GR and CAT under waterlogged condition in tolerant and susceptible pigeonpea genotypes reveal a continuous increase in all the 4 enzymes upto 6 d of waterlogging in ICP 301 (tolerant genotype), while in case of Pusa 207 (susceptible genotype) the increase in antioxidative enzymes were noticed only in 2-d water-logged plants and at subsequent stages there was decline in activity of all the antioxidant enzymes as compared to control and 2 d waterlogged plants. It has been suggested by various workers that the reason for the increase in antioxidative enzyme activities during waterlogging is primarily to take care of posthypoxia oxidative stress. Monk *et al.* (1987) observed a continuous increase in SOD activity in rhizomes of *Iris pseudacorus* under waterlogging stress. The results obtained from this study and the study of Blokhina *et al.* (2001) suggested that there indeed is an increase in oxidative stress during waterlogging, and the increase in antioxidative enzymes were to scavenge build up ROS. The plants can also suffer by ROS production when they are returned to aerobic condition and this explains overall higher antioxidant enzymes activity in tolerant genotype ICP 301 not only during waterlogging but also during recovery as compared to control plants, and also compared to susceptible Pusa 207. The increase in activity of various antioxidative enzymes under waterlogging have also been reported by other workers, viz., SOD (Monk *et al.* 1987, VanToai and Bolles 1991, Biemelt *et al*. 2000), APX (Ushimaru *et al*. 1997, Biemelt *et al*. 1998), GR (Albrecht and Wiedenroth, 1994, Ushimaru *et al*. 1997) and CAT (Ushimaru *et al.* 1997).

ROS produced during hypoxia (*e.g*. H_2O_2) can also serve signal not only for the induction of antioxidant enzymes (Agarwal *et al.* 2005), but also for alcohol dehydrogenase, which is involved in the regeneration of NAD, and consequently the continuation of glycolysis and energy supply during hypoxia (Fukao and Bailey-Serres 2004).

From the results obtained it is clear that pigeonpea plants sufferred oxidative stress during waterlogging, which increased with duration of exposure, and we experimentally established that this oxidative stress was primarily due to increase in the activity of DPI-inhibitable-NADPH oxidase. Tolerant genotype ICP 301 has better ROS scavenging system in terms of greater activity of SOD, APX, GR, and CAT, while the susceptibility of Pusa 207 can be attributed to its failure to augment the activity of important antioxidative enzymes during waterlogging stress. Secondly the increase in the activity of antioxidant enzymes during waterlogging is required to scavenge not only the post hypoxic ROS build up, but also to detoxify cellular system of ROS produced during hypoxia itself.

References

Aebi, H.: Catalase *in vitro*. - Methods Enzymol. **105**: 121-126, 1984.

Agarwal, S., Sairam, R.K., Srivastava, G.C., Tyagi, A., Meena, R.C.: Role of ABA, salicylic acid, calcium and hydrogen peroxide on antioxidant enzymes induction in wheat seedlings. - Plant Sci. **169**: 559-570, 2005.

Albrecht, G., Wiedenroth, E.M.: Protection against activated oxygen following re-aeration of hypoxically pre-treated wheat roots. The response of the glutathione system. - J. exp. Bot. **45**: 449-455, 1994.

Arnon, D.I.: Copper enzymes in isolated chloroplasts. Polyphenol oxidase in *Beta vulgaris*. - Plant Physiol. **24**: 1-15, 1949.

Beauchamp, C., Fridovich, I.: Superoxide dismutase. Improved assays and an assay applicable to acrylamide gels. - Anal. Biochem. **44**: 276-287, 1971.

Biemelt, S., Keetman, U. Albrecht, G.: Re-aeration following hypoxia or anoxia leads to activation of the antioxidative defense system in roots of wheat seedlings. - Plant Physiol. **116**: 651-658, 1998.

Biemelt, S., Keetman, U., Mock, H.P., Grimm, B.: Expression and activity of isoenzymes of superoxide dismutase in wheat roots in response to hypoxia and anoxia. - Plant Cell Environ. **23**: 135-144, 2000.

Blokhina, O.B., Chirkova, T.V., Fagerstedt, K.V.: Anoxic stress leads to hydrogen peroxide formation in plant cells. - J. exp. Bot. **52**: 1-12, 2001.

Blokhina, O.B., Fagerstedt, K.V., Chirkova, T.V.: Relationships between lipid peroxidation and anoxia tolerance in a range of species during post-anoxic reaeration. - Physiol. Plant. **105**: 625-632, 1999.

Bowler, C., Montague, M.V., Inze, D.: Superoxide dismutase and stress tolerance. - Annu. Rev. Plant Physiol. Plant mol. Biol. **43**: 83-116, 1992.

Chaitanya, K.S.K., Naithani, S.C.: Role of superoxide, lipid peroxidation and superoxide dismutase in membrane perturbation during loss of viability in seeds of *Shorea robusta* Gaertnf. - New Phytol. **126**: 623-627, 1994.

Chirkova, T.V., Novitskaya, L.O., Blokhina, O.B.: Lipid peroxidation and antioxidant systems under anoxia in plants

differing in their tolerance to oxygen deficiency. - Russ. J. Plant Physiol. **45**: 55-62, 1998.

Collaku, A., Harrison, S.A.: Loses in wheat due to waterlogging. - Crop Sci. **42**: 444-450, 2002.

Crawford, R.M.M., Braendle, R.: Oxygen deprivation stress in a changing environment. - J. exp. Bot. **47**: 145-159, 1996.

Crawford, R.M.M., Walton, J.C., Wollenweber-Ratzer, B.: Similarities between post-ischaemic injury to animal tissues and post anoxic injury in plants. - Proc. roy. Soc. Edinburgh **102B**: 325-332, 1994.

De Carvalho, M.C.C.G., Da Silva, D.C.G., Ruas, P.M., Medri, M.E., Ruas, E.A., Ruas, C.F.: Flooding tolerance and genetic diversity in populations of *Luehea divaricata*. - Biol. Plant. **52**: 771-774, 2008.

Dhindsa, R.A., Plumb-Dhindsa, P., Thorpe, T.A.: Leaf senescence correlated with increased permeability and lipid peroxidation, and decreased levels of superoxide dismutase and catalase. - J. exp. Bot. **126**: 93-101, 1981.

Drew, MC.: Oxygen deficiency and root metabolism: injury and acclimation under hypoxia and anoxia. - Annu. Rev. Plant Physiol. Plant mol. Biol. **48**: 223-250, 1997.

Else, M.A., Davies, W.S., Malone, M., Jackson, M.S.: A negative hydraulic message from oxygen-deficient roots of tomato plant? - Plant Physiol. **109**: 1017-1024, 1995.

Elstner, E.F.: Metabolism of activated oxygen species. - In: Davies, D.D. (ed.): The Biochemistry of Plants. Biochemistry of Metabolism. Vol. 11. Pp. 253-315. Academic Press, San Diego 1986.

Fukao, T., Bailey-Serres, J.: Plant responses to hypoxia is survival a balancing act. - Trends Plant Sci. **9**: 449-456, 2004.

Gambrell, R.P., Patrick, W.H.: Chemical and microbiological properties of anaerobic soils and sediments. - In: Hook, D.D., Crawford, R.M.M. (ed.): Plant Life in Anaerobic Environments. Pp. 375-423. Ann Arbor Scientific Publications, Ann Arbor 1978.

Heath, R.L., Packer, L.: Photoperoxidation in isolated chloroplast. I. Kinetics and stoichiometry of fatty acid peroxidation. - Arch. Biochem. Biophys. **125**: 189-198, 1968.

Hiscox, J.D., Israelstam, G.F.: A method for extraction of chloroplast from leaf tissue without maceration. - Can. J. Bot. **57**: 1332-1334, 1979.

Jackson, M.B., Herman, B. Goodenogh, A.: An examination of the importance of ethanol in causing injury to flooded plants. - Plant Cell Environ. **5**: 163-172, 1982.

Jackson, M.B., Drew, M.C.: Effects of flooding on growth and metabolism of herbaceous plants. - In: Kozlowski, T.T. (ed.): Flooding and Plant Growth. Pp. 47-128. Academic Press, Orlando 1984.

Kalashnikov, Yu.E., Balakhnina, T.I., Zakrzhevsky, D.A.: Effect of soil hypoxia on activation of oxygen and the system of protection from oxidative destruction in roots and leaves of *Hordeum vulgare*. - Russ. J. Plant Physiol. **41**: 583-588, 1994.

Kramer, P.J., Jackson, W.T.: Causes of injury to flooded tobacco plants. - Plant Physiol. **29**: 241-245, 1954.

Laemmli, U.K.: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. - Nature **227**: 680-685, 1970.

Min, X.J., Bartholomew, D.P.: Effects of flooding and drought on ethylene metabolism, titratable acidity and fruiting of pineapple. - Acta Hort. **666**: 135-148, 2005.

Monk, L.S., Fagerstedt, K.V., Crawford, R.M.M.: Superoxide dismutase as an anaerobic polypeptide - a key factor in recovery from oxygen deprivation in *Iris pseudacorus*? - Plant Physiol. **85**: 1016-1020, 1987.

Monk, L.S., Fagerstedt, K.V., Crawford, R.M.M.: Oxygen toxicity and superoxide dismutase as an antioxidant in physiological stress. - Physiol Plant. **76**: 456-459, 1989.

Naidoo, G.: Effects of flooding on leaf water potential and stomatal resistance in *Bruguiera gymporrhiza* (L.) Lam. - New Phytol. **93**: 369-376, 1983.

Nakano, Y., Asada, K.: Hydrogen peroxide is scavenged by ascorbate specific peroxidase in spinach chloroplasts. - Plant Cell Physiol. **22**: 867-880, 1981.

Oberson, J., Pavelic, D., Braendle, R., Rawler, A.: Nitrate increases membrane stability of potato cells under anoxia. - J. Plant Physiol. **155**: 792-794, 1999.

Rao, M.V., Paliyath, G., Ormrod, D.P. Murr, D.P., Watkins, C.B.: Influence of salicylic acid on H_2O_2 production, oxidative stress and H_2O_2 metabolizing enzymes. - Plant Physiol. **115**: 137-149, 1997.

Rawyler, A., Arpagaus, S., Braendle, R.: Impact of oxygen stress and energy availability on membrance stability of plant cells. - Ann. Bot. **90**: 499-507, 2002.

Richard, B., Couce, I., Raymond, P., Saglio, P.H., Saint-Ges, V., Pradet, A.: Plant metabolism under hypoxia and anoxia. - Plant Physiol. Biochem. 32: 1-10, 1994.

Sairam, R.K.: Effect of moisture stress on physiological activities of two contrasting wheat genotypes. - Indian J. exp. Biol. **32**: 594-593, 1994.

Sairam, R.K., Kumutha, D., Ezhilmathi, K., Deshmukh, P.S., Srivastava, G.C.: Physiology and biochemistry of waterlogging tolerance in plants. - Biol. Plant. **52**: 401-412, 2008.

Sairam, R.K., Srivastava, G.C.: Water stress tolerance of wheat (*Triticum aestivum* L.): Variations in hydrogen peroxide accumulation and antioxidant activity in tolerant and susceptible genotypes. - J. Agron. Crop Sci. **186**: 63-70, 2001.

Sairam, R.K., Rao, K.V., Srivastava, G.C.: Differential response of wheat genotypes to long term salinity stress in relation to oxidative stress, antioxidant activity and osmolyte concentration. - Plant Sci. **163**: 1037-1046, 2002.

Sairam, R.K., Srivastava, G.C., Saxena, D.C.: Increased antioxidant activity under elevated temperatures: a mechanism of heat stress tolerance in wheat genotypes. - Biol. Plant. **43**: 245-251, 2000.

Sandalio, L.M., Palma, P.M., Del-Rio, L.A.: Localization of manganese superoxide dismutase in peroxisomes isolated from *Pisum sativum* L. - Plant Sci. **51**: 1-8, 1987.

Singh, K., Sharma, S.P., Singh, T.K., Singh, Y.: Effect of waterlogging on growth, yield and nutrient concentration of black gram and green gram under subtropical condition of Varanasi. - Ann. agr. Res. **7**: 169-177, 1986.

Smith, I.K., Vierheller, T.L., Thorne, C.A.: Assay of glutathione reductase in crude tissue homogenates using 5, 5'-dithiobis (2-nitrobenzoic acid). - Anal. Biochem. **175**: 408-413, 1988.

Sorte, N.V., Deotah, R.D., Meshram, J.H., Chanekar, M.A.: Tolerance of soybean cultivars of waterlogging at various growth states. - J. Soil Crops **6**: 68-72, 1996.

Tadege, M., Dupuis, I., Kuhlemeier, C.: Ethanolic fermentation: new functions for an old pathway. - Trends Plant Sci. **4**: 320-325, 1999.

Ushimaru, T., Maki, Y., Sano, S., Koshiba, K., Asada, K., Tsuji, H.: Induction of enzymes involved in the ascorbate-dependent antioxidative system, namely ascorbate peroxidase, mono dehydroascorbate reductase and dehydroascorbate reductase, after exposure to air of rice (Oryza sativa) seedlings germinated under water. - Plant Cell

Physiol. **38**: 541-549, 1997.
Van Toai, T.T., Bolles, C.S.: Postanoxic injury in soybean (*Glycine max*) seedlings. - Plant Physiol. **97**: 588-592, 1991.
Vartapetian, B.B., Jackson, M.B.: Plant adaptations to anaerobic stress. - Ann. Bot. **79** (Suppl. A): 3-20, 1997.
Weatherley, P.E.: Studies in the water relations of cotton plants. I. The field measurement of water deficit in leaves. - New Phytol. **49**: 81-97, 1950.
Yan, B., Dai, Q., Liu, X., Huang, S., Wang, Z.: Flooding-induced membrane damage, lipid oxidation and activated oxygen generation in corn leaves. - Plant Soil **179**: 261-268, 1996.
Yu, Q., Rengel, Z.: Drought and salinity differentially influence activities of superoxide dismutase in narrow-leafed lupines. - Plant Sci. **142**: 1-11, 1999.

Campbell, A., Jones, E.W., Schupbach, G. (ed.): **Annual Review of Genetics. Vol. 42.** - Annual Reviews Inc., Palo Alto 2008, 772 pp. ISBN 978-0-8243-1242-8

The Introductory chapters deal with the history of population genetics (J.F. Crow) and with the work of the Nobel prize winner Joshua Lederberg (L. Herzenberg *et al*.). The readers of Biologia Plantarum may find interesting the reviews: Molecular determinants of a symbiotic chronic infection (K.E. Gibson *et al*.), Evolutionary genetics of genome merger and doubling in plants (J.J. Doyle *et al.*), The dynamics of photosynthesis (S. Eberhard *et al*.), and Genomic insights into marine microalgae (M.S. Parker *et al*.).

Other reviews covered in this Volume: How *Saccharomyces* responds to nutrients, Silica biomineralization in Diatoms, Multicellular patterns in *Myxococcus xanthus,* Future of mouse, QTL mapping, Retroviral restriction factors, Heritable symbionts of insects, Rhomboid proteases and their biological functions, Organization of the bacterial genome, The origins of multicellularity, Individuality in bacteria, Transposon Tn5, Selection on codon bias, How shelterin protects mammalian telomeres, Design features of a mitotic spindle, Genetics of sleep, Cleavage plane in *Caenorbabditis elegans*, Planar cell polarity signaling, Quorum sensing in *Staphylococci*, Evolution of vertebrates sex chromosomes, Retrotransposable elements and their hosts, The bacteriophage DNA packaging motor, Genetics and cell biology of *Wolbachia*-host interaction, Retroviral genomic effects, and Mammalian dosage compensation.

Annual Reviews is a nonprofit scientific publisher, so that the Annual Reviews are reasonably priced. Current individual subscriptions include online access to full-text articles, PDFs, reviews in advance (as much as 6 months ahead of print publication), bibliographies, and other supplementary material in the current volume and the prior 4 year´s volumes. All articles are written by the foremost experts in the field.

The Annual Review of Genetics is online at http://genet.annualreviews.org

T. GICHNER (*Praha*)