Ultraviolet-B radiation (280-315 nm) invoked antioxidant defence systems in *Vigna unguiculata* (L.) Walp. and *Crotalaria juncea* L.

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Abstract

A crop legume *Vigna unguiculata* L. (Walp.) and a wild legume *Crotalaria juncea* L. were evaluated for their relative responses to the oxidative stress injury induced by various doses of UV-B radiation (UV-B, 280–315 nm; 0, 1.0, 1.4, 4.7, and 6.0 kJ m^{-2} d^{-1}). A dose-dependent damage in lipid peroxidation was determined as an index of membrane injury caused by UV-B. The impact was significantly higher in *V. unguiculata* than in *C. juncea*. The specific activities of superoxide dismutase, ascorbate peroxidase, monodehydroascorbate reductase, and dehydroascorbate reductase increased directly proportional to UV-B doses. However, the activities of these enzymes were significantly higher in *V. unguiculata* than in *C. juncea* indicating that *V. unguiculata* was inflicted with more severe oxidative stress injury under UV-B. In *C. juncea* the glutathione reductase and ascorbate oxidase activities were 35 and 40 % greater than in *V. unguiculata*, respectively. Further, the non-enzymatic antioxidants ascorbate and glutathione, and their reduced/ oxidizes ratios in *C. juncea* were much greater than *V. unguiculata* indicating *C. juncea* has an inherently greater anti-oxidative potential than *V. unguiculata*. Thus *C. juncea* is better adapted to oxidative stress than *V. unguiculata* by means of efficient cellular antioxidant mechanisms helping to combat the photooxidative stress injury elicited by UV-B.

Additional key words: ascorbate-glutathione cycle; ascorbate oxidase and peroxidase; catalase; dehydroascorbate reductase; glutathione transferase; monodehydroascorbate reductase; oxidative stress; peroxidase; superoxide dismutase.

Introduction

Recent measurements of ozone concentrations show that the stratospheric ozone layer is being depleted as a result of indiscriminate release of anthropogenic pollutants such as the chlorofluorocarbons and other ozone antagonists into the atmosphere. As a consequence, the increased ultraviolet-B irradiation (UV-B, 280–315 nm) is currently penetrating the biosphere (Kerr and McElroy 1993, Russell *et al*. 1996). UV-B inhibits a variety of growth and metabolic processes in crop plants diminishing agri-cultural productivity (Caldwell *et al*. 1986, Balakumar *et al*. 1999a). While the metabolic target sites of UV-B radiation in plants have been well documented (Renger *et al*. 1989, Balakumar *et al*. 1999a,b), the mechanisms of oxidative stress injury caused by UV-B radiation in plants have not been elucidated in detail.

The active oxygen species (AOS) such as singlet oxygen ($^1O_2$), superoxide radical (O_1^-), hydroxyl radical ($OH^\cdot$), and hydrogen peroxide (H_2O_2) are present in all plants in varying degrees as a result of normal aerobic metabolism. Under biotic or abiotic stress, production and removal of AOS are controlled by an array of enzymatic and non-enzymatic antioxidant mechanisms in plants reducing damage to DNA, proteins, and lipids (Foyer *et al*. 1994b, Asada 1999, Singh *et al*. 2006). The antioxidant enzymes function cooperatively, and any change in one of them may affect the equilibrium of oxidative stress. The non-enzymatic antioxidant defence systems consist of low molecular mass compounds such as ascorbate (AA), glutathione (G), α-tocopherol, and carotenoids (Asada 1999). Enzymatic defence enzymes

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Abbreviations: AA – ascorbate; AOS – active oxygen species; APX – ascorbate peroxidase (EC 1.11.1.11); Car – carotenoids; CAT – catalase (EC 1.11.1.6); Chl – chlorophyll; DHA – dehydroascorbate; DHAR – dehydroascorbate reductase (EC 1.8.5.1); FM – fresh mass; G – glutathione; GR – glutathione reductase; POD – peroxidase (EC 1.11.1.7); MDHA – monodehydroascorbate; MDHAR – monodehydroascorbate reductase (EC 1.6.5.4); SOD – superoxide dismutase (EC 1.15.1.1).
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include superoxide dismutase (SOD) which converts O_1^- to H_2O_2 that is then subsequently scavenged by catalase (CAT), peroxidase (POD), and ascorbate peroxidase (APX). H_2O_2 scavenging is accomplished by AA–G cycle that involves a series of coupled redox reactions involving four enzymes – ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), and glutathione reductase (GR) (Asada 1999, Mittler 2002). The AA-G cycle is an efficient pathway for plant cells to dispose of H_2O_2 in certain cellular compartments (Halliwell and Gutteridge 1989). This cycle also functions in most of the sub-cellular compartments of both photosynthetic and non-photosynthetic tissues (Asada 1999, Foyer and Noctor 2003).

The overproduction of AOS and the induction of oxidative stress by UV-B radiation have been documented in tomato plants (Balakumar *et al*. 1997) and the marine macroalgae of *Ulva fasciata* (Shiu and Lee 2005). In plant breeding programs, efficiency of AOS management strategy is one of the primary selection criteria, since the generation of AOS is elicited under all stress environments. For the acquisition of effective resistance to oxidative stress injury, a balanced increase in scavenging and regenerating enzymes, and also for targeting enzymes to cell organelles or to the cell compartments where the AOS is generated, is required. Moreover, the mechanisms in the regulation of the antioxidant defence system in plants under UV-B stress are unknown. Therefore, I studied UV-B radiation induced oxidative stress resistance strategies in a crop legume *Vigna unguiculata* and a wild legume *Crotalaria juncea*. The major objectives of the present work are three: (*1*) to find the dose responses of cultivated and wild legumes to UV-B; (*2*) to identify the various kinds of enzymatic antioxidants which serve as defence against oxidative stress injury caused by UV-B; and (*3*) to investigate the nature of non-enzymatic antioxidants which come into play under UV-B treatment.

Materials and methods

Plants and treatments: Seeds of *Vigna unguiculata* (L.) Walp. and *Crotalaria juncea* L. were obtained from the National Pulses Research Centre, Vamban 622 303, Pudukkottai, India. The methods of growing *V. unguiculata* and *C. juncea* seedlings, UV-B radiation milieu, and measurements are described in Balakumar *et al*. (1999a). Five seedlings of uniform size were grown in each container. After germination on the third day the seedlings were subjected to doses of UV-B of 1.0, 1.4, 4.7, and 6.3 kJ m^{-2} d^{-1} for one week. On the 7th d of treatment, the activities of defence antioxidant enzymes and non-enzymatic antioxidants were estimated in fully expanded unifoliate leaves. The enzyme assays were carried out using crude enzyme extract of leaves obtained by cutting them into small pieces (250 mg) and homogenizing in a mortar and pestle at 4 ^oC in 50 mM Tris-HCl (pH 7.8), 10 mM $MgCl_2$, 10 mM 2-mercaptoethanol, and 0.25 mM EDTA. The homogenate was centrifuged at 2 000×*g* for 5 min and the clear yellowish-green supernatant was collected.

Growth parameters and photosynthetic pigments: The leaf thickness was measured using a screw gauge at the completion of the experiment. Total leaf area of 10 randomly selected plants per treatment was determined with a leaf area meter, and the areas exhibiting chlorotic symptoms were also determined as leaf injury. Chlorophyll (Chl) was extracted in pre-chilled acetone (80 %, v/v) and estimated according to Arnon (1949). The amount of carotenoids (Car) present in the acetone extract was quantified by measuring the absorbance at 480 nm after correction for Chl interference (Kirk and Allen 1965) using the extinction coefficient proposed by Ridley (1979).

Preparation of membrane-rich fraction from leaves and NADPH-oxidase determination: Leaves were homogenized at 4 °C in buffer containing 100 mM potassium phosphate buffer (pH 7.5), 1.0 mM EDTA, and 0.5 mM sucrose. The homogenate was filtered through four layers of cheesecloth and centrifuged at 12 000×*g* for 20 min. The supernatant was centrifuged at 180 000×*g* for 60 min and the pellet was re-suspended in the homogenizing buffer and re-centrifuged at 180 000×*g* for 60 min. The pellet was re-suspended in homogenizing buffer and used to analyze NADPH-oxidase activity. NADPH-oxidase enzyme activity was measured spectrophotometrically (*UV160U* UV-V-visible recording spectrophotometer, *Shimadzu*, Kyoto, Japan) according to the method of Askerlund *et al*. (1987).

Determination of *in vivo* oxidative damage on protein and lipid peroxidation: The i*n vivo* oxidative damage of protein carbonyl content was measured with 2,4-dinitrophenyl hydrazine as described by Levine *et al*. (1990). Lipid peroxidation in the leaf tissue was measured as formation of malondialdehyde (MDA, a product of lipid peroxidation) determined by thiobarbituric acid reaction; a minor modification of the method of Dhindsa *et al*. (1981). The concentration of MDA was calculated using its extinction coefficient of 155 mM^{-1} cm^{-1}. Protein content was estimated by the method of Bradford (1976) using bovine serum albumin as standard.

Assays for antioxidant enzymes: The contents of SOD, CAT, DHAR, GR, APX, and MDHAR were measured in cytosolic fractions following the standard analytical methods of Beauchamp and Fridovich (1971), Kar and Mishra (1976), Hossain and Asada (1984), Foyer and Halliwell (1976), and Nakano and Asada (1981), respectively.

AA determination: AA and dehydroascorbate (DHA) were determined by the colour development with 2,4-dinitrophenylhydrazine with 1 cm^3 of 6 % metaphosphoric acid and 2.8 M acetic acid. The homogenate was diluted to 50 cm^3 with a solution of 3 % metaphosphoric acid and 1.4 M acetic acid, which was centrifuged at 2 000×*g* for 10 min. AA and DHA in the supernatant as well as in the standard solution were determined immediately. The reaction mixture containing 1 cm^3 of sample solution, 1 cm^3 of 0.26 M dinitrophenylhydrazine in 4.5 M H_2SO_4, was incubated at 37 °C for 3 h. After cooling on ice, 2.5 cm^3 of 16 M H_2SO_4 was added to the assay medium and incubated at room temperature for 30–40 min. Colour development was monitored spectrophotometrically at 540 nm. Total AA was determined as DHA, after oxidation with one drop of 7 mM Na-2,6-dichlorophenol indophenol. AA was calculated as the difference between total AA and DHA.

Determination of reduced (GSH) and oxidized (GSSG) glutathione contents: G content was measured fluorometrically by the method of Hissin and Hilf (1976). Leaves were ground with 25 % (v/v) metaphosphoric acid and potassium phosphate buffer (pH 8.0) and the homogenate was sonicated for 10 min and centrifuged at 30 000×*g* for 30 min. The supernatant was kept at 0 °C used for estimating GSH and GSSG. For the estimation of GSH content, fluorescence intensity was monitored with a *Hitachi MPF4* spectrofluorimeter at 420 nm (excitation at 350 nm) after incubating 0.2 cm^3 of the supernatant in 1.7 cm^3 potassium phosphate-EDTA buffer (pH 8.0) and 1.0 cm^3 of the fluorescence reagent *o*-phthaldialdehyde (OPT) (1 mg cm^{-3}) for 15 min. For the estimation of GSSG content, 0.5 cm^3 of the supernatant was incubated in 0.2 cm^3 of 40 mM N-ethylmaleimide for 30 min at room temperature. After adding 4.3 cm^3 of 0.1 M NaOH and 0.1 cm^3 OPT solution (1 mg cm^{-3}), fluorescence intensity was monitored for 15 min similarly as for GSH estimation. The contents were determined using standard curves.

Statistical analysis: A completely randomized block of a 2×4 factorial design with two treatments and three replicates was executed. Growth parameters were determined with at least ten independent replicates. The experiments for all the markers of oxidative stress and antioxidant enzymes were repeated at least three times. Variations between control and treatments were statistically analyzed by Student's *t*-test and the significance was defined at 5 % probability (Gomez and Gomez 1984). Karl Pearson's correlation coefficient (*r*) was calculated for the related parameters measured.

Results and discussion

UV-B treatment caused significant inhibition in overall plant growth and development. Normal (control) leaves of *C. juncea* are 2.7-fold thicker than *V. unguiculata* leaves. UV-B increased the leaf thickness by 70 % in *V. unguiculata* whereas in *C. juncea* there was no significant difference between the control and treated plants (Table 1). Leaf injury (estimated based on the chlorotic portions in the leaves of treated plants) showed a dose dependent response in *V. unguiculata* whereas in *C. juncea* only the highest dose of UV-B (6.4 kJ m^{-2} d^{-1}) induced leaf injury (Table 1). The impact of UV-B on vital plant processes such as photosynthesis (Allen *et al*. 1998) and nitrate assimilation (Balakumar *et al*. 1999a,b) has been extensively investigated. Among the various mechanisms proposed to inhibit plant growth and metabolism in an UV-B environment, the oxidative stress injury is of main importance (Foyer *et al*. 1994b, Rao *et al*. 1996). Growth responses of *V. unguiculata* and *C. juncea* recorded under UV-B established that both plants are sensitive to UV-B radiation (Table 1), but vary in the degree of sensitivity to UV-B.

Table 1. Changes in leaf thickness and leaf injury in *V. unguiculata* and *C. juncea* under various doses of UV-B radiation. Means±SE (n = 12); means followed by the same letter within the columns are not significantly different ($^*p$<0.05) according to Duncan multiple range test. *Values in parentheses* are % over control.

UV-B [kJ m^{-2} d^{-1}]	Leaf thickness [mm] *V. unguiculata*	*C. juncea*	Leaf injury [%] *V. unguiculata*	*C. juncea*
Control	18.60±0.29 a	49.70±0.81 a	0.00±0.00 a	0.00±0.00 a
1.0	-	-	0.00±0.00 a	0.00±0.00 a
1.4	-	-	10.40±0.46 b	0.00±0.00 a
4.7	31.80±0.76 b (170)*	50.20±0.98 a (101)	27.60±0.96 c	0.00±0.00 a
6.4	-	-	40.40±1.11 d	13.20±0.32 b

Chl and Car contents: Both plant species showed a progressive decline in the content of the photosynthetic pigments proportional to the UV-B dose, but the magnitude of inhibition was less in *C. juncea* than in *V. unguiculata* at all the four doses of UV-B radiation employed. At the highest dose of UV-B treatment (6.4 kJ m^{-2} d^{-1}), *C. juncea* showed only a 20 % reduction in the total Chl content, but in *V. unguiculata* the reduction was as high

as 49 % (Table 2). Although UV-B treatment decreased Car content of both species in a dose-dependant manner, there were no significant differences between them.

Table 2. Photosynthetic pigment responses, chlorophyll (Chl) [g kg^{-1}(FM)] and carotenoids (Car) [mmol kg^{-1}(FM)], of *V. unguiculata* and *C. juncea* under various doses of UV-B radiation for seven days. Means±SE (n = 3). Means followed by the same letter within the columns are not significantly different ($^{*}p$<0.05) according to Duncan multiple range test (1955). *Values in parentheses* are % over control.

UV-B	*V. unguiculata*				*C. juncea*			
[kJ m^{-2} d^{-1}]	Chl *a*	Chl *b*	Chl *a*+*b*	Car	Chl *a*	Chl *b*	Chl *a*+*b*	Car
Control	0.77±0.05 a	0.37±0.02 a	1.14±0.00 a	0.32±0.01 a	0.34±0.05 a	0.16±0.00 a	0.50±0.11 a	0.24±0.01 a
1.0	0.56±0.09 b (73)*	0.36±0.05 (95)	0.92±0.18 a (80)*	0.35±0.05 a (110)	0.46±0.08 a (133)	0.15±0.06 a (89)*	0.61±0.05 a (122)	0.35±0.01 (145)
1.4	0.45±0.010 b (58)*	0.28±0.08 ab (76)*	0.76±0.16 b (66)*	0.21±0.06 b (66)*	0.28±0.08 ab (81)*	0.16±0.06 a (00)	0.44±0.14 a (88)*	0.17±0.05 b (71)*
4.7	0.35±0.09 c (46)*	0.23±0.05 b (62)*	0.61±0.13 bc (53)*	0.19±0.01 bc (59)*	0.28±0.07 ab (81)*	0.16±0.05 a (97)	0.43±0.02 ab (86)*	0.17±0.05 b (68)*
6.4	0.39±0.05 bc (50)*	0.20±0.07 bc (53)*	0.58±0.11 bc (51)*	0.18±0.05 bc (56)*	0.26±0.05 b (75)*	0.14±0.00 ab (89)*	0.40±0.05 ab (80)*	0.15±0.00 b (63)*

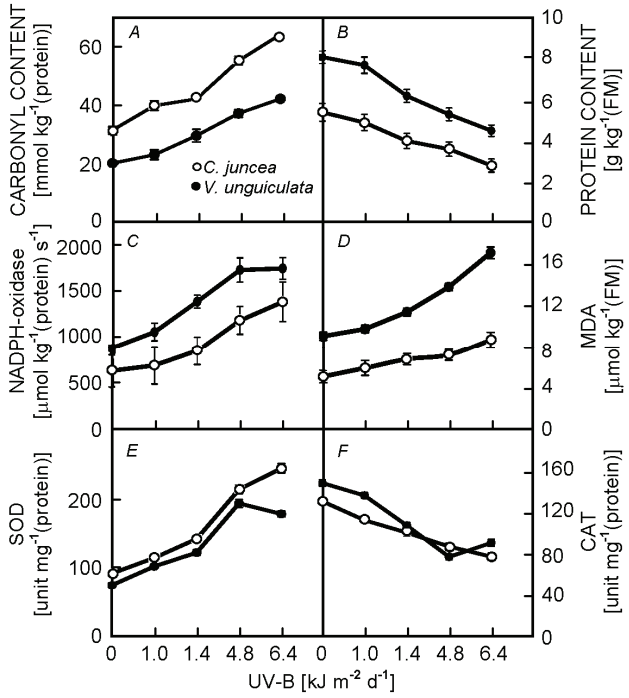

Fig. 1. Changes in i*n vivo* oxidative damage on protein (*A*), total soluble protein (*B*), activity of NAPDH-oxidase (*C*), content of malondialdehyde, MDA (*D*), and activities of superoxide dismutase, SOD (*E*) and catalase, CAT (*F*) in the leaves of *V. unguiculata* and *C. juncea* exposed to various doses of UV-B radiation. *Vertical bars* indicate mean±SE of three separate experiments (n = 3).

Foliar carbonyl content, total soluble proteins, and NADPH-oxidase: Foliar carbonyl groups are indicators for assessing the oxidative damage to proteins (Pacifici and Davies 1990). UV-B doses proportionally increased the foliar carbonyl content in both *V. unguiculata* and *C. juncea* (Fig. 1*A*). At the highest dose of UV-B employed (6.4 kJ m^{-2} d^{-1}), both plant species showed a two-fold increase in the carbonyl accumulation. The oxidative damage recorded in terms of the foliar carbonyl content was detectable in the total protein content. In both *V. unguiculata* and *C. juncea*, the protein content was reduced and the extent of reduction was dependent on the intensity of UV-B radiation (Fig. 1*B*). At 6.4 kJ m^{-2} d^{-1} of UV-B both plants had only half of the total protein content compared to the respective controls.

The involvement of the membrane-bound enzyme NADPH-oxidase in the generation of AOS has been established in various plant species (Chen *et al.* 2004). I found that UV-B radiation has stepped up the enzyme activity in both *V. unguiculata* and *C. juncea* (Fig. 1*C*) compared to the respective controls. Although in both species a dose-dependent enhancement in NADPH-oxidase activity was recorded, different UV-B exposure showed no difference between *V. unguiculata* and *C. juncea*. The increased NADPH-oxidase activity indicated the generation of AOS by UV-B. Plants recognize UV-B through mechanisms identical with those adopted to detect pathogenic infection. Upon such infections, activation of membrane-localized NADPH-oxidase and the consequent generation of AOS in plants was established (Vera-Estrella *et al.* 1994, Lamb and Dixon 1997). The AOS generated thus, in turn, may act as a signalling molecule (Chen *et al.* 2004). These observations along with my results confirm the involvement of the membrane-bound enzyme NADPH-oxidase in the generation of AOS under UV-B stress.

Lipid peroxidation: In plants, oxidative stress causes Chl and protein loss, leaf damage, increased lipid peroxidation, and changes in cell membrane permeability. All of them contribute to a progressive decline in photosynthetic capacity. The *V. unguiculata* and *C. juncea* plants that were irradiated with UV-B exhibited all these

symptoms. This is why I measured the MDA content which is a reliable indicator of free radical formation in plant tissue. Both plant species exhibited a proportional increase in peroxidation according to the quantum of UV-B treatment given. In *V. unguiculata* a greater lipid peroxidation had taken place than in *C. juncea*, particularly at the three highest doses of UV-B exposure (Fig. 1*D*). At 6.4 kJ m^{-2} d^{-1} of UV-B, *V. unguiculata* showed a 19 % higher peroxidation than *C. juncea*, exhibiting greater susceptibility to UV-B mediated lipid peroxidation (Fig. 2*D*). High lipid peroxidation coupled with high H_2O_2 content might have damaged chloroplasts, decreased plant biomass, and inhibited Chl synthesis, leading to lower Chl content (Table 2) and lower protein content in both plants (Fig. 1*B*). Singh *et al*. (2006) also observed an increase in the content of thiobarbituric acid-reactive substances' (TBARS) formation measured as an index of membrane damage in the fronds of *Pteris vitata* under arsenate treatment.

Antioxidant defence enzymes: Plants have several mechanisms to defend themselves against UV-B induced stress, mainly by invoking enzymatic and non-enzymatic antioxidant defence systems (Asada 1992, Foyer *et al*. 1994b). SOD is the primary enzyme in this process (Bowler *et al*. 1994). In both plant species the cytosolic SOD activity increased with UV-B treatment (Fig. 1*E*). Also the magnitude of enzyme activity increased proportional to the dosage of UV-B administered in both *V. unguiculata* and *C. juncea*. In *C. juncea* at 6.4 kJ m^{-2} d^{-1} there was 28 % higher SOD activity than in *V. unguiculata* (Fig. 1*E*). The gradual UV-B dose-dependent increase in SOD activity in *V. unguiculata* and *C. juncea* indicated the generation of superoxides (O_2^-) in the UV-B treated plants. Chilling stress in tomato plants also produces superoxide which induces the synthesis of SOD (Sui *et al*. 2007). In my experiments a similar kind of induction of SOD biosynthesis might have occurred during the irradiation. Although the precise role of O_2^- in the induction of SOD under UV-B treatment remains obscure, the increase in O_2^- caused by UV-B may trigger *de novo* synthesis of SOD, since the amount of SOD present under normal conditions may not suffice to scavenge the higher contents of AOS generated during UV-B treatment. The intricate mechanism, by which the synthesis of SOD is triggered, remains to be elucidated. UV-B mediated increase in SOD activity might maintain the metabolic stability of plants under UV-B treatment.

On the contrary, the CAT activity in leaf homogenate declined in relation to the dose of UV-B employed (Fig. 1*F*). A differential behaviour of the cytosolic CAT under UV-B treatment in both *V. unguiculata* and *C. juncea* is rather surprising. Since CAT is not a robust enzyme, it is susceptible to photoinactivation and degradation under prolonged exposure to UV-B (Streb *et al*. 1993) and its effectiveness is limited by a relatively poor affinity for H_2O_2 (Foyer *et al*. 1994a). Foyer *et al*. (1994b) have also suggested that the endogenous CAT may not be effective in the decomposition of H_2O_2, while a positive role has been proposed for CAT in protecting the mitochondrial components from oxidative damage (Anderson *et al*. 1992). However, I presume that the cytosolic CAT may be more readily accessible to photoinactivation and photodegradation by UV-B radiation than the enzyme localized within the chloroplasts.

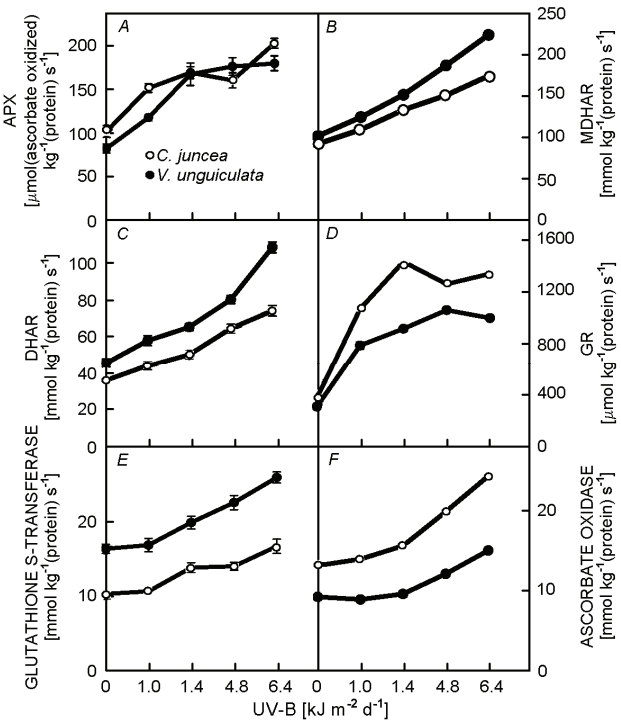

Fig. 2. Changes in activities of ascorbate peroxidise, APX (*A*), monodehydroascorbate reductase, MDHAR (*B*), dehydroascorbate reductase, DHAR (*C*), glutathione reductase, GR (*D*), glutathione-S-transferase (*E*), and ascorbate oxidase (*F*) in the leaves of *V. unguiculata* and *C. juncea* exposed to various doses of UV-B radiation. *Vertical bars* indicate mean±SE of three separate experiments (n = 3).

When the intracellular concentrations of H_2O_2 accumulate due to the depression of the CAT scavenger, peroxidases are induced to detoxify H_2O_2. APX is the key enzyme that comes to the rescue under such situations (Siminis *et al*. 1994). I found in *V. unguiculata* a dose-dependent enhancement of APX activity in leaf homogenate in response to UV-B. At 6.4 kJ m^{-2} d^{-1}, a 115 % higher APX activity was recorded compared to the control (Fig. 2*A*). *C. juncea* also showed a similar response in the enzyme activity, but the increase in enzyme activity at each level of UV-B was less than in *V. unguiculata*. A higher APX activity (Fig. 2*A*) is correlated with physiological processes such as the removal of H_2O_2, biosynthesis of lignin, and general plant development (Asada 1992). Though elevated activities of APX result in the depletion of AA, it has to be regenerated for

maintaining the redox status of the cells. In wheat the changes in SOD, CAT, and POD activities under water deficit are important anti-drought mechanisms enabling the plant to cope with the oxidative stress (Selote and Khanna Chopra 2006). These antioxidant enzymes may play similar roles under UV-B treatment.

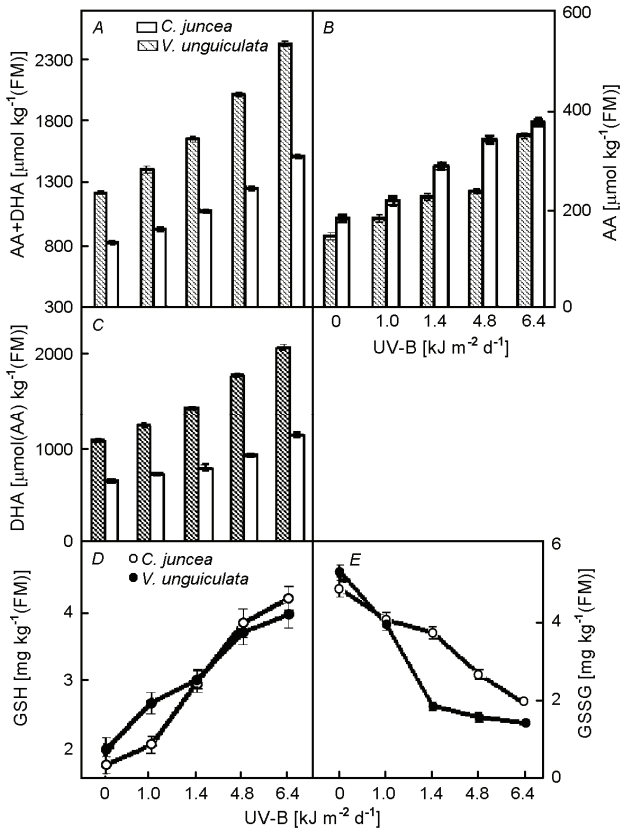

Fig. 3. Changes in contents of (*A*) total ascorbate (AA+DHA) or (*B*) ascorbate content (AA) and (*C*) dehydroascorbate, (*D*) reduced glutathione, GSH, and (*E*) oxidized glutathione, GSSG in the leaves of *V. unguiculata* and *C. juncea* under various doses of UV-B radiation. *Vertical bars* indicate mean±SE of three separate experiments (n = 3).

AA has to be regenerated by cells. This is accomplished by the AA-G cycle or the Halliwell-Asada pathway (Halliwell and Gutteridge 1989). The AA-G cycle is an efficient way for plants to dispose of H_2O_2 in certain cellular compartments where no CAT is present. To accomplish the cellular regeneration of AA from MDHA produced by the activity of APX, three enzymes function in a concerted manner. They are MDHAR, DHAR, and GR (Hossain and Asada 1984). Both *V. unguiculata* and *C. juncea* had increased MDHAR and DHAR specific activities under UV-B compared to the control and the increment in activities was proportional to the intensity of UV-B (Fig. 2*B,C*). Nevertheless, the magnitude of increase in the enzyme activity in *C. juncea* was consistently less compared to *V. unguiculata* at all levels of UV-B radiation employed. GR, which is the terminal enzyme in the regeneration of AA-G cycle, was greatly enhanced under UV-B treatment in both plants tested at all doses of UV-B employed (Fig. 2*D*). Surprisingly, in *C. juncea* the GR activity was greater than in *V. unguiculata* at all levels of UV-B. At 6.4 kJ m^{-2} d^{-1} *C. juncea* had 35 % higher GR activity than *V. unguiculata* (Fig. 2*D*). UV-B enhances the expression of GR genes in pea plants (Strid 1993). Rao *et al*. (1996) recorded enhanced GR activity in *Arabidopsis thaliana* mutants irradiated with UV-B, and low temperature stress also brought about similar responses in GR in maize (Foyer *et al*. 1998). Sub-cellular fractionation of pea tissue showed distinct isoforms of GR localized in mitochondria, chloroplasts, and cytosol (Foyer and Noctor 2003). Increased GR activity may ensure adequate protection against oxidative conditions encountered under UV-B.

My experiments established that UV-B is capable of generating AOS and inflicting oxidative damage on proteins and vital processes in general in both plant species. Nevertheless, both plant species invoked their enzymatic antioxidant mechanisms to metabolize the AOS, *viz*. the SOD-CAT system and the AA-G cycle enzymes. However, there were distinct differences between them in the activities of various enzymes triggered by UV-B radiation. The *C. juncea* was better adapted to oxidative stress than *V. unguiculata* by means of efficient cellular antioxidant mechanisms under UV-B. Moreover, an array of non-enzymatic antioxidants was also present in plant cells which supplemented the enzymatic ones in the removal of AOS.

AA pool: AA is the most important metabolite, universal in photosynthetic eukaryotes (Smirnoff 1996). UV-B treatment increased proportionally the total AA content of both plants, depending on the dose used (Fig. 3*B*). However, the *V. unguiculata* plants showed more severe oxidative stress injury under UV-B, which was evident from the larger pools of total AA in their leaves than in the leaves of *Crotalaria*. At 6.3 kJ m^{-2} d^{-1} of UV-B, in *V. unguiculata* leaves 98 % higher content of total AA was recorded compared to its control, while in *C. juncea* the increase in total AA content was only 84 % (Fig. 3*A*). The AA content also registered a similar trend in *V. unguiculata* and *C. juncea* under UV-B exposure (Fig. 3*A*). The UV-B inflicted oxidative damage in *V. unguiculata* was more severe than in *C. juncea* and was discernible from the higher content of AA noted in *V. unguiculata* leaves. At 6.3 kJ m^{-2} d^{-1} of UV-B, *V. unguiculata* leaves registered 142 % higher AA content compared to the control, while in *C. juncea* the same dose of UV-B brought only 108 % higher AA content (Fig. 3*A*). The increase in AA content of UV-B treated leaves may be related to the increase in MDHAR and DHAR activities since these enzymes are involved in the recycling of AA from its oxidation products through the AA-POD-catalyzed reaction (Hossain and Asada

1985). Increase in AA content in leaves may signify one of the mechanisms evolved by the plants for protection against reactive oxygen species generated in response to UV-B radiation exposure.

In the AA-G cycle which is the most important machinery for scavenging the free radicals in plants, DHA and AA are the major partners (Asada and Takahashi 1987). The AA oxidase activity, which leads to the production of DHA, showed a dose-dependent promotion in *V. unguiculata* plants under UV-B (Fig. 2*F*). The response of AA oxidase activity in *C. juncea* was also similar to that of *V. unguiculata.* However, in *C. juncea* a strikingly higher enzyme activity was noticed at all levels of UV-B employed when compared to *V. unguiculata* (Fig. 2*F*). The increased AA oxidase activity brought about by UV-B treatments in both plants was reflected in the DHA content in leaves. The DHA content increased proportionally to UV-B dose in both the plants, compared to their respective control (Fig. 3*C*). DHA produced *via* the non-enzymatic disproportion of MDHA may be involved in the detoxification of H_2O_2 in chloroplasts (Dipierro and Borraccino 1991). Because the production of DHA is non-enzymatic, it is unlikely affected by the activities of other enzymes of the AA-G cycle. Further, the relative contents of AA and DHA in plant cells are crucial for the maintenance of the redox status of the cell. Plants tend to protect their cellular redox status under environmental stresses to minimize the damage that would be caused by the production of AOS. This is substantiated by the consistent retention of the AA/DHA ratio under all UV-B doses given to the test plants (Table 3). Nevertheless, the AA/DHA ratio was strikingly higher in *C. juncea* in both the control and UV-B treated variants compared to *V. unguiculata*.

Table 3. Changes in ascorbate (AA) to dehydroascorbate (DHA) ratio and glutathione reduced (GSH) to oxidized (GSSG) ratio in *V. unguiculata* and *C. juncea* under various doses of UV-B radiation. *Means followed by the same letter* within the columns are not significantly different ($p<0.05$) according to Duncan multiple range test.

UV-B [kJ m^{-2} d^{-1}]	AA/DHA *V. unguiculata*	*C. juncea*	GSH/GSSG *V. unguiculata*	*C. juncea*
Control	0.13 a	0.28 a	0.36 a	0.37 a
1.0	0.15 a	0.30 a	0.55 b	0.68b
1.4	0.16 a	0.30 a	0.80 bc	1.59 bc
4.7	0.13 a	0.37 b	1.44 c	2.30 c
6.4	0.17 b	0.33 a	2.19 d	2.73 d

G pool: Besides AA, thiol tripeptide (γ-glu-cys-gly) G (GSH) is another antioxidant metabolite (Rao *et al*. 1996, Matamoros *et al*. 1999) implicated in many metabolic processes and constituting an important plant defence system against environmental stress including that by heavy metals (May *et al*. 1998). G and its oxidized form GSSG drive the G component of the AA-G cycle. UV-B increased the GSH content in *V. unguiculata* and *C. juncea* leaves depending on the UV-B dose employed (Fig. 3*D*). However, the GSH accumulation in the UV-B irradiated leaves of *C. juncea* was larger than that of *V. unguiculata* which was more pronounced at the higher doses of UV-B. On the contrary, in both plant species the GSSG content was inhibited by UV-B treatment in a dose-dependent manner (Fig. 3*E*). Here *V. unguiculata* also showed smaller GSSG accumulation than *C. juncea* at all intensities of UV-B tested. The observed increase in the GSH content with the concomitant decrease in GSSG content under UV-B was discernible in the GSH/GSSG ratio, which stepped up with an added UV-B dose (Table 3). The progressive increase in GSH/GSSG ratio brought in by UV-B was very high in *C. juncea* compared to *V. unguiculata*. Increase in total GR activity during cold hardening in conifers and broadleaf plants is well documented (Doulis *et al*. 1993). Along with accumulated GSH, this probably ensures adequate protection against oxidative conditions encountered at low temperatures. Moreover, in the AA-G cycle the GR activity which favours the production of GSH seems to outweigh the activity of DHAR which leads to the formation of GSSG in the UV-B treated plants. This apparent lack of pace between GR and DHAR might influence the regeneration of AA. There are also other means of recycling AA through MDHAR activity and *de novo* synthesis, so that equimolar levels of AA and G are sustained. Equimolar concentrations of AA and G are essential to sustain a high redox state in the AA-G cycle. The ability of a plant to maintain a high redox state of AA and G has been attributed to coordination between SOD that generates H_2O_2 and GR-APX that metabolizes H_2O_2 (Foyer *et al*. 1994a). High redox state of the AA-G cycle is believed to provide resistance against oxidative stress in plants (Smirnoff 1996, Singh *et al*. 2006).

In plant cells the content of G is regulated by additional pair of enzymes, G-peroxidase and G-S-transferase (Drotar *et al*. 1985). When the activity of G-peroxidase was estimated in the leaf homogenate of control and UV-B treated plants, no detectable activity was recorded. The existence of G-peroxidase in higher plants has been discussed even though its activity was detected in the leaves of plant regenerated from the callus of salt-adapted *Citrus sinensis* (Holland *et al*. 1993, Gueta-Dahan *et al.*

1997). Drotar *et al*. (1985) were successful in showing G-peroxidase activity in extracts of cultured plant cells of spinach, maize, and *Acer*, but they could not demonstrate any activity in soybean using H_2O_2 as the substrate. My experience with this enzyme was similar, when I assayed enzyme activity with H_2O_2 as the substrate. Presumably G-peroxidase is either not present in legumes or is so labile that can not be assayed. However, this proposal warrants further experimentation using structural analogues of H_2O_2 as substrate. On the contrary, UV-B treatment promoted G-S-transferase activity in a dose-dependent manner in both *V. unguiculata* and *C. juncea* (Fig. 2*E*). Rajaguru *et al*. (1999) also noticed an increase in the GST activity in salt stressed cotton leaves. GST is largely attributed to the detoxification of xenobiotics through the formation of the G-conjugates with a variety of electrophilic compounds that are also involved in the synthesis of phytoalexins, thus its function as an antioxidant is insignificant.

Conclusion: My study shows that the UV-B mediated oxidative injury in plants is efficiently abated by the enzymatic antioxidants with additional support from the non-enzymatic defence metabolites. The wild legume *C. juncea* posseses a more efficient AOS scavenging mechanism than *V. unguiculata* in terms of its SOD-CAT system, the Halliwell-Asada cycle enzymes, and the active pools of low molecular mass antioxidants AA and G. *C. juncea* has strikingly higher AOS management efficiency which offers a potential germplasm resource for genetic improvement of crops for oxidative stress tolerance using transgenic technologies. The induction of SOD synthesis by UV-B, the mechanism of suppression of cytosolic CAT under UV-B, *de novo* synthesis of AA in the UV-B environment, and the role of AA-G metabolism in AOS detoxification were elucidated.

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