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BRIEF COMMUNICATION

Increased antioxidant activity in *Cassia* seedlings under UV-B radiation

S. AGARWAL

Stress Physiology Laboratory, *Department of Botany*, *JNV University*, *Jodhpur*, *Rajasthan-342011*, *India*

Abstract

Cassia auriculata L. seedlings were irradiated with ultraviolet B (UV-B) in an environment-control chamber. The two doses assayed (7.5 and 15.0 kJ m^{-2}) induced oxidative damage with an increase in lipid peroxidation and hydrogen peroxide and a decrease in chlorophyll and total phenol contents. The ascorbate and dehydroascorbate content as well as the reduced glutathione/oxidized glutathione content and ratio were significantly increased. The UV-B stress led to significant increases of the activity of superoxide dismutase, catalase, peroxidase and polyphenol oxidase. It is suggested that *Cassia* seedlings try to counteract high concentrations of oxygen species produced under UV-B stress through a co-ordinated increase in the contents and activities of antioxidants involved in their detoxification.

Additional key words: ascorbate, catalase, glutathione, hydrogen peroxide, oxidative stress, peroxidase, phenol, polyphenol oxidase, superoxide dismutase, thiobarbituric acid reactive substances.

Plants have evolved different defensive systems against environmental stresses such as salinity, drought, temperature, pollutants, metal toxicity or ultraviolet-B (UV-B) radiation which generate highly-reactive oxygen species (Agarwal and Pandey 2003a,b, 2004) Enhanced levels of UV-B (290 - 320 nm) radiation reaching the earth's surface due to stratospheric ozone depletion (*e.g.* Madronich *et al*. 1998) may damage DNA, proteins and lipids, impair chloroplast function and reduce photo-synthesis, growth and development (*e.g.* Jansen *et al*. 1998). UV-B radiation also produces oxidative stress (Panagopoulos *et al*. 1990) although the mechanism of reactive oxygen species (ROS) generation in UV-B irradiated plants is not known (Strid 1993, Rao *et al*. 1996). These ROS (superoxide radical, hydrogen peroxide, hydroxyl radical and other free radicals) are extremely reactive and cytotoxic (Wilhelmová *et al*. 2004). Plants have evolved protective mechanisms including enzymatic and non-enzymatic antioxidants (Rao *et al*. 1996).

The main enzymatic antioxidants are superoxide dismutase (SOD), catalase (CAT), peroxidase (POX), polyphenol oxidase (PPO) and non-enzymatic antioxidants ascorbate (ASA) and glutathione (GSH). ASA and GSH react with singlet oxygen, superoxide and hydroxyl radicals. Phenols are oxidized by peroxidase and primarily by PPO, this latter enzyme catalyzing the oxidation of the *O*-diphenols to *O*-diquinones, using molecular oxygen as an electron acceptor (Somner *et al*. 1994).

The antioxidant system of this plant species is unknown and hence the aim of this study was to evaluate the antioxidant response of *Cassia auriculata* L. seedlings to UV-B radiation.

Cassia auriculata L. seeds were surface sterilized by treating with 0.1 % mercuric chloride solution for 5 min and then thoroughly washed with double distilled water. Thirty sterilized seeds were placed in 9 cm Petri dishes lined with double layers of *Whatman No.1* filter paperand were irrigated with Hoagland nutrient. Seedlings were grown at 25/20 °C, with a 14-h photoperiod under white fluorescent lamp (160 μmol m^{-2} s^{-1}) in a controlled environmental growth chamber. After 7 d of germination, the seedlings were separated into two sets, one used as control and the other subjected to UV-B (290 - 320 nm) irradiation with a *Philips* 25 W UV-B lamp with filter (irradiance of 2.60 W m^{-2}). UV-B doses were adjusted by exposure of seedlings for 50 and 100 min exposure to

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Abbreviations: ASA - ascorbate; CAT - catalase; DHA - dehydroascorbate; DTNB - 5,5'- dithio-bis-(2-nitrobenzoic acid); EDTA - ethylenediaminetetraacetic acid (disodium salt); f.m. - fresh mass; GSH - reduced glutathione; GSSG - oxidized glutathione; NADPH - reduced nicotinamide adenine dinucleotide phosphate; NBT - nitroblue tetrazolium; POX - peroxidase; PPO - polyphenol oxidase; PVP - polyvinylpyrrolidone; ROS - reactive oxygen substances; SOD - superoxide dismutase; TBA - thiobarbituric acid; TBARS - thiobarbituric acid reactive substances.

Fax: (+ 91) 0291 2512248, e-mail: sheelaagarwal@yahoo.com

UV-B source. Using the generalized plant response function and normalizing the spectral irradiance at 300 nm, according to Caldwell (1971), the biologically effective UV-B doses used were 7.5 and 15 kJ m^{-2}.

Ascorbate (ASA) and dehydroascorbate (DHA) were estimated according to the modified procedure of Law *et al*. (1983). Seedlings (1 g) were extracted with 10 cm^3 of 6 % trichloroacetic acid (TCA). NaOH (0.01 cm^3, 5 M) was added to 0.4 cm^3 of the extract, mixed and centrifuged for 2 min at 3 500 *g*. To a 0.2 cm^3 sample of the supernatant was added 0.2 cm^3 of 150 mM NaH_2PO_4 buffer, pH 7.4, and 0.2 cm^3 of water. To another 0.2 cm^3 of supernatant, 0.2 cm^3 of buffer and 0.1 cm^3 of 10 mM dithiothreiotol were added and after 15 min at room temperature 0.1 cm^3 of 0.5 % (m/v) *N*-ethylmaleimide was added. To each sample was then added 0.4 cm^3 of 10 % (m/v) TCA, 0.4 cm^3 of 44 % (v/v) H_3PO_4, 0.4 cm^3 of 4 % (m/v) bipyridyl in 70 % (v/v) ethanol and 0.2 cm^3 of 3 % (m/v) $FeCl_3$. After vortex-mixing, samples were incubated at 37 ºC for 40 min and the absorbance was recorded at 525 nm in a *Beckman model 36* UV-visible spectrophotometer (Geneva, Switzerland). A standard curve of ASA or DHA was used for calibration.

Thiols were extracted by homogenizing 0.3 g of seedlings in 3 cm^3 of 0.1 M HCl (pH 2) and 1 g polyvinylpyrrolidone (PVP) according to the method of Schupp and Rennenberg (1988). The homogenate was centrifuged at 10 000 *g* for 10 min at 4 °C and the supernatants were used for analysis. Thiol content was determined measuring absorbance at 412 nm, in the presence of 0.5 mM 5,5'-dithiobis-2-nitrobenzoic acid (DTNB), 0.5 U cm^{-3} glutathione reductase and 0.2 mM NADPH. GSSG was determined by the same method in the presence of 2-vinylpyridine and GSH content was calculated from the difference between total glutathione and GSSG.

The level of lipid peroxidation was measured as the amount of thiobarbituric acid reactive substances (TBARS) determined by the thiobarbituric acid (TBA) reaction as described by Heath and Packer (1968). The absorbance of the supernatant was recorded at 532 nm. The TBARS content was calculated using its absorption coefficient of 155 mM^{-1} cm^{-1}.

Hydrogen peroxide was estimated with titanium reagent as described by Teranishi *et al*. (1974). Sample preparation for H_2O_2 estimation was done as described by Mukherjee and Choudhuri (1983).

The chlorophyll content was analyzed as described by Wintermans and De Mots (1965). Chlorophyll was extracted by 80 % ethanol and measured spectrophotometrically at 654 nm.

Phenolics were extracted with methanol and assayed according to the method of Sadasivam and Manickam (1992). The absorbance was recorded at 630 nm with Folin-Ciocalteau reagent. The reference curve was prepared using catechol (0.1 mg cm^{-3}).

Enzyme extracts for determination of SOD, POX, CAT, and PPO activities were prepared by grinding tissue with chilled phosphate buffer (for SOD pH 7.8, for CAT, POX and PPO pH 6.8). The homogenate was filtered through cheese cloth and the filtrate was centrifuged in a refrigerated centrifuge at 10 000 *g* for 20 min. The supernatant served as enzyme extract. All operations were carried out at 4 °C. Total soluble protein content was determined according to Bradford (1976) using bovine serum albumin as a standard.

The SOD (EC 1.15.1.1) activity was estimated according to the method of Beauchamp and Fridovich (1971) and modified by Giannopolitis and Ries (1977). Catalase (CAT, EC 1.11.1.6), peroxidase (POX, EC 1.11.1.7) and polyphenol oxidase (PPO, EC 1.10. 3.1) activities were assayed according to Chance and Maehly (1955) with modifications (for detail see Agarwal and Pandey 2004).

Each data point is the mean of three replicates obtained from two independent experiments. The analysis was carried out using statistical package *MINITAB* version *13.1*. All data were subjected to a one-way analysis of variance (*ANOVA*) and the significance of differences between control and each treatment was analyzed using Tukey's test. Levels of significance used were $P = 0.05$ and $P = 0.01$.

In the seedlings exposed to 7.5 and 15.0 kJ m^{-2} UV-B radiation the contents of TBARS were higher (22 and 34 % over control values). H_2O_2 showed a significant increase over control (48 and 76 %) (Table 1). This enhancement of TBARS and H_2O_2 indicated that UV-B radiation induced oxidative stress. These results are in good agreement with the results of Dai *et al*. (1997) in rice leaves, Jain *et al*. (2003) in cucumber cotyledons and Agarwal and Pandey (2003a,b) in *Cassia* species.

Chlorophyll content was reduced in both treatments (8 and 19 % with respect to control) revealing a possible damage in the photosynthetic capacity of chloroplasts (Malanga *et al*. 1997, Kakani *et al*. 2004, Santos *et al*. 2004). Strid (1993) and Mackerness *et al*. (1999) suggested that under UV-B stress plants sacrifice their chloroplasts in order to protect the rest of the cell. Also total phenol content decreased (9 and 25 % with respect to control).

ASA content increased significantly (160 and 113 %) at lower and higher UV-B doses, respectively. Similarly a significant increase in DHA content (182 and 118 %) was found. GSH contents also increased over control values when the seedlings were exposed to two UV-B treatments. GSSG content increased 7-fold for 7.5 and 5-fold for 15 kJ m^{-2} UV-B compared to control. The GSH/GSSG ratio was enhanced (43 and 12 % at the two doses).

ASA together with GSH protect plant tissues by direct scavenging of ROS (Halliwell and Gutteridge 1989) and by the maintenance of the redox status in plant cells and organs (Horemans *et al*. 2000). The impressive increase in both ASA and DHA content and GSH/GSSG ratio in the *Cassia* seedlings could represent defence against UV-B radiation. However, the significant increase at the lower irradiation dose indicate that at this dose seedlings were more effective in coping with the UV-B stress.

Table 1. Effect of UV-B treatments on the contents of TBARS, H_2O_2, chlorophyll, total phenol, ASA, DHA, GSH, GSSG, GSH/GSSG, and the activities of SOD, CAT, POX and PPO. Means of three replicates ± SE; ** - significant differences at $P < 0.01$ according to Tukey's test.

Parameter	Control	UV-B 7.5 kJ m^{-2}	UV-B 15 kJ m^{-2}
TBARS [nmol g^{-1}(f.m.)]	34.40 ± 0.90	42.10 ± 1.10**	46.10 ± 1.10**
H_2O_2 [µmol g^{-1}(f.m.)]	15.03 ± 0.77	22.03 ± 0.80**	26.50 ± 1.00**
Chlorophylls [µg g^{-1}(f.m.)]	248.30 ± 3.40	228.20 ± 2.20**	201.60 ± 2.80**
Phenols [nmol g^{-1}(f.m.)]	24.20 ± 0.90	21.50 ± 1.00*	17.40 ± 0.80**
ASA [µmol g^{-1}(f.m.)]	1.50 ± 0.03	3.90 ± 0.05**	3.20 ± 0.03**
DHA [µmol g^{-1}(f.m.)]	1.10 ± 0.08	3.10 ± 0.10**	2.40 ± 0.01**
GSH [µmol g^{-1}(f.m.)]	2.30 ± 0.05	28.00 ± 0.90**	12.00 ± 0.30**
GSSG [µmol g^{-1}(f.m.)]	0.50 ± 0.06	3.60 ± 0.11**	2.30 ± 0.17**
GSH/GSSG	4.60 ± 0.11	8.00 ± 0.25**	5.20 ± 0.21**
SOD [U mg^{-1}(protein)]	2.48 ± 0.07	3.62 ± 0.07**	4.56 ± 0.11**
CAT [U g^{-1}(f.m.)]	2.16 ± 0.03	4.08 ± 0.09**	3.68 ± 0.09**
POX [U g^{-1}(f.m.)]	2.40 ± 0.05	3.40 ± 0.10**	3.80 ± 0.10**
PPO [U g^{-1}(f.m.)]	3.05 ± 0.08	4.30 ± 0.06**	4.70 ± 0.13**

The activity of SOD showed a significant increase at the two UV-B radiation treatments (46 and 84 %), with respect to the control values. The CAT activity significantly increased at 7.5 and 15 kJ m^{-2} UV-B treatments (88 and 70 % over control values).The activity of POX increased at both UV-B radiation treatments over control values (41 and 58 %). The PPO activity notably increased at 7.5 and 15 kJ m^{-2} UV-B treatments (41 and 54 % over controls, respectively).

Santos *et al*. (1999) have emphasized that UV-B radiation interferes with the SOD similarly as do other stresses and also affects the isoenzymes of SOD differently. The tolerance of *Cassia auriculata* L. seedlings to UV-B is certainly due to the enhancement of SOD activity and other antioxidative enzymes which is consistent with the results of Santos *et al*. (1999, 2004) in potato, wheat and maize, Mackerness *et al*. (1999) in pea, Kondo and Kawashima (2000) in cucumber, and Prasad and Zeeshan (2005) in *Plectonema boryanum*. The UV-B enhancement of CAT and POX activities which are both responsible for detoxification of H_2O_2 are probably equally important in the detoxification of H_2O_2 generated by SOD in *Cassia* seedlings. Increases in activities of peroxidases by UV-B radiation have been observed in several species including *Cassia* species (Agarwal and Pandey 2003b), *Arabidopsis thaliana* (Rao *et al*. 1996), cucumber (Krizek *et al*. 1993), sugarbeet (Panagopoulos *et al*. 1990), and potato (Santos *et al*. 2004). In addition, the metabolism of phenolic compounds also includes the action of oxidative enzymes such as POX and PPO, which catalyze the oxidation of phenols to quinones (Thypyapong *et al*. 1995). The decreased total phenol contents as well as the enhanced PPO activity under UV-B radiation contradicts an earlier report where an increase in phenol content and a decrease in PPO activity was reported (Balakumar *et al*. 1997). It seems possible that oxidoreductases PPO and POX involved in phenol oxidation may play an important role as defense against UV-B oxidative stress.

A perusal of the results suggests that the two UV-B doses induced the antioxidant defenses protecting the plant against major deleterious effects of ROS. The damage due to these radicals as indicated by increase in TBARS, H_2O_2 and decrease in chlorophyll and total phenol contents was limited indicating that the water soluble non-enzymatic antioxidants ASA/DHA and GSH/GSSG and enzymatic antioxidant defense system represented by SOD, CAT, POX and PPO is functional in *Cassia* seedlings. Thus *Cassia* seedlings are able to cope with UV-B stress.

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