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Comparative Sensitivity of Two Vigna Species to UV-B Stress: Impacts on Oxidative Stress and Antioxidant Responses

Rajiv Dwivedi[1] and Raghvendra Pratap Narayan[2]
[1]Pt. Deen Dayal Upadhyay Government P.G. College, Sitapur, UP
[2]Deen Dayal Upadhyay Government P.G. College, Saidabad, Prayagraj, UP
Email: rajivdau@gmail.com

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Abstract

The study investigated the differential effects of ultraviolet-B (UV-B) radiation (0.4 W m^{-2}; 280–315 nm) on hydroponically grown seedlings of two Vigna species, Vigna radiata and Vigna unguiculata, focusing on growth, reactive oxygen species (ROS), lipid peroxidation, membrane stability, and antioxidant responses. UV-B exposure inhibited growth in both species in a dose-dependent manner, with V. unguiculata showing greater sensitivity. The radiation triggered increased ROS production—specifically superoxide radicals (SOR) and hydrogen peroxide—along with elevated lipid peroxidation and electrolyte leakage, effects that were more pronounced in V. unguiculata. Antioxidant enzyme activities, including superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT), were higher in V. unguiculata and initially increased under UV-B stress; however, higher doses led to a sharp decline in CAT and SOD activities. In contrast, V. radiata exhibited significantly higher levels of ascorbic acid and UV-B-absorbing flavonoids, which increased with UV-B exposure, while V. unguiculata accumulated more proline. The findings suggest that V. radiata's greater tolerance to UV-B stress stems from its elevated ascorbic acid and flavonoid content in the epidermal layer, acting as effective UV-B screens. Conversely, V. unguiculata's heightened sensitivity likely results from the rapid drop in CAT and SOD activities coupled with excessive ROS accumulation under UV-B stress.

Key words: *UV-B radiation; Antioxidants; Superoxide dismutase; Peroxidase; Catalase; Ascorbic acid; Proline; UV-B absorbing pigments*

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Introduction

The stratospheric ozone layer is vital to life on the Earth as it absorbs the harmful ultraviolet radiations. During last two decades, the continuous release of chlorofluorocarbons and other potent ozone depleting gases have shifted the balance, favouring large scale destruction of ozone, which has increased the influx of ultraviolet-B radiation [1, 2, 3, 4, 5]. Plants are vulnerable to increased UV-B radiation. Many cellular components such as nucleic acids, proteins and lipids can absorb UV-B radiation directly [6]. Several studies have indicated that UV-B radiation can deleteriously affect physiological processes and over all growth in plants [7,8,9]. Such a decrease in growth of the plants may be due to direct effects of UV-B on various cell components or indirectly through enhanced generation of reactive oxygen species (ROS). The photosynthetic electron transport systems, the electron transport chain in the mitochondria, photorespiration pathway and plasma membrane have been regarded as potential source of ROS for the oxidative burst [10]. ROS play different roles *in vivo*. Some are positive and related to their involvement in energy production, phagocytosis, regulation of cell growth, intercellular signaling and synthesis of biologically important compounds [11]. However, all ROS can be extremely harmful to organism at high concentration [6]. $O_2^{\bullet -}$ is moderately reactive short-lived ROS that cannot cross the biological membrane and is dismutated to H_2O_2. Hydrogen peroxide is moderately reactive and is a relatively long-lived molecule that can diffuse some distance from its production site. Hydrogen peroxide is potent inhibitor of photosynthesis and its destruction is vital for the function of chloroplast [12]. Although neither superoxide nor H_2O_2 at physiological concentrations seems particularly harmful and their toxicity arises by a metal ion dependent conversion into hydroxyl radical, which is able to mutate DNA and to initiate chain reaction of lipid peroxidation leading to loss of function and tissue destruction [12, 13, 14]. Dai et al. (1997) [15] showed involvement of oxiradicals in UV-B injury to plants as UV-B exposure caused significant increase in lipid peroxidation and membrane permeability. Lipid peroxidation is a natural metabolic process under normal aerobic conditions, and it is one of the most investigated consequences of ROS action on membrane structure and function [16].

Because of the highly cytotoxic and reactive nature of ROS, their accumulation must be under tight control. The antioxidant defense systems allow scavenging of ROS and protect cells from oxidative damage. The enzymatic antioxidants superoxide dismutase (SOD), catalase (CAT) and

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peroxidase (POD) are efficient scavengers of $O_2^{\bullet-}$ and H_2O_2. The scavenging of $O_2^{\bullet-}$ is achieved through an upstream enzyme superoxide dismutase, which catalyzes the dismutation of superoxide radicals to H_2O_2. The enzyme is present in all sub cellular components susceptible to oxidative stress [13]. SOD activity determines the concentration of $O_2^{\bullet-}$ and H_2O_2, and is therefore called cell's first line of defense against ROS [17]. The scavenging of H_2O_2 is brought about by catalase and a number of peroxidases. Catalase decomposes H_2O_2 to water and molecular oxygen without consuming reductant thus may provide plant cells with an energy efficient mechanism to remove H_2O_2 [18]. Peroxidases are monomeric haemoproteins that catalyze the oxidation of a range of substrates by hydrogen peroxide. Apart from enzymatic antioxidants, plants also contain an important array of non-enzymatic antioxidants such as ascorbic acid, proline, flavonoids etc. The ascorbic acid is one of the most studied and powerful antioxidant [16,19]. Under physiological conditions, ascorbic acid intercellular concentration can build up to millimolar range (20 mM in cytosol and 20-300 mM in chloroplast stroma) [20]. The ascorbate deficient mutant of *Arabidopsis thaliana* was found to be more sensitive to UV-B radiation than wild plants [21]. The role of proline in higher plant cells in response to environmental stress is well documented [22]. Proline provides less than 5 % of the total pool of free amino acids in plants under stress free condition, whereas, the concentration may increase up to 80 % of the amino acid pool during stress [23]. The function of proline in stressed plants is often explained by its property as an osmolyte, able to balance water stress but recent researches have shown its involvement in plant protection against oxidative stress [23,24]. Flavonoids are mostly localized in the epidermal cells, where they screen out the UV-B radiation. Formation of UV-B absorbing compounds significantly increased following UV-B radiation [25]. Xu et al. [26] reported enhanced UV-B sensitivity due to absence of flavonoid epidermal screening compounds in one of the two soybean lines.

Keeping above facts in mind, an experiment was conducted in controlled environment chambers to evaluate the sensitivity of *Vigna unguiculata* and *Vigna radiata* seedlings to UV-B radiation.The crops are helpful in maintaining the nitrogen economy of agricultural field and the nutritive value in vegetarian diet due to high protein content. The relative sensitivity of the two species exposed to UV-B stress was investigated by analyzing growth and status of enzymatic and low molecular weight non-enzymatic antioxidants, reactive oxygen species and oxidative damage.

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2. Materials and methods

2.1 *Plant materials and growth conditions*

The seeds of *Vigna radiata* (L.) Wilczek cv. Samrat (PDM-139), and *Vigna unguiculata* (L.) Walp cv. Gomti (VU-89) were obtained from Indian Institute of Pulses Research (IIPR), Kanpur and Indian Institute of Vegetable Research (IIVR), Varanasi, U.P., India, respectively. Healthy uniform sized seeds were surface sterilized in sodium hypochlorite solution for 15 min. Sodium hypochlorite solution was decanted followed by several rinses with sterilized double distilled water. Thoroughly washed seeds were soaked for 2 h in distilled water, wrapped in moistened cotton cloth and left overnight in dark for germination. The germinated seeds were sown in plastic trays containing acid washed sterilized sand and incubated in dark at 28 ± 2 ºC for a day. The seedlings were grown in a growth chamber at 28 ± 2 ºC under 13:11 light and dark periods (150 µmol photon m^{-2} s^{-1}, PAR) with relative humidity of 60-80 %. The seedlings were watered daily with double distilled water. After 3 days of growth, equal sized seedlings were gently transferred in plastic glasses containing 0.2 strength Rorison nutrient medium (pH 7.5). The nutrient medium was aerated intermittently with sterile air to avoid the anaerobic condition around roots.

2.2 *UV-B treatment*

After acclimatizing in nutrient medium for two days, the seedlings were given two exposures of UV-B on 5th and 7th day with the help of single fluorescent UV tube (TL - 40 W/12, Philips, Holland) with its main output at 312 nm together with white light (75 µmol photon m^{-2} s^{-1}, PAR). The radiation was filtered through 0.127 nm cellulose acetate sheet (Johnston Industrial plastics, Toronto, Canada) to remove all incident UV-C (< 280 nm). The UV-B exposure influence rate was 0.4 Wm^{-2} (simulating 15 % ozone depletion at Varanasi, adjoining to Allahabad). The seedlings were exposed to UV-B radiation for 15, 30, 45, 60, and 90 min (corresponding to 0.36, 0.72, 1.08, 1.44 and 2.16 kJ m^{-2} d^{-1}, respectively). The radiation intensity was measured with a Power meter (Spectra Physics, Model 407, A-2, USA). On 8th day seedlings of each set were harvested and various parameters were analyzed.

2.3 *Measurement of growth*

After 24 h of last UV-B exposure (on 8th day) seedlings of each set were harvested and plant fresh mass and dry mass were determined by single pan electronic balance (Contech- CA 223, India). For

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dry mass measurement, the seedlings were wrapped in butter paper and dried in an oven at 120 ºC for 8 h. The leaf area of treated and untreated seedlings was determined by leaf area meter (Model 211, Systronics, India).

2.4 *Estimation of reactive oxygen species*

Superoxide radical (SOR)was measured by the method of Elstner and Heupel [27] with some modifications as described by Jiang and Zhang [28]. One gram of washed frozen leaf segments from UV-B treated and untreated seedlings were homogenized with 3 ml of 65 mM potassium phosphate buffer (pH 7.8) and centrifuged at 10,000 g for 10 min. Leaf extract was incubated with 1ml of hydroxylamine (10 mM). After incubation at 25 ºC for 20 min, 17 mM sulfanilamide and 7 mM napthylethylene diamine dihydrochloride were added to the incubation mixture. The components were mixed and separated into two layers using same volume of diethyl ether to eliminate the interference caused by the pigments. The absorption of pink water phase of the lower layer was measured at 530 nm. The SOR content was quantified from a standard curve.Hydrogen Peroxide (H_2O_2) content in the treated and untreated samples was estimated by following ferrithiocyanate method as described by Sagisaka [29]. One gram of the fresh frozen leaves was homogenized in 3.5 ml of ice cold 5 % TCA, centrifuged at 8,000 g for 15 min and supernatant obtained was used for H_2O_2 estimation. The reaction mixture contained 50 % TCA, 2.5 M potassiumthiocyanate (KSCN), 10 mM ferrous ammonium sulfate. The absorbance of the solution was read at 480 nm against blanks, and the level of H_2O_2 in each sample was calculated from standard curve.

2.5 *Estimation of oxidative damage indices*

Oxidative damage to lipids was estimated by measuring the content of malondialdehyde (MDA) in leaf homogenates, prepared in 10 % trichloroacetic acid containing 0.65 % 2-thiobarbituric acid (TBA) and heated at 95 ºC for 25 min as described by Hodges et al. [30]. MDA content was calculated by correcting for compounds other than MDA which absorb at 532 nm by subtracting the absorbance at 600 nm of a reaction mixture containing leaf extract incubated without TBA from an identical solution containing TBA.The intactness of plasma membrane in leaves of UV-B treated and untreated seedlings was measured as the leakage of electrolytes as described by Gong et al. [31]. Leaves from treated and untreated plants (0.200 gm) were cut into pieces of 5 mm length and placed

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in test tubes containing 20 ml deionized water at 30 ºC for 2 h. The sample was centrifuged and the initial electrolyte conductivity (EC_1) was measured by digital conductivity meter (Systronic-607, India). One set of control sample was boiled at 100 ºC for 15 min to release all electrolytes and electrical conductivity (EC_2) of supernatant was measured. The percentage of electrolyte leakage was calculated as (EC_1 / EC_2 x 100).

2.6 ***Estimation of the activity of enzymatic antioxidants***

Superoxide dismutase (SOD, EC 1.15.1.1) activity was estimated by measuring the inhibition of the reduction of *p*-nitroblue tetrazolium chloride (NBT) by method of Giannopolitis and Ries [32]. For extraction of SOD, fresh washed leaves (0.500 gm) were homogenized under ice cold condition with 100 mM EDTA-phosphate buffer (pH 7.8). The homogenate was centrifuged at 10,000 g for 20 min and used for enzyme assay. The reaction was performed in a total volume of 3 ml containing 1.3 µM riboflavin, 13 mM L-methionine, 0.05 M Na_2CO_3 (pH 10.2), 63 µM NBT and 0.1 ml of crude extract. The reaction mixture in similar test tubes was irradiated with visible light (250 µmol m^{-2} s^{-1}) for 5 min. The initial rate of reaction as measured by the difference in absorbance at 560 nm, in the presence and absence of extract was proportional to the amount of enzyme. One unit of SOD was defined as the amount of enzyme that inhibited NBT reduction by 50 % under the specified conditions. Peroxidase (POD, EC 1.11.1.7) activity in leaf homogenates of treated and untreated seedlings was determined according to the method of Zhang [33]. Fresh leaf segments (0.050 gm) were homogenized in 2 ml 50 mM phosphate - buffer (pH 6.1). The homogenate was centrifuged at 10,000 g for 15 min and the supernatant was used as enzyme extract. Peroxidase activity was measured with guaiacol as the substrate in a total volume of 3 ml. The reaction mixture consisted of 50 mM potassium phosphate buffer (pH 6.1), 1 % (w/v) guaiacol, 0.4 % (w/v) H_2O_2 and enzyme extract. Increase in absorbance due to oxidation of guaiacol (E = 25.5 mM cm^{-1}) was measured at 470 nm. Enzyme activity was calculated in terms of Unit (gmfresh mass)$^{-1}$. One unit of the POD activity is the amount of enzyme oxidizing 1 µM guaiacol min^{-1} at 28 ºC. Catalase (CAT, EC 1.11.1.6) activity was determined polarographically at 28 ºC with Clark type oxygen electrode (Rank Brother, UK) as described by Sgherri et al. [34]. Fresh frozen leaves (0.250 gm) from UV-B treated and untreated seedlings were homogenized in 2.5 ml of 100 mM phosphate buffer (pH 7.0). The homogenate was centrifuged at 10,000 g for 15 min and supernatant was used as enzyme extract. Catalase activity was determined by O_2 released from

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enzymatic dissociation of H_2O_2 in darkness for 1 min after the addition of 5 ml of 50 mM phosphate buffer (pH 7.0) containing 25 mM H_2O_2 directly to 1 ml of enzyme extract in reaction vessel. Oxygen produced by enzymatic reaction was calculated after correction for auto production of O_2 from H_2O_2. One unit of catalase is the amount of enzyme producing 1 µmol O_2 min^{-1}.

2.7 *Estimation of non-enzymatic antioxidants*

Ascorbic acidcontent in leaf homogenates of treated and untreated seedlings was estimated using the method given by Oser (1979). Fresh leaves (0.500 gm) were homogenized in 2 ml of 5 % sulfosalicylic acidthen the homogenate was centrifuged at 10,000 g for 15 min. The reaction mixture consisted of 2 ml 2 % sodium molybdate, 2 ml 0.15 N H_2SO_4, 1 ml 1.5 mM K_2HPO_4 and 1 ml tissue extract. The reaction mixture was incubated at 60 ºC in water bath for 40 min, cooled, centrifuged at 3,000 g for 10 min and absorbance was recorded at 660 nm. The amount of ascorbic acid was calculated by comparing with standard curve. Proline content in leaf homogenates of UV-B treated and untreated seedlings was estimated according to the method of Bates et al. [35]. Fresh leaves (1 gm) were crushed in 3 % aqueous sulfosalicylic acid centrifuged at 10,000 g for 10 min and then reacted with 3 % glacial acetic acid and acid ninhydrin. Samples were heated for 1 h in a water bath at 95 ºC, cooled and extracted with 4 ml toluene by vortexing for 15 sec with a test tube mixer. The toluene layer was then aspired and the absorbance was read at 520 nm using toluene as blank. The proline content in each sample was calculated from the standard curve. UV-B absorbing compounds in fully expanded leaves exposed to different doses (exposure time) of UV-B were estimated as described by Mirecki and Teramura [36]. Samples were taken from the middle of fully exposed leaves. UV-B absorbing pigments were extracted from leaf discs by keeping them in acidified methanol (methanol: water: HCl, 78: 20: 2, v/v) for 24 h at 4 ˚C. The filtered extract was then used for measuring the absorbance at 320 nm, which is indicative of relative concentration of UV-B absorbing pigments. Flavonoids content are expressed as A_{320nm} (gm fresh mass)$^{-1}$.

2.8 *Statistical analysis*

Values presented in the text indicate mean values ± SE of three replicates. The significance of differences between control and treated plants were analyzed using the student's *t*-test at the level of significance of $P \leq 0.01$ and $^*P \leq 0.05$.

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3. Results

3.1 *Growth*

Growth parameters were analyzed to determine the sensitivity and the level of tolerance between the seedlings of *Vigna unguiculata* and *Vigna radiata* exposed to UV-B radiation. UV-B exposure resulted in dose dependent decrease in biomass accumulation in the seedlings of both the species (Table 1). The decrease in plant dry mass was more pronounced in *V. unguiculata* than *V. radiata*, showing 25 % decline in *V. radiata* and 33 % in *V. unguiculata* after 90 min of UV-B exposure. Similar dose of UV-B radiation also caused about 22 % reduction in fresh mass in *V. radiata* while there was 29 % decrease in *V. unguiculata*. UV-B exposure for 90 min diminished the leaf area by 34 % in *V. radiata* and 43 % in *V. unguiculata* (Table 2). The leaf fresh mass and dry mass also exhibited similar declining trend with significantly more effect on *V. unguiculata*.

3.2 *Reactive oxygen species*

The results pertaining to superoxide radical (SOR) content in leaves of both the species of *Vigna* seedlings exposed with different doses of UV-B radiation are represented in Fig. 1a. There was progressive rise in SOR content with increasing doses (15-90 min exposure) of UV-B as *V. radiata* leaves exhibited 2.5 folds rise and only 1.4 folds increment in *V. unguiculata* over the values of respective controls. The results also clearly reveal that SOR content in leaves of *V. unguiculata*, before and after UV-B treatment, was substantially higher than that of *V. radiata*. Similar to SOR content, the amount of H_2O_2in leaves of *V. unguiculata* was significantly high and the increasing doses of UV-B caused progressive rise in H_2O_2 content in both the species as it was enhanced by 3 folds in *V. radiata* and by 3.6 folds in *V. unguiculata* seedlings following 90 min of UV-B exposure (Fig. 1b).

Table 1: Effect of different doses of UV-B radiation on plant fresh mass (PFM, mg plant^{-1}) and plant dry mass (PDM, mg plant^{-1}) of *Vigna radiata* and *Vigna unguiculata* seedlings.

UV-B exposure (min	Growth parameters			
	Vigna radiata		*Vigna unguiculata*	
	PFM	PDM	PFM	PDM
Control	273.6 ± 2.9	23.7 ± 0.2	617.5 ± 7.2	49.3 ± 0.6

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15	259.9 ± 1.7 (5)*	22.7 ± 0.2 (4)	568.1 ± 5.2 (8)	46.3 ± 0.4 (6)
30	251.7 ± 1.3 (8)	21.5 ± 0.2 (9)	542.5 ± 4.2 (12)	42.5 ± 1.0 (14)
45	240.7 ± 1.9 (12)	20.6 ± 0.3 (13)	520.5 ± 5.9 (16)	40.9 ± 1.0 (17)
60	224.3 ± 1.1 (18)	18.9 ± 0.3 (20)	472.9 ± 6.4 (23)	37.0 ± 0.4 (25)
90	213.4 ± 2.7 (22)	17.7 ± 0.3 (25)	438.7 ± 4.2 (29)	33.2 ± 0.4 (33)

The values are means ± SE (n=3). Values in parenthesis are [%] decrease. All the treatments are significantly different ($p< 0.01$, $P^*<0.05$) from their respective controls (student's *t*-test).

Table 2: Effect of different doses of UV-B radiation on leaf area (LA, mm^2 $plant^{-1}$), leaf fresh mass (LFM, mg $plant^{-1}$) and leaf dry mass (LDM, mg $plant^{-1}$) of *Vigna radiata* and *Vigna unguiculata* seedlings.

UV-B exposure (min)	Growth parameters					
	Vigna radiata			*Vigna unguiculata*		
	LA	LFM	LDM	LA	LFM	LDM
Control	318.2 ± 2.1	43.0 ± 0.8	5.6 ± 0.2	818.2 ± 10.2	116.6 ± 2.4	13.8 ± 0.2
15	308.6 ± 1.2 (3)*	42.1 ± 0.5 (2)^{ns}	5.4 ± 0.1(3)^{ns}	770.4 ± 8.0 (6)*	110.8 ± 1.4 (5)*	13.0 ± 0.1 (6)
30	286.3 ± 2.4 (10)	39.1 ± 0.4 (9)*	5.0 ± 0.1(11)*	695.4 ± 8.2 (15)	99.1 ± 1.5 (15)	11.6 ± 0.2 (16)
45	270.4 ± 2.3 (15)	36.1 ± 0.6 (16)	4.7 ± 0.1(16)*	640.9 ± 8.4 (22)	92.1 ± 2.0 (21)	11.0 ± 0.3 (20)
60	248.1 ± 3.1 (22)	33.1 ± 0.7 (23)	4.2 ± 0.1 (25)	595.9 ± 7.8 (27)	81.6 ± 1.4 (30)	9.4 ± 0.3 (32)
90	210.0 ± 2.9 (34)	30.5 ± 0.8 (29)	3.9 ± 0.2 (30)	470.4 ± 7.9 (43)	72.3 ± 1.3 (38)	8.3 ± 0.2 (40)

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The values are means ± SE (n=3). Values in parenthesis are [%] decrease. All the treatments are significantly different ($p < 0.01$, $P^{*}<0.05$) from their respective controls (student's *t*-test). ns = not significant.

3.3 *Lipid peroxidation and electrolyte leakage*

The data presented in Fig. 1c reveal that UV-B radiation caused substantial damage to cellular membrane in both species as malondialdehyde (MDA) content rose progressively following the exposure of leaves with increasing doses (15-90 min) of UV-B radiation. The accumulation of MDA content was significantly high in *V. unguiculata* and after 90 min of UV-B exposure the level of MDA was raised by 1.9 and 1.6 folds in *V. unguiculata* and *V. radiata* respectively over their respective controls. Further, the damaging effect was also analyzed by recording the electrolyte leakage from the leaves of both the species. The results presented in Fig. 1d depict that the exposure of leaves with increasing doses (15-90 min) of UV-B radiation caused progressive rise in electrolyte leakage (%) and the leakage was comparatively more in *V. unguiculata* than *V. radiata.*

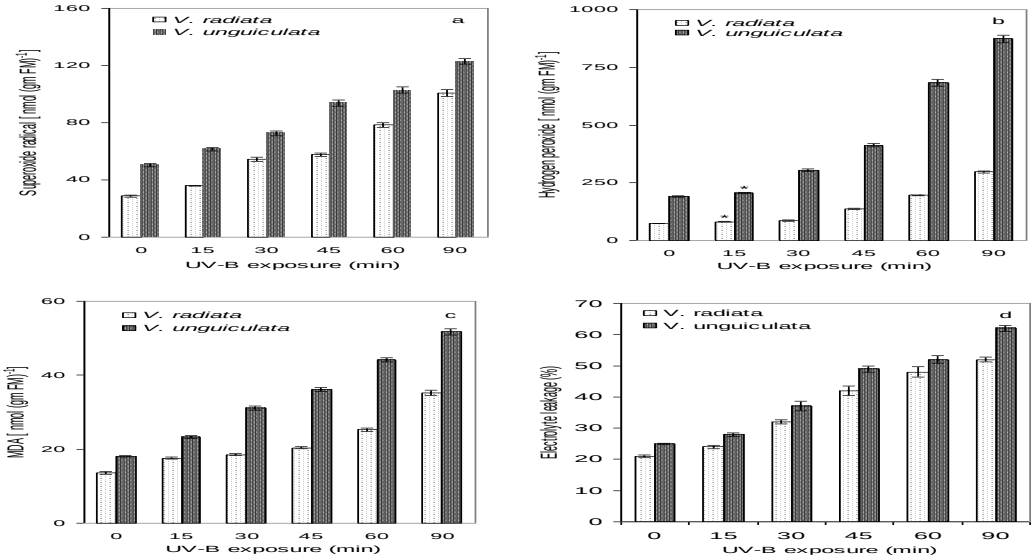

Fig.1. Superoxide radical (a), hydrogen peroxide (b), malondialdehyde (c)contents and electrolyte leakage (d) in the leaves of *Vigna radiata* and *Vigna unguiculata* seedlings exposed to UV-B radiation. The values are means ± SE (n=3). All the treatments are significantly different ($p \leq 0.01$, $*P \leq 0.05$) from their respective controls (student's*t*-test).

3.4 *Enzymatic antioxidant*

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The results presented in Fig. 2a exhibit that superoxide dismutase (SOD) activity was significantly high in leaves of *V. unguiculata* than that of *V. radiata* and following UV-B treatment there was substantial rise in the activity in both the species. The maximum enhancement in SOD activity was noticed following 15 min of UV-B exposure in *V. unguiculata* and after 45 min treatment in *V. radiata*, and thereafter further extension in UV-B exposure time produced declining trend in the activity. Catalase activity in untreated *V. unguiculata* seedlings was significantly higher (2.8 folds) than that of *V. radiata* (Fig. 2b). The leaves of *V. unguiculata* seedlings exposed with UV-B radiation for 15 min exhibited 35 % enhancement in catalase activity and further rise in UV-B exposure time showed decreasing trend and the activity even declined by 31 % over the value of control after 90 min of UV-B exposure. Notwithstanding with *V. unguiculata*, in *V. radiata* catalase activity exhibited marginal increase up to 30 min of UV-B exposure and further extension in exposure time resulted into appreciable depression in CAT activity. Peroxidase (POD) activity in untreated leaves of *V. unguiculata* was nearly 2 folds higher than its other counterpart (Fig. 2c). Unlike SOD and CAT activity, POD activity in this species was substantially high and showed progressive rise in activity following UV-B exposure and even 90 min of UV-B treatment accelerated the activity by 222 % over the control. In *V. radiata* 15-30 min UV-B exposure caused 27-47 % up in POD activity and further increase in UV-B dose did not show appreciable change in the activity.

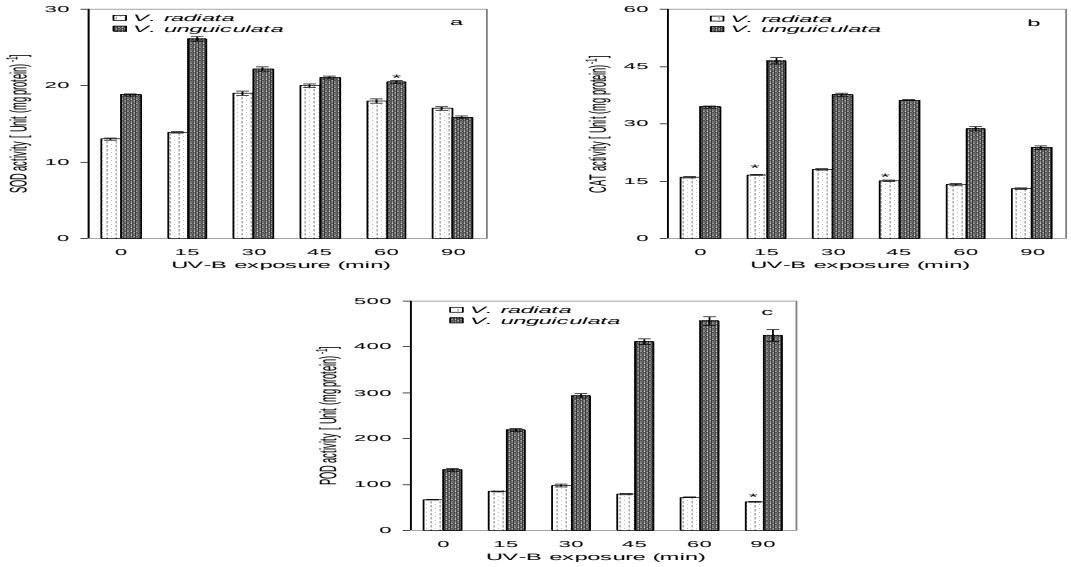

Fig. 2. Superoxide dismutase (a), catalase (b) and peroxidase (c) activity in the leaves of *Vigna radiata* and *Vigna unguiculata* seedlings exposed to UV-B radiation. The values are means ± SE (n=3). All

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the treatments are significantly different ($p \leq 0.01$, $^*P \leq 0.05$)from their respective controls (student's *t*-test).

3.5 *Non-Enzymatic Antioxidants*

The untreated leaves of *V. radiata* contained substantially high amount [952 ± 10 µg $(gmFM)^{-1}$] of ascorbate (Fig. 3a) and a progressive rise (7- 62 %) was noticed with increasing doses (15-90 min) of UV-B exposure. Compared to *V. radiata*, the untreated leaves of *V. unguiculata* showed quite low amount [306 ± 7.3 µg $(gmFM)^{-1}$] of ascorbate and the content exhibited similar increasing trend as 15-90 min UV-B exposure caused 20-87 % enhancement. Fig. 3b shows the impact of different UV-B doses on proline accumulation in leaves of two *Vigna* species seedlings. Untreated leaves of *V. radiata* and *V. unguiculata* contained 25.3 ± 0.9 and 42.8 ± 1.4 µg $(gmFM)^{-1}$ proline, respectively. Enhanced UV-B exposure increased proline content; however, a declining trend was noticed at high UV-B doses. The amount of proline in *V. unguiculata* increased from 30-118 % after 15-60 min UV-B exposure; thereafter the content declined and about 89 % increase in the amount over the control after 90 min of UV-B exposure was noticed. In *V. radiata* seedlings, proline content showed an enhancement of 21-63 % following 15-45 min of UV-B exposure then it started receding but it was still higher than that of control. Similar to ascorbate content, UV-B absorbing pigments (flavonoids) in epidermal layer of untreated leaves of *V. radiata* was fairly high [155 ± 4.2 A_{320nm} (gm fresh mass)$^{-1}$] than that of *V. unguiculata* [77.2 ± 2.1 A_{320nm} (gm fresh mass)$^{-1}$] and

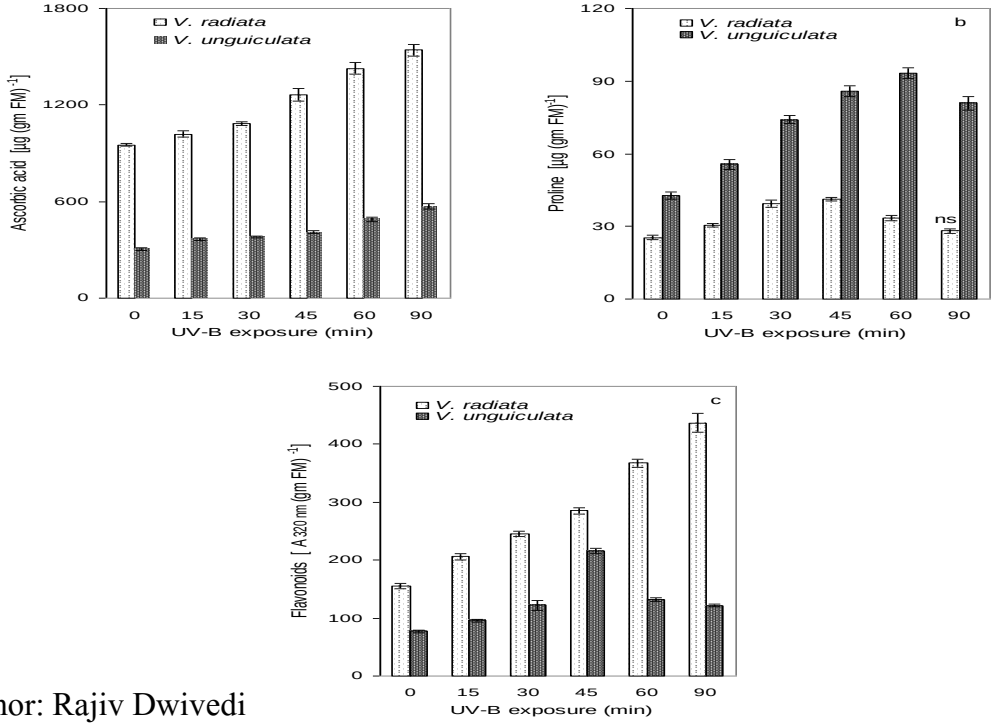

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Fig. 3. Ascorbic acid (a), proline (b) and flavonoids (c) contents in the leaves of *Vigna radiata* and *Vigna unguiculata* seedlings exposed to UV-B radiation.The values are means ± SE (n=3). All the treatments are significantly different ($p \leq 0.01$, $P^{*} \leq 0.05$)from their respective controls (student's*t*-test). ns = not significanttheir accumulation in *V. radiata* enhanced (by 33-182 %) progressively (Fig. 3c) when UV-B exposure time raised from 15 to 90 min. In *V. unguiculata* flavonoids content increased steadily up to 45 min and thereafter the content showed declining trend though the content was still higher than that of control.

4. Discussion

UV-B dose dependent reduction in growth parameter i.e. biomass accumulation in *Vignaradiata* and *Vigna unguiculata* seedlings was recorded (Table 1, 2). Besides this, visual symptoms such as dwarfing of plants, distortion of shoots, and reduction in leaf dimension in the seedlings of both the species were also observed. The UV-B exposure declined the growth, however, the effect was more pronounced in *Vigna unguiculata.* Our results are in agreement with earlier finding where elevated level of UV-B radiation significantly declined the growth parameters in *Vignaunguiculata*, a native and cultivated plant of Southern Africa [7]. Such reduction in growth has been correlated with UV-B induced suppression in photosynthesis rate [9] destruction of growth promoting hormone indole acetic acid (IAA) [37], and also with excessive generation of free radicals that cause damaging effect on structural and functional components of cells [38]. It has also been suggested that UV-B induced stunting and dwarfing of plants is probably associated with changes in cell division or cell elongation [39]. Greater damaging effects in *V. unguiculata* compared to *V. radiata* could be attributed to more generation / accumulation of ROS in leaves (Fig. 1a, b). Furthermore, high dose of UV-B induced strong reduction in growth of *Vigna* species particularly in *V. unguiculata* might have taken place due to irreparable damage to biomolecules i.e. DNA, proteins and lipids, and photosynthetic apparatus particularly thylakoid membrane associated photosynthetic electron transport carriers and C_3 cycle enzymes as observed by Melis et al. [40], Friso et al. [41] and Bischof et al. [42].

UV-B exposure may stimulate the generation of reactive oxygen species in plants by diverting the normal path of electron as it is known to cause lesion in the photosynthetic and respiratory electron transport systems[43,44]. Disrupted electron transport system in various cellular compartments led to

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enhanced generation / accumulation of ROS, which may further deteriorate the cellular systems previously damaged by the direct effects of UV-B. Superoxide radical (SOR) and hydrogen peroxide (H_2O_2), the potent inhibitors of a number of key metabolic processes, increased substantially in UV-B exposed *V.* species seedlings (Fig. 1a, b) and the increment was found to be UV-B dose dependent. Compared to *Vignaradiata* the contents of SOR and H_2O_2 were found to be appreciably high in *V. unguiculata* untreated leaves and the amount of ROS increased progressively with rising doses of UV-B. The heavy accumulation of ROS particularly H_2O_2 in *V. unguiculata* leaves could be correlated with UV-B induced substantial damaging effects on photosynthetic electron transport and CO_2 fixation as reported in *V. unguiculata* in our earlier finding [9] and this probably led to the leakage of electron to O_2 resulting in the accelerated generation of ROS. Another reason for substantial amount of ROS accumulation, probably occurred due to sharp decline in the activity of antioxidant enzymes, SOD and CAT in *V. unguiculata* leaves at higher doses of UV-B (Fig. 2a, b). ROS produce damaging effects on functional biomolecules and if there were excess ROS formed within the cells, one would expect impairment in several metabolic processes that could eventually lead to cell death [45,46]. H_2O_2 is known to inactivate enzymes by oxidizing their thiol groups. The studies have confirmed that H_2O_2 at elevated level (µM concentration) inactivates Calvin cycle enzymes [47] and superoxide dismutases [13]. However, the real threat of SOR and H_2O_2 is their potential to act as precursors of the hydroxyl radical. The hydroxyl radical can readily oxidize amino acid residues of proteins, fatty acids of phospholipids and deoxyribose and bases in DNA [43]. UV-B induced heavy accumulation of ROS has been shown to affect a wide range of plant cellular activities, however, action on membrane biogenesis and integrity is of prime importance [48,49](Kramer et al., 1991, Mahdavian et al., 2008). H_2O_2, $^{\bullet}OH$and other ROS are responsible for the lipid peroxidation of membrane (Sairam, 2005), thus disrupting its functional ability. In the present study even untreated seedlings of two *Vigna* species showed appreciable difference in MDA content (Fig. 1c) which could be attributed to generation / accumulation of SOR and H_2O_2 (Fig. 1a, b). Further, UV-B exposure increased the MDA content in both the species of *Vigna* in UV-B dose dependent manner and it was appreciably high in UV-B exposed *V. unguiculata* leaves compared to *V. radiata* (Fig. 1c). This difference in MDA content occurred due to faster accumulation of ROS in *V. unguiculata* leaves (Fig. 1a, b). The electrolyte leakage from leaf discs of *V.* species seedlings also showed similar trend as observed in MDA content. Lower lipid peroxidation and higher membrane stability (lower electrolyte leakage) in *V. radiata*

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seedlings may be linked with relative tolerance of this species to UV-B, while the reverse is true for *V. unguiculata* seedlings. Our results are in agreement with those of Kramer et al. [15, 48, 49, 50, 51] who found increased MDA content with elevated level of UV-B stress in *Oryza sativa*, barley seedlings, *Capsicum annum*, cucumber leaves and *Ulva fasciata*.

Under non stressful condition, the antioxidative defense system provides adequate protection against active oxygen and free radicals; however, under severe stress condition the antioxidant capacity may not be sufficient to mitigate the harmful effects of oxidative injury [14]. Oxidative stress is essentially a regulated process. The equilibrium between the oxidative and antioxidative capacities determines the fate of the plant [12]. The antioxidant enzymes induced by UV-B are likely to be specific to the plant species. In cucumber, activities of SOD, CAT, APX and POD were reported to be enhanced by UV-B radiation [38]. Enhanced ROS formation under stress condition induces both protective and damaging responses within the cell. Plant cell contains an impressive array of antioxidant metabolites and enzymes that scavenge or protect from the formation of the most aggressive ROS, thus protecting cells from oxidative damage. Adaptation to oxidative stress involves not only the regulation of the synthesis and repair of the proteins but also enhanced oxidative capacity. The ability of plants to metabolize $O_2^{\bullet -}$ and H_2O_2 has been reported to be largely dependent on the coordination of several interrelated antioxidant enzymes such as SOD, CAT and POD, GR and APX [28]. In the present study, the response of antioxidant enzymes SOD, CAT and POD varied with the duration of UV-B exposure and species tested. Though the leaves of untreated *V. unguiculata* seedlings exhibited significantly high SOD, CAT and POD activities, UV-B exposure caused substantial decrease in growth (Table 1) and photosynthetic processes, reported in our earlier finding [9]. This might have occurred due to direct effect of UV-B on energy gaining processes or following greater accumulation of the ROS due to the imbalance between generation of ROS and their scavenging by antioxidants at the site. UV-B at lower doses accelerated the activity of SOD and CAT in the two *Vigna* species; however, at higher UV-B doses, a declining trend was noticed. The decrease in activity might have resulted due to either down regulation of the genes coding for these enzymes or ROS mediated damaging effect on antioxidant enzymes. Our results are in consonance with earlier observations where SOD activity increased in UV-B exposed rice plants initially but long-term exposure declined the activity appreciably [15]. Further, ROS may act as secondary messenger to regulate gene expression and protein biosynthesis involved in defense process under sub-toxic

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condition [28]. H_2O_2 is found to induce the expression of genes potentially involved in its synthesis, such as NADPH oxidase [46] and also of those encoding proteins / enzymes involved in its degradation, implying a complex mechanism for cellular regulation of oxidative status. Besides this, H_2O_2 is also reported to induce the expression of genes encoding H_2O_2 degrading enzymes, ascorbate peroxidase and catalase in rice and maize [52]. Blokhina et al. [16] noticed that SOD genes are sensitive to environmental stresses, presumably as a consequence of increased POD formation. In present study under UV-B stress, there was substantial increase in POD activity in *V. unguiculata* seedlings and thus, a possible sharp decline in SOD activity was noticed (Fig. 2a, c). The accelerated POD activity could also be correlated with reduced growth and biomass accumulation in *V. unguiculata* seedlings as POD has been reported to initiate the catabolism of growth promoting hormone indole acetic acid (IAA). Increased activity of POD is not only linked to an increase in UV-tolerance but also to decreased auxin levels in tobacco transgenic plants [53]. The significant increase in POD activity was shown in UV-B exposed, bitter gourd, cucumber, wheat and *Cucumis sativus* [54,55,56,57]. Along with SOD and POD activity, lower UV-B doses increased CAT activity in *V. radiata* and *V. unguiculata* but further rise in UV-B doses showed declining trend, and at high UV-B doses (60-90min exposure) the activity declined to the level that was even less than the value of control plant. Similarly, Ambasht and Agrawal [58] found significant decline in CAT activity in UV-B exposed soybean seedlings, while POD exhibited reverse trend. In another finding, Zancan et al. [59] also recorded sharp decline in catalase activity in iron deficient barley seedlings due to UV-B exposure and thus, the plant became more sensitive to UV-B. Sharp decline in catalase activity following the increase in UV-B exposure time probably led to faster accumulation of H_2O_2 in *Vignaunguiculata*. Increased CAT activity is related with stress tolerance of plants. In earlier study, the regulation of antioxidant system under UV-B was determined in a marine macroalga *Ulva fasciata*, which showed enhanced H_2O_2 content; lipid peroxidation and various antioxidative enzymes including SOD, CAT, POD, APX, and GR [50]

Apart from enzymatic antioxidants, plant cells also contain an important array of non-enzymatic antioxidants such as ascorbic acid, proline, flavonoids, glutathione etc. for mitigating the toxic effects of free radicals and ROS under oxidative stress. These chemicals are helpful in scavenging some of the reactive species, however, for singlet oxygen enzymatic scavenging system has not evolved. Ascorbic acid is one of the most studied powerful antioxidant scavenging the damaging H_2O_2 and

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other ROS profoundly. Possibly the protective effect of ascorbic acid is more related to reduce ROS induced damage to essential proteins and /or nucleic acids [60]. In present study significantly high ascorbic acid content was recorded in leaves of *V. radiata* control seedlings, while *V. unguiculata* leaves showed quite low amount (Fig. 3a). This may be one of the strong reasons for more H_2O_2 accumulation in *V. unguiculata*, in spite of high CAT and POD activities. Thus, low level of ascorbic acid content in *V. unguiculata* probably made this species more sensitive towards UV-B stress while reverse effect was noticed in *V. radiata.* There are two possibilities regarding increased ascorbic acid content; either its synthesis has been amplified or its regeneration through Asada-Halliwell pathway has been increased as observed in *Ulva fasciculata* [50]. Rao et al. [61] also found enhancement in ascorbic acid content in *Arabidopsis thaliana* exposed to UV-B radiation. Thus, ascorbic acid participates in removal of H_2O_2 as a substrate of ascorbate peroxidase, directly reduces superoxide, quenches singlet oxygen and regenerates reduced α-tocopherol [50]. Agarwal and Pandey [62] concluded the increased ascorbic acid content in *Cassia* seedlings as a key factor to control the oxidation at the membrane level limiting the increase in H_2O_2 content and lipid peroxidation. Ascorbate and glutathione both are constituents of the Ascorbate-Glutathione Cycle, which detoxify H_2O_2 through a series of enzyme reactions [63]. Higher amount of ascorbic acid in cells is expected to provide greater protection to sulphydryl group, a functional integrity of protein molecules thus, *V. radiata* seedlings exhibited greater tolerance to tested stress. Like ascorbic acid, proline content has also been shown to be up-regulated in plants exposed to various stresses such as drought, salt and UV-B [49,64,65]. Unlike ascorbic acid, even in untreated leaves proline content was significantly high in *V. unguiculata* (Fig. 3b). Enhanced UV-B exposure caused substantial increase in the proline content in the leaves of both the *Vigna* species seedlings; however, a declining trend was recorded at high UV-B dose (90 min exposure). In consonance with our finding, increased content of proline in response to UV-B stress in plants was also noticed [24]. The accumulation and protective effect of proline has been observed in plants and bacteria as well as in protozoa, algae and marine invertebrates [22,23]. It may act as a regulatory or signaling molecule to activate multiple responses that are part of the adaptative process [24]. In contrast to this, several researchers have the view that proline accumulation is a symptom of injury, which does not confer tolerance against stresses [25].

The UV-B absorbing pigments (flavonoids) offer the first line of defense particularly against UV-B as they are primarily located in leaf epidermal layers, preventing its penetration to the leaf

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interior. This view was further confirmed by the experimental results obtained by Bornman and Vogelmann [66] where they have applied a fiber-optic microprobe to understand the importance of UV-B absorbing pigments in leaf epidermal layers. Duan et al. [67] noticed that drought stress induced accumulation of UV-B absorbing pigments in leaves of *Populus yunnanensis* and thus, the plants exhibited more resistance to UV-B radiation. In present study significantly high flavonoid contents was noticed in leaves of *V. radiata* control seedlings, while *V. unguiculata* leaves showed quite low amount (Fig. 3c). There was a progressive rise in the level of UV-B absorbing pigments in *V. radiata* throughout tested UV-B exposure time, while in *V. unguiculata* after 45 min of UV-B exposure, a declining trend was noticed. Similarly, a remarkable increase in the flavonoid contents of the UV-B exposed *V. unguiculata*, wheat seedlings, barley and *Vaccinium uliginosum* in field and control conditions was also reported by earlier workers[7, 51, 68, 69] .

The study concludes that UV-B exposure caused significant reduction in growth in both the species of *Vigna*; however, the diminishing effects were more prominent in *V.unguiculata.* The heavy accumulation of ROS and associated membrane damage confirm the higher sensitivity of *V. unguiculata* seedlings to UV-B irradiation. The UV-B exposure at low dose enhanced the antioxidant enzyme activities, while high doses were found to be inhibitory in both the *Vigna* species seedlings. In spite of high SOD, CAT and POD activities; ROS accumulation was appreciably high in *V. unguiculata* leaves. Appreciably high amount of ascorbic acid and UV-B absorbing pigments in *V. radiata* leaves render it as the resistant species towards UV-B while the reverse is apparently true for *V. unguiculata*.

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