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ANTIOXIDANT POTENTIAL OF INDIAN MEDICINAL PLANT *PHYLLANTHUS AMARUS* L. UNDER SUPPLEMENTARY UV-B RADIATION

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Abstract

The stratospheric ozone depletion and enhanced solar ultraviolet-B (UV-B) irradiance may have adverse impact on living organism. The impact of UV-B radiation (UV-B, 280~320nm) on growth, biochemical and antioxidant enzymes activity was studied in *Phyllanthus amarus* (L.) seedling, commonly used as a green manure. The supplementary UV-B radiation significantly decreased the growth, development and changes in UV-B absorbing compounds such as anthocyanin and flavonoids. The antioxidant enzymes were unaffected and showed an enhanced activities in peroxidase, superoxide dismutase, Polyphenoloxidase and phenylalanine ammonia- lyase except catalase in UV-B irradiated seedling. *Phyllanthus amarus* seedling tries to counteract high level of reactive oxygen species produce under UV-B stress through the increased activities of antioxidant enzyme. We suggest that *Phyllanthus amarus* is resistant against UV-B radiation damage and the possible negative effect of additional UV-B radiation on the growth of seedling may have been effectively balanced by the UV-B radiation stress through increase in UV absorbing compound and antioxidant enzymes.

Key Words: Antioxidant; Flavonoids; *Phyllanthus amarus*; Oxidative stress; UV-B radiation.

Introduction

The stress affecting plants are numerous and often species or even variety or location specific. They induce drought, high salinity, temperature extremes, water logging, mineral nutrients deficiency, metal toxicity, pollutants and ultraviolet–B (UV-B) radiation [1]. It has been established that depletion of the stratosphere ozone layer is increasing the level of ultraviolet-B radiation reaching the earth surface [2]. Scenarios based chemistry-climate models shows that in the middle of 21st century, UV-B radiation at ground level is enhanced due to high concentration of greenhouse gases and halogenated species [3].

A wide range of morphological, growth, biochemical, and physiological responses of plant have been reported to elevate UV-B radiation [2, 4, 5]. Plant have developed a complex biochemical defense system that including carotenoids and flavanoids. Flavonoid compounds, as secondary metabolites are considered to play a major role in protecting plants from UV-B damage [6]. These flavonoids generally absorb the light in the region of 280~320 nm and thus are capable of acting as a UV filter, thereby protecting the photosynthetic tissues from damage [7]. Flavaonoids stabilize and protect the lipid phase of the thylakoid membrane, and are quenchers of the excited triplet state of chlorophyll and singlet oxygen [8]. A part from the flavonoids, carotenoids also having antioxidant property, which act as an internal filter against UV-B radiation. Plants scavenge reactive oxygen species by detoxification mechanism produced by enzymatic antioxidant such as catalase, Peroxidase, Superoixde dismutase and Phenylalanins ammonia–lyase etc [9]. Some plants are more tolerant to UV-B than other because they produce a variety of secondary metabolites that effectively absorb UV-B and prevent it from penetrating in to the leaf mosophyll cells. The aim of this work was to investigate growth, UV-absorbing pigments, and antioxidant defense mechanism of *Phyllanthus*

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amarus seedling against UV-B radiation under field condition.

Materials and Methods

Plant material and UV-B exposure

Seeds were surface sterilized with 0.2% $CuSo_4$ for 12h and grown in plastic trays containing a soil mixture of sand (70 per cent) and compost (30 per cent). Seeds were grown in 16/8h light /dark photoperiod. After germination the selected seedlings were transferred in to field and subjected to ultraviolet B radiation with Philips sun lamps (Philips TL 20 W/12, N.V. Philips Gloelampenfabrickan, Holland) was installed 15 cm above the seedling and oriented in an east west direction. UV radiation was filtered through cellulose acetate filter paper to avoid transmission of wave length below 290 nm, and controls were exposed to normal day light. Seedlings were irradiated for 2 hours per day (11.00 to 13.00h) for 8 days.

Growth parameters and photosynthetic pigments

Total length of the seedlings and fresh weight were measured immediately after removing the seedling from the experimental field. Leaf area was calculated by using the Licor 3100 leaf area meter (LICOR, model LI- 3100, Lincoln, USA). For leaf thickness, leaf bits of 0.5X1.5 cm were cut and cross sections of leaves were prepared by using a rotary microtome. Chlorophyll content was estimated by the procedure of moran and Porath [10] using the formulae suggested by Inskeep and Bloom [11]. Fresh leaf disc of 100mg were cut and placed in a test tube containing 10 ml of N, N' – dimethyl formamide (DMF) and stored for 24h at 4ºC. The colored supernatant was used for chlorophyll estimation. By reading the absorbance 647 and 666 nm in a spectrophotometer with DMF as blank for chlorophyll.

Anthocyanin content was extracted in acidified methanol (1:99 – HCl: methanol) in 100 mg of leaf material. Extract was kept at 0ºC for 24 hours. After 24h, the content was made up to 10ml and the absorbance was read at 530 nm, as described by mancinelli et al.,[12]. Flavonoids were extracted and quantified by mirecki and Teramara [13] method. 100 mg of leaves were placed in 80 per cent acidified methanol (methanol: water: HCl– 80: 20:1) for 12 hours in dark at 4ºC to extract flavonoids and the absorbance was read at 315 nm.

Measurement of the Activities of Catalase, Peroxidase and Superoxide dismutase

Catalase (CAT, EC 1. 11.1.6) activity was measured by the method of machly and chance [14]. One gram of leaf tissue was homogenized in 10ml of 0.1 mM sodium phosphate buffer, pH 7 and centrifuged at 4ºC for 10 minutes at 10,000g. An aliquot of 1ml of supernatant of the enzyme extract was added to the reaction mixture containing 1ml of 0.01 M H_2O_2, 3ml of 0.1M sodium phosphate buffer, pH 6.8. The reaction was stopped after on incubation of 5 minutes at 20ºC by addition of 10ml of 1 per cent H_2SO_4. The acidified medium without or with the enzyme extract was titrated against 0.005N $KMnO_4$.

Peroxidase (POX, EC 1.11.17) activity was assayed by the method of Kumar and Khan [15] and Superoxide dismutase (SOD, EC 1.15.1.1) activity by Beauchamp and Fridouich [16]. 1g of leaves were homogenized with 20ml of ice cold extraction medium containing 2M on $MgCl_2$, 1mM EDTA, 10mM β - Mercaptoethanol, 7 per cent PVP and 10 mM sodium metabisulphate. The homogenate was strained through two layers of cheesecloth and centrifuged at 10,000 g for 5 minutes and the supernatant was made up to 20ml with the same buffer and it was used as the source of enzyme.

Polyphenoloxidase (EC 1.10.3.1)

Polyphenoloxidase activity was assayed by the method of Kumar and Khan [15]. Assay mixture of polyphenoloxidase contained 2ml of phosphate buffer (pH 6.0) 1ml of 0.1M catechol 3.6 and 0.5 ml of enzyme extract. This was incubated for 5 minutes at 25ºC, and then the reaction was stopped by adding 1ml of 2.5 N sulphuric acid. The absorbance of the purpurogallin formed was recorded at 495nm. The enzyme activity was expressed in units. One unit is defined as the amount of purpurogallin formed, which raised the absorbance by 0.1 per minute under the assay condition.

Phenylalanine ammonia- lyase (EC 4.3.1.5)

Phenylalanin ammonia- lyase was determined by following the method of Bruseke [17]. 500 milligram fresh leaves were homogenized in 5 ml at cold 25 mM

mercaptoethanol and centrifuged at 12,000 g for 20 minutes. The supernatant was used for assay. An aliquot of 0.2 ml of enzyme extract was added with 0.5 ml borate buffer and 1.3 ml distilled water. The reaction of was initiated by adding 1ml of 0.1 m phenylalanine solution and incubated for 30-60 minute at 32ºC. After incubation the reaction was terminated by adding 0.5 ml of 1M trichloroacetic acid and measured the absorbance at 290 nm, against blank. The reaction rate was expressed as micro mole trans cinnamic acid formed per mg protein per minute.

Statistical Analysis

Each value is the mean of five replicate experiments ($\pm$ SE). The analysis was carried out using statistical package SPSS. All data were subjected to one-way analysis of variance (ANOVA) and the significance of difference between control and each treatment was analyzed using student's t test. Levels of significance used were $P<0.05$.

Results and Discussion

Growth Parameters

Ambient UV radiation exerted a clear effect on the growth parameters, biochemical and antioxidant enzyme of *Phyllanthus amarus* seedling. In our study, foliar symptoms such as glazing, wrinkling, chloretic lesions and nercosis are observed and similar observations were also noticed by Feng et al., [18,55]. These foliar damages might be due to reduction in photosynthetic pigments [19] and cell and tissue damage in upper epidermis [20]. According to Teramura [21] interveinal wrinkling and leaf chlorosis were observed in UV-B treated plants. These observation conclude that interveinal wrinkling is the result of UV-B effect on cell division and development.

In the present study after eight days of UV-B treatment, the shoot length, fresh weight, dry weight and leaf area were reduced to 23.44%, 37.3%, 28.9% and 28.7% respectively. However, leaf thickness increased with increasing day after UV-B radiation treatment. The highest increase of 16 per cent was observed after 8 days in treated seedling. Krause et al., [22,55] observed that UV-B radiation reduced the height of spring wheat. However, field study indicates that plant growth is less sensitive to UV-B radiation. Teramura and Murali [23,55] observed that the reduction on plant growth under UV-B radiation in soybean is between 26 and 38 per cent in green house and between 11 to 22 per cent in field study. Increase in shoot length is due to IAA, which absorbs in the UV-B range and is readily destroyed by UV-B *in vitro* and *in Vivo* [24].

UV-B radiation significantly depressed the biomass accumulation of many species such as rice [25], *Vigna mungo* [26] and *Trifolium repens* [27]. Decrease in dry weight observed in the present study may be caused by reduced photosynthesis rates and enzyme activities. The changes in the leaf area measurements observed under supplemental UV-B radiation were similar to those seen for fresh and dry weight. In musfard, leaf area reduction was observed under UV-B treatment [28]. Similar trend in leaf area reduction have also been observed in Potato [29], Cotton plans [30] and spring wheat [18]. According to Krizek et al., [31] reduction in leaf area could be an adaptive mechanism to minimize the exposure to UV-B radiation. There are also report correlating the tolerance of the species to their ability to increase leaf thickness in response to UV-B exposure [18, 32].

Biochemical Constituents

The supplemental UV-B radiation significantly reduced the chlorophyll content (20.1%) and carotonoid (30.3%) throughout the study period except during the initial period. The chloroplast is the first organize to show injury response when irradiated with UV-B radiation [33]. In a field experiments with *Vigna radiata,* Pat et al., [34] observed an initial increase and subsequent decrease in chlorophyll content, which is also reflected in the present study. In our study, an initial increase in chlorophyll was observed and this might be due to accumulation of UV-B absorbing pigments. The carotenoid content gradually declined due to UV-B radiation treatment. Previous work demonstrated that carotenoid content decreased under UV-B radiation [35]. The reduction in carotenoid content may result either from inhibition of synthesis or from break down of the pigments [36]. Since, the carotenoid are involved in the light harvesting and protection of chlorophyll from photo oxidative destruction, any reduction in carotenoid could have serious consequences of chlorophyll pigments.

Table: 1. Effect of UV-B radiation on shoot length, fresh weight, dry weight, leaf area and leaf thickness in *Phyllanthus amarus* L. Seedlings

Day (s)	Treatments	Shoot length (cm)	Fresh weight (g plant $^{-1}$)	Dry weight (g plant^{-1})	Leaf area (cm^2 plant $^{-1}$)	Leaf thickness (mm)
2	Control	3.6 ± 0.18	0.42* ± 0.021	0.25 ± 0.01	0.32 ± 0.02	0.07 ± 0.03
	UV-B	3.9 ± 0.19	0.45 ± 0.023	0.26* ± 0.01	0.31 ± 0.02	0.08 ± 0.03
4	Control	5.8* ± 0.29	0.50 ± 0.03	0.29 ± 0.02	0.35* ± 0.02	0.08 ± 0.03
	UV-B	5.1 ± 0.25	0.47 ± 0.03	0.28 ± 0.01	0.33 ± 0.02	0.10* ± 0.04
6	Control	7.5* ± 0.38	0.55* ± 0.03	0.33* ± 0.02	0.40* ± 0.03	0.85 ± 0.045
	UV-B	5.8* ± 0.29	0.49 ± 0.03	0.31* ± 0.02	0.35* ± 0.02	1.09 ± 0.07
8	Control	8.9* ± 0.44	0.64* ± 0.04	0.38* ± 0.03	0.46* ± 0.03	0.92* ± 0.06
	UV-B	6.1* ± 0.31	0.52* ± 0.03	0.32* ± 0.02	0.37* ± 0.03	1.18* ± 0.08

Results are means ± S.E. of 5 replicates. Significant level (p) for Student 't' test is shown of *p<0.05

Table: 2. Effect of UV-B radiation on Chlorophyll, Carotenoid, Anthocyanin and Flavonoid in *Phyllanthus amarus* L. Seedlings

Day (s)	Treatments	Chlorophyll (mg g^{-1} fr. wt.)	Carotenoids (mg g^{-1} fr.wt.)	Anthocyanin (A_{530} g fr.wt.)	Flavonoid (A_{315} g fr. wt.)
2	Control	1.82 ± 0.99	0.12 ± 0.06	0.03 ± 0.01	0.100* ± 0.05
	UV-B	1.83 ± 0.99	0.13* ± 0.07	0.05 ± 0.02	0.200* ± 0.10
4	Control	1.91* ± 0.09	0.135* ± 0.07	0.037 ± 0.02	0.115* ± 0.05
	UV-B	1.84* ± 0.10	0.132 ± 0.08	0.070* ± 0.03	0.280* ± 0.14
6	Control	2.12* ± 0.12	0.148* ± 0.08	0.044* ± 0.02	0.123* ± 0.06
	UV-B	1.95* ± 0.10	0.138* ± 0.09	0.097* ± 0.05	0.356* ± 0.17
8	Control	2.40* ± 0.13	0.162* ± 0.09	0.054 ± 0.02	0.133* ± 0.077
	UV-B	2.03* ± 0.11	0.145* ± 0.09	0.132* ± 0.06	0.448* ± 0.02

Results are means ± S.E. of 5 replicates. Significant level (p) for Student 't' test is shown of *p<0.05

Table: 3. Effect of UV-B radiation on Catalase, Peroxidase, Polyphenol oxidase, superoxide dismutase and phenylalanine ammonia-lyase in *Phyllanthus amarus* L. Seedlings

Day (s)	Treatments	Catalase (μ mol H_2O_2 decomposed min^{-1} g^{-1} fr.wt.)	Peroxidase (μ mol pupurogallin formed min^{-1} g^{-1} fr.wt.)	Polyphenol oxidase (μ mol purpurogallin min^{-1} g^{-1} fr.wt.)	Superoxide dismutase (μ mol annamic acid min^{-1} g^{-1} fr.wt.)	Phenylalanine ammonia-lyase (units h^{-1} mg^{-1} protein fr.wt.)
2	Control	7.8 ± 0.39	22.6 ± 1.13	19 ± 0.95	12.7 ± 0.64	8.4 ± 0.42
	UV-B	6.9 ± 0.34	28.9 ± 1.45	22 ± 1.10	14 ± 0.70	11.2 ± 0.56
4	Control	9.3* ± 0.46	26.4* ± 1.32	22.7* ± 1.14	14.9 ± 0.75	9.9 ± 0.49
	UV-B	8.1* ± 0.40	36.4* ± 1.82	32.5* ± 1.63	18.2* ± 0.91	14.8* ± 0.74
6	Control	10.3 ± 0.52	27.4* ± 1.37	23.5* ± 1.18	15.6* ± 0.78	10.2* ± 0.51
	UV-B	8.4* ± 0.42	40.9* ± 2.05	33.5* ± 1.68	21.5* ± 1.08	16.4* ± 0.82
8	Control	11.6* ± 0.58	28.5 ± 1.43	25.1* ± 1.26	16.5* ± 0.83	10.9* ± 0.55
	UV-B	8.7* ± 0.44	47* ± 2.35	38* ± 1.90	24.5* ± 1.23	19.1* ± 0.96

Results are means ± S.E. of 5 replicates. Significant level (p) for Student 't' test is shown of *p<0.05

UV- Absorbing pigments

Increased anthocyanin and flavonoid content was observed in UV-B treated seedling. The highest accumulation of anthocyanin (84%) and flavonoids (91%) were noticed in UV-B treated seedling after eight days of treatment when compared to control seedlings. Anthocyanin concentration was significantly increased in UV-B radiation treatment, when compared to control seedlings. Several studies have shown that increased anthocyanin was mainly due to UV-B irradiation effect [37,38]. Ravindran et al., [39]demonstrated over 171 per cent increase in anthocyanin content in a halophyte species, *Suaeda maritima* under field study. Ambasht and Agarwal [40] observed that over 275 per cent increase in the anthocyanin content in maize. Anthocyanin have very weak absorption in the UV-B regions and regarded as UV screens only at very high concentration. Olssen et al., [41] suggested that UV-B induced accumulation of anthocyanin which protect the photosynthetic apparatus from the damaging effect of UV-B radiation. Similar to anthocyanin, flavonoid concentration was also increased in UV-B treated seedlings after eight days of treatment. In general, the flavonoid accumulation is linearly depends on UV-B fluence [18, 42]. Feng et al., [22] concluded that flavonoid concentration can reduce the UV-B penetration and protect the photosynthetic apparatus upto some extent, but if depends up on a threshold level, which may vary in different species. Accumulation of flavonoids is also considered a defense mechanism against UV-B radiation and protect the mesophyll tissue through epidermal screening. UV-B screening by epidermal flavonoids is often proposed as an adaptive mechanism to prevent this radiation from reaching the mesophyll.

Antioxidant enzymes

Most physiological stresses including UV-B enhancement to disturb plant metabolism and cause oxidative injury by enhancing the production of reactive oxygen species. The metabolism of reactive oxygen species depend on low molecular anti-oxidant systems as well as enzymes such as super oxide dismutase, peroxidase, polyhenoloxidase and phenylalanine ammonia-lyase. In the present study the catalase activity was inhibited under supplemental UV-B radiation treatment. The highest decrease of catalase activity (22.7 per cent) was observed after eight days in treated seedlings. In contrast to catalase activity, peroxidase, polyphenol oxidase, superoxide dismutase and phenylalanine Ammonia- lyase activities were increased (36.5%, 39.9%, 41.5% and 40.7% respectively) under supplemental UV-B radiation. This reduction trend in catalase activity under UV-B radiation was also observed previously by Baumbush et al., [43] and Yang et al., [44]. Catalase is the most efficient antioxidant enzyme which protects plants by scavenging free radicals and H_2O_2 [45]. Decreased catalase activity in the vtc1 mutants of Arabidopsis thaliana during course of the UV-B exposure experiment was observed and it could be due to destruction of the peroxisome via rampant lipid peroxidation [46].

Along with catalase activity, peroxidase activity was also an important component of antioxidant defense system for scavenging H_2O_2. In the present study, the peroxidase activity increased with increasing treatment period of supplemental UV-B radiation. Yannarelli et al., [47] demonstrated that phenol–oxidizing peroxidase unlikely contribute to UV tolerance as a result of their oxygen radical scavenging activity.

Polyphenol oxidase is also responsible for the oxidation of phenolic compound [48]. Several studies have shown increased polyphenol oxidase due to UV-B irradiation [49, 50]. Enhanced UV-B radiation activates antioxidant enzymes and induces polyamines but also cause damage in the leaves, exemplified by an increase in polyphenol oxidase activity and a decrease in chlorophyll concentration. Superoxide dismutase showed a linear increase in activity throughout the study period. UV-B caused 325 per cent increase in superoxide dismutase activity when compared to control [51]. Similar to superoxide dismutase, Phenylalanine ammonia-lyase activity was also increased under supplemental UV-B radiation treatment. Studies in cucumber [52] have shown that exposure of seedlings to supplemental UV-B radiation caused 78 per cent increase in the activity of phenylalanine ammonia- lyase [53]. Similar enhancement of phenylalanine ammonia-lyase was also observed by Brawn and Tevini [54]. Phenylalanine ammonia-lyase is an important enzyme in regulating flavonoid biosynthesis and transcriptionally induced by UV-radiation [67]. Thus, it concluded in the present study, increase in Phenylalanine ammonia-lyase activity, stimulate the synthesis of flavonoid and anthocyanin.

Our results shows that after eight days of treatment, *Phyllanthus amarus e*xhibits a different sensibility to supplemental UV-B radiation. UV-B adversely affected the growth parameters and photosynthetic pigments.

However, accumulation of UV-B absorbing compounds and antioxidant enzymes content indicates that supplemental UV-B radiation induces a photosynthetic protection mechanism in a short time growth period. Finally, it suggests that antioxidant enzymes and UV-B absorbing compounds provide protection during oxidative injury. The balance between the antioxidant enzymes and UV-absorbing compounds contribute the growth and development of *Phyllanthus amarus* seedling under the oxidative stress condition.

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