

EFFECT OF ENHANCED UV-B RADIATION ON GERMINATION, SEEDLING GROWTH AND BIOCHEMICAL RESPONSES OF *VIGNA MUNGO* (L.) HEPPEL

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Abstract

The study focuses on the effect of supplemental UV-B radiation on germination, seedling growth, chlorophyll a and b contents, soluble phenols, anthocyanins, flavones contents, phenylalanine ammonia lyase (PAL) activity and tyrosine ammonia lyase (TAL) activity of mash-bean (*Vigna mungo* (L.) Hepper). Even though the germination velocity was substantially increased, the final germination percentage remained significantly suppressed by UV-irradiance. Both root and shoot growth of the seedlings were markedly reduced by enhanced UV-B radiation. UV-B irradiation substantially decreased both chlorophylls a and b and the total amount of chlorophyll a plus b compared to controls. However, chlorophyll a/b ratio was generally elevated. Remarkable accumulation of total soluble phenols occurred in response to UV-B radiation. PAL activity increased markedly as a result of UV-stress in the beginning, subsequently, however, it declined, whereas, TAL activity consistently increased over the controls following UV-B irradiation up to 8 days of treatment. The levels of anthocyanins and flavones increased in treatments over the controls as they provide a protective mechanism to UV-B radiations. In general, the growth and physiological responses to UV-B radiation were more pronounced at greater exposure period.

Introduction

Thinning of Stratospheric ozone layer and global climatic changes are the major threats to the life on earth. The ozone layer exists at an altitude of 10-30 Km around the earth. The depletion of stratospheric ozone has led to enhanced penetration of solar UV-B (280-320 nm) radiation through the atmosphere, reaching earth's surface (McKenzie *et al.*, 1999). Unfortunately, the protective ozone layer is continuously being damaged because of human activity i.e., by ozone depletion substances including chlorofluorocarbons and other industrial products that contain halogens (Kerr, 1988). The resulting increased levels of UV radiation can be harmful for all life forms, plants, animals and even microorganisms. Madronich *et al.*, (1998) showed that 2% biologically effective UV radiation can be increased by 1% depletion of ozone layer. Exposure to enhanced levels of UV-B radiation has been demonstrated to damage DNA (Landry *et al.*, 1997), proteins (Strid *et al.*, 1994), cell membranes and the chloroplasts and its associated pigment system (Day & Vogelmann, 1995; Greenberg *et al.*, 1997) in many plants. In human beings UV-light may cause cataracts, skin cancer, herpes suppression of immune system etc. (Brian & Taylor, 2001).

A number of studies have demonstrated that UV-B can induce some general stress responses and other physiological and photomorphogenic responses (Mackerness, 2000; Jansen, 2002; Ravindran *et al.*, 2008). Numerous studies have demonstrated significant impact of enhanced UV-B radiation on growth, development, biomass accumulation, yield and metabolism of plants (Rozema *et al.*, 1997; Gao *et al.*, 2003; Ravindran *et al.*, 2008). Some studies have also shown the inhibition of stem growth thereby altering the shoot morphology (Kim *et al.*, 1998; Kobzar *et al.*, 1998). Mechanisms such as increased leaf thickness alterations in cuticle and

increased production of UV-B protective pigments have been investigated in different plant species (Gwynn-Jones, 2001). Enhanced UV-B radiation due to 5% simulated ozone resulted in the depletion of biomass and leaf area (Barnes *et al.*, 1993). Greenberg *et al.*, (1997) stated that UV-B absorbing compounds and chlorophylls (physiological parameters) have been found to be useful indicators of UV-B sensitivity and tolerance. If protective mechanism fails to protect the genome and photosynthetic machinery against UV-B, repair mechanisms are relied upon (Takeuchi *et al.*, 1998). One protective mechanism which seems to be common under stress conditions is the increase in the phenol content (Kozłowska *et al.*, 2007; Ravindran *et al.*, 2008). Exposure to near ambient UV-B results in increase in leaf phenolic content in soybean plants (Zavala *et al.*, 2001). UV-B can accelerate the biosynthesis of plant flavonoids and anthocyanins (Ravindran *et al.*, 2008) and other phenolic compounds (Figuerola *et al.*, 2009) which serve to protect the sensitive tissues from UV-B radiations (Beggs & Wellman, 1994).

L-Phenylalanine ammonia lyase (PAL) which catalyses the formation of trans-cinnamate from L-phenylalanine by non-oxidative deamination occurs in most plants and in some fungi (Kalghatgi & Subba-Rao, 1975). Consequently, the importance of PAL is that it catalyses the first committed step in the biosynthesis of defense related phenylpropanoids. Thus stress conditions generally result in increased PAL activity of plant tissue (Zucker, 1965; Pegg & Sequeira, 1968; Chmielowski *et al.*, 2008). Lavola *et al.*, (2008) demonstrated that UV-B radiation significantly increased PAL activity in birch seedlings. The products of PAL and TAL (tyrosine ammonia lyase) are modified through phenylpropanoid metabolism including, lignin, flavonoids and pigments and phytoalexins that play a key role in a range of diseases and stresses (Morrison & Buxton, 1993).

Despite several studies on the physiological responses of plants to UV-B radiation, no literature is available on the growth and physiological responses of mash-bean *Vigna mungo* (L.) Hepper, an important bean crop in the Indo-Pakistan sub-continent. This study focuses on germination, seedling growth, development and physiological and biochemical responses of mash-beans (*V. mungo*) to supplemental UV-B radiation.

Materials and Methods

Germination conditions: The seeds of mash-bean (*Vigna mungo* (L.) Hepper variety Mash-2 (NARC) used in the current study were obtained from Pakistan Agricultural Research Council, Karachi. Mash-bean is an important pulse (bean) crop that is rich in proteins and cultivated widely in Asia and Africa. Clean seeds of *Vigna mungo* were first surface sterilized with 0.5% Sodium Hypochlorite for 2 min., rinsed and soaked in distilled water for 2 h and then 20 seeds were placed in 9 cm diameter sterile Petri plates fitted with two discs of Whatman No.1 filter paper, subsequently transferred to radiation chamber and exposed to fluorescent UV-B tube. The chamber was covered by wooden lid for safety reasons. Within the chamber a UV-B fluorescent tube (TL40W/12, Philips, Eindhoven, The Netherlands) was installed which exhibited its emission >280nm to a maximum at 312 nm (the actual UV-B range is 280-320nm). Acetate paper was fitted above the plant canopies that cuts off any radiations below 280nm. The Petri plates containing 20 mung-bean seeds, moistened with distilled water for 0.5 h, were exposed for 10, 20, 30, and 40 minutes to UV-B radiation. There were five replicates for each treatment and control. Five ml sterile distilled water was added to each Petri plate. For germination study Petri plates were kept at 28°C and 50% humidity on a laboratory bench. Day light was supplemented by light from two fluorescent tubes (1000 Lux). Observations on germination were recorded daily. Small amounts of distilled water were added periodically to keep the Petri plates moist. Germination was recorded daily up to 10 days. At the end of experiment root and shoot lengths, of the seedlings and their fresh weights were recorded. Germination velocity (GV) was measured using the index proposed by Khandakar & Bradbeer (1983), as follows:

$$GV = [N_1/1 + N_2/2 + N_3/3 + \dots + N_n/n] \times 100/1$$

where $N_1, N_2, N_3, \dots, N_n$ are the proportion of seeds that germinated on day 1, 2, 3, ..., n respectively.

Estimation of chlorophyll: Two-hour soaked seeds were exposed to UV-B radiation for 0 (control), 10, 20, 30 and 40 min and the seedlings grown as outlined above, subsequently at 12th day chlorophyll in the first leaf and the cotyledons was determined. Chlorophyll a and b were extracted from the irradiated shoots and estimated by the method of Maclachlan & Zalik (1963). For extraction 0.5g of shoots were grounded in 10ml of 80% (v/v) acetone and centrifuged at 2000 rpm for 15 minutes to obtain clear suspension. Supernatant, which contained soluble

pigments, was used for determination of chlorophylls. Absorbance of the extract was recorded at 663 and 645 nm on Shimadzu UV-1201 spectrophotometer against 80% (v/v) acetone blank.

Total soluble phenols: Total soluble phenol contents of treatments and controls were measured in the root tissue of *V. mungo* according to Gonzalez *et al.*, (2003) with minor modifications. Root tissues (500 mg) were taken from seedlings in each Petri dish and homogenized in an ice bath with 2 ml cold 80% ethanol (v/v). The homogenate was centrifuged at 8000 g for 3 min. One hundred μ l of the supernatant was added to 0.5 ml Folin-Ciocalteu reagent and 1 ml of 20% Sodium carbonate. Subsequently, distilled water was added to make a final volume of 10 ml. The mixture was incubated at 40° C for 30 min., and the absorbance of the developed blue color was read at 750 nm using a Shimadzu UV-1201 spectrophotometer. Catechol was used as standard.

Phenylalanine ammonia lyase (PAL) and tyrosine ammonia lyase (TAL) activity: Fresh root tissue were homogenized with chilled 50 mmol Tris-HCl (pH 8.8, 1/10 w/v) supplemented with 0.5 mmol EDTA and 1% Polyvinyl pyrrolidone. The homogenized suspension was obtained by centrifuging at 12000 rpm for 10 min at 4°C. The supernatant was used for the assay of PAL and TAL activity. PAL activity was measured as the rate of conversion of L- phenylalanine to trans-cinamic acid in accordance with Dickerson *et al.*, (1984). The enzyme extract 0.4 ml was incubated at 37°C in 0.5 ml of 0.1 M borate buffer (pH 8.8) to which was also added 0.5 ml of 12 mM L-phenylalanine. The reaction was terminated with 0.3 ml of 6N HCl and the absorbance was recorded at 290nm. The extraction and incubation procedures for tyrosine ammonia lyase (TAL) were the same as described above. TAL activity was measured using L-tyrosine as the substrate (Beaudin-Eagan & Thorpe, 1985). The product, p-coumaric acid was measured spectrophotometrically recording absorbance at 333nm.

Anthocyanins and flavonoids: UV-absorbing pigment anthocyanins were determined spectrophotometrically. Fresh leaves from seedlings were ground and extracted in acidified methanol (1: 99 HCl: methanol, v/v) using 100mg of leaf tissue. The extract was kept at 0°C for 24h., the content was made up to 10 ml and the absorbance was noted at 530nm as described by Mancinelli *et al.*, (1975). Flavonoids were extracted and measured as described by Mirecki & Teramura (1984). 100 mg of fresh leaves were placed in 80% acidified methanol (methanol: water: HCl–80:20:1 v/v/v) for 12 min., in dark at 4°C to extract flavonoids and the absorbance recorded at 315 nm.

Statistical analysis: The data were subjected to appropriate statistical analysis which included the two-way analysis of variance (ANOVA) using UV=B radiation and time as factors, and a post-hoc test viz., Duncan's multiple range test (DMRT) following Zar (1999). Computer programs for all the statistical analyses

were developed by the first author (S.S.S.) in C++ and are available on request at a nominal cost.

Results

Seed germination: Table 1 shows that the final germination percentage was significantly reduced (p at the most 0.05) at 20, 30 and 40 min., exposures to UV-B radiation compared to the controls (Table 1). On the other hand, germination velocity (GV) increased markedly at 10 and 20 min., of irradiation to UV-B radiation though it was markedly retarded at 30 and 40 min., exposure relative to controls (Table 1). It is worth mentioning that the cotyledons and first leaf growth of the seedlings were suppressed. In addition, the UV-B exposed seedlings were never straight but slightly twisted with brown spots (numerical values for these remained unrecorded).

Table 1. Germination percentage and germination velocity (GV) of mash-bean *Vigna mungo* wet seeds exposed to various durations (min) of UV-B radiation.

Treatments UV-B exposure	Final germination (%)	Germination velocity (GV)
0 (control)	94.7a \pm 1.5	28.4
10 min	89.5a \pm 2.5	32.7
20 min	81.5b \pm 2.8	33.3
30 min	73.3c \pm 3.5	23.2
40 min	70.5c \pm 2.7	20.5

Mean \pm 1 SE. Means not followed by the same letter are significantly different from each other at $p \leq 0.05$. Each mean is of five replicates

Seedling growth: Although root growth remained unaffected at 10 min., exposure to UV-B radiation, it was significantly retarded in the treatments (20, 30, and 40 min., UV-B irradiation ($p < 0.05$) compared to unexposed controls (Table 2). By contrast, shoot growth was significantly (P at the most 0.05) suppressed at all the UV-B exposure periods. Both root and shoot dry weights declined significantly (P at the most 0.05) compared to controls.

Chlorophylls: As a result of exposure of mash-been seeds to UV-B radiation, both chlorophyll a and b were significantly declined (p at the most 0.05) relative to controls (Fig. 1). The total chlorophyll (a plus b) were also reduced markedly (P at the most 0.05) relative to controls. The chlorophyll a/b ratio, was however, depressed owing to the fact that reduction in chlorophyll a content was more pronounced compared to that of chlorophyll b (Fig. 1).

Anthocyanins, flavonoids and soluble phenols: Following exposure to UV-B irradiation both the anthocyanin and flavone contents of the seedlings were elevated significantly (p at the most 0.05) at all the exposure periods except for 10 min (Table 3). Interestingly, the increase in flavonoid content over the controls was more pronounced than that of anthocyanins. About four-fold increase in flavonoid content was observed in response to UV-B radiation exposure for 40 min. when compared with the controls. For anthocyanins the increase at 40 min., exposure was about three-fold that of control. A remarkable accumulation of total soluble phenol in the seedlings was recorded over the controls at all the UV-B radiation treatment periods (p at the most 0.05). The rate of accumulation of total soluble phenols was found to be directly related to UV-B exposure period.

Table 2. Effect of UV-B radiation exposure for different duration (min) on root and shoot growth of *Vigna mungo*.

Treatments UV-B exposure	Root length cm	Shoot length cm	Root wt. g	Shoot wt. g
0 (controls)	5.8a \pm 0.25	9.2a \pm 0.82	0.79a \pm 0.045	1.84a \pm 0.26
10 min	5.3a \pm 0.33	7.1b \pm 1.16	0.62b \pm 0.035	1.24b \pm 0.14
20 min	3.9b \pm 0.28	6.4b \pm 0.41	0.54b \pm 0.015	0.90c \pm 0.13
30 min	3.4b \pm 0.22	5.2b \pm 0.44	0.39bc \pm 0.017	1.02c \pm 0.09
40 min	2.5c \pm 0.16	3.8c \pm 0.55	0.36c \pm 0.012	0.95c \pm 0.14

Means \pm 1 SE. Means not followed by the same letter are significantly different at $p < 0.05$. Each value is mean of 5 replicates

Table 3. Effect of UV-B radiation on UV-B absorbing pigments and total soluble phenols.

Treatments UV-B exposure	Anthocyanins A_{530} g ⁻¹ fr. wt.	Flavonoids A_{315} g ⁻¹ fr. wt.	Total soluble Phenols μ g/g
0 (Control)	0.08a \pm 0.03	0.17a \pm 0.11	48.9a \pm 4.2
10 min	0.07a \pm 0.03	0.20b \pm 0.13	68.3b \pm 4.8
20 min	0.12b \pm 0.04	0.41c \pm 0.18	80.2c \pm 5.8
30 min	0.15b \pm 0.07	0.48c \pm 0.20	77.4 c \pm 5.4
40 min	0.22c \pm 0.09	0.67d \pm 0.23	86.6d \pm 7.9

Means not followed by the same letters are significantly different from each other at $p \leq 0.05$

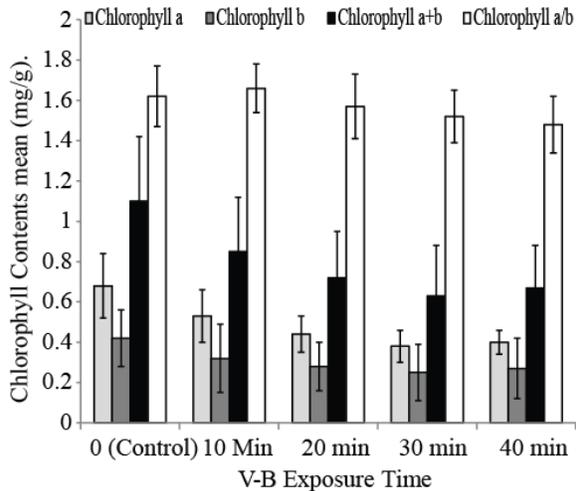


Fig. 1. Effect of UV-B radiation exposure chlorophyll contents (mg/g) of *Vigna mungo* seedlings.

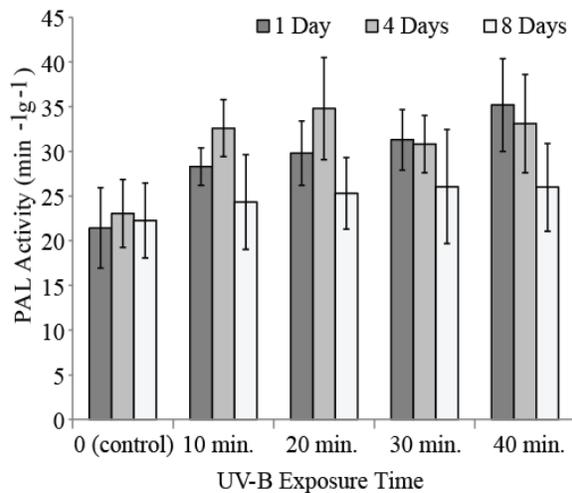


Fig. 2. Effect of UV-B radiation on PAL activity (ca=cinnamic acid) in *V. mungo* seedlings nmoles ca/min/g.

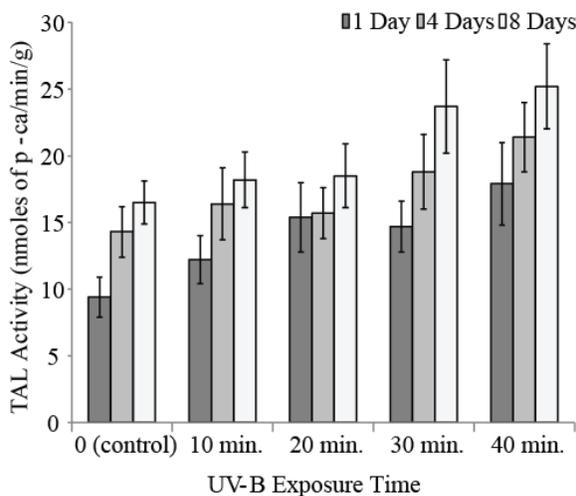


Fig. 3. Effect of UV-B radiation on tyrosine ammonia lyase (TAL) activity (p-ca=p-coumaric acid) in *Vigna mungo* seedlings.

PAL and TAL activities: The results of time course study of phenylalanine ammonia lyase (PAL) activity are outlined in Fig. 2. In response to UV-B exposure (20, 30 and 40 min) the PAL activity was enhanced over the controls up to 4 days after treatment. However, the PAL activity increased both in controls and treatments. Subsequently, it diminished and became closer to the initial values i.e., not differing significantly with that at 1 day after exposure. Compared to controls PAL activity increased from 1 to 4 days but at 8 days of experimental period it did not differ from controls. On the other hand, TAL activity in general tended to increase in both the controls as well as treatments from 1 to 8 days (Fig. 3). UV-B radiation exposure enhanced the TAL activity at all time periods over the controls. Greater increase occurred at greater exposure time to UV-B irradiation.

Discussion

Studies on the effects of UV-B radiations on plants have consistently demonstrated that increasing intensities of UV-B radiations induce several morphological (Day & Demchik, 1996; Furness *et al.*, 1999; Bilger *et al.*, 2001; Frohnmeyer & Staiger, 2003) and a number of physiological/biochemical changes in higher plants (Bilger *et al.*, 2001; Jansen, 2002; Warren *et al.*, 2003; Ranjbarfordoei *et al.*, 2009; Kozłowska *et al.*, 2007; Ravindran *et al.*, 2008). In the present study, UV-B exposure of hydrated seeds of mash-bean for 20, 30, 40 min significantly reduced the final germination percentage but the speed of germination (GV) was substantially increased at 20 and 30 min exposure to UV-B radiation. Our result pertaining to final germination percentage contradicts the findings of many other workers (Tosserams *et al.*, 1997; Dai & Upadhyaya, 2002). On the other hand, Wagne (1966) investigated the effect of UV light on lettuce seeds and noted improvement in germination (at 254-405 nm). Noble (2002) investigated the effect of UV-B irradiation on seeds of four species and found that germination was not affected. However, he showed that the speed of germination was increased which is consistent with the results of the present study.

The results of the experiments clearly demonstrated the deleterious effects of UV-B radiation on mash-bean (*Vigna mungo*) seedlings in terms of resulting physical and biochemical changes. UV-B radiation exposure not only caused decrease in root and shoot growth and their corresponding weights but also resulted in curling and twisting of roots and shoots. These results corroborate the findings of earlier workers (Barnes *et al.*, 1990; Greenberg *et al.*, 1997; Furness *et al.*, 1999; Bilger *et al.*, 2001; Warren *et al.*, 2003; Kozłowska *et al.*, 2007) who reported marked changes in the morphological traits such as reduction in plant height, decreased leaf expansion, curling of leaves, etc. However, plant response to UV-B radiation varies among species (Barnes *et al.*, 1990; Musil, 1995; Cybulski & Peterjohn, 1999) and even in different species of the same genus (Johanson *et al.*, 1995). The differences among species, though not examined here, can be attributed to the mechanism whereby the plants reduce or tolerate the damage inflicted by UV-B radiation. In a comparative study, Furness *et al.*,

(1999) examined the effect of UV-B radiation on three weeds (*Cyanoglossum officinale* L., *Centaurea diffusa* Lam. and *Tragopogon pratensis* L.), the UV-B radiation decreased the growth and leaf area in all three weeds while most susceptible was *Cyanoglossum officinale*. The results of the current experiment show that the level of UV-B radiation used has measurable suppressive impact on root and shoot growth of mash-bean (*V. radiata*) seedlings. The fresh weights of the seedling were consistently reduced following exposure to UV-B radiation which was presumably due to inhibition of photosynthesis (Fegueroa *et al.*, 2009). The suppression of seedling growth could also result due to the accumulation of phenolic compounds synthesized in the plant in response to UV-B stress (Warren *et al.*, 2003).

A significant reduction in chlorophyll a and b content in response to UV-B exposure was recorded in the present study. Similarly, Day & Vogelmann (1995), Ambasht & Agarwal (1998), Skorska (2000) and Ravindran *et al.*, (2008) reported a marked reduction in total chlorophyll (about 50% of the controls). Strid *et al.*, (1990) and Hoffman (1999) also demonstrated significant reduction in chlorophyll content following UV-B irradiation. The decrease in chlorophyll a/b ratio, also reported by Gitz *et al.*, (2004), could be explained on the grounds that the degradation pathway of chlorophyll a is different from that of chlorophyll b. Chlorophyll a may undergo degradation under stress condition first prior to degradation of chlorophyll b. This may account for decreased a/b ratios with increasing UV-B stress.

Our results showed an increase in the content of anthocyanin pigments that play an important role in radiation absorption, following exposure of plants to UV-B radiation. Both anthocyanins and flavonoids are protective pigments that depict marked changes in response to UV-B radiation. The increase in anthocyanin pigments in response to UV-B radiation corroborates the findings of Tevini *et al.*, (1991), Pinter *et al.*, (2007) and Ravindran *et al.*, (2008). Likewise, flavonoid production was promoted in response to UV-B exposure. This is in accordance with the observations of several workers (Warren *et al.*, 2003; Ravindran *et al.*, 2008; Ranjbarfordoei *et al.*, 2009). Accumulation of UV-B absorbing pigments such as flavonoids provides one of the fundamental mechanisms whereby plants alleviate the harmful effects of UV-B radiations.

Exposure of mash-bean hydrated seeds to UV-B radiation resulted in accumulation of soluble phenols in the seedlings. Accumulation of phenols as a result of exposure of plants to UV-B radiation has also been recorded by Ambasht & Agarwal (1998), Gitz *et al.*, (2004), Kozłowska *et al.*, (2007) and Ravindran *et al.*, (2008) which provides a protection against UV radiation. It has been established that phenol metabolism is activated in plants as a reaction to abiotic or biotic stresses (Abreu & Mazzafera, 2005; Olenchenko & Zagorskina, 2005; Ganeva & Zozikova, 2007; Shaikat *et al.*, 2009). Shaikat *et al.*, (1999, 2010) demonstrated that the exposure of plants to heavy metals such as Cd, Cr, Pb and Zn results in the accumulation of soluble phenols. Plant phenolics have been regarded as defenses against pathogens and herbivores (Dixon & Paiva,

1995; Shaikat & Khan, 2009) and provide protective device against a variety of abiotic stresses including stress due to heavy metals, pathogens and UV radiations. Our results provide additional support to this conjecture. Secondary metabolic pathway is physiologically important as it provides the means of channeling and storing carbon compounds, accumulated from photosynthesis during periods when nitrogen is limiting and whenever leaf growth or cotyledons are suppressed, e.g., by UV-B radiations. In this connection it is noteworthy that the cotyledons and first leaf growth was suppressed by the UV-B radiation. The protective role of phenolics may be due to structural stabilization of cell wall through condensation-polymerization of phenols and quinines. Secondly, they can provide photoprotective mechanism *i.e.*, by altering the absorbance of visible and UV-radiation. Thirdly, they act as powerful antioxidant and antiradical agents (Harborne, 2007; Edreva *et al.*, 2008).

PAL activity was enhanced in response to UV-B stress. An increase in PAL activity is symptomatic of plant tissue subjected to some kind of stress (heavy metals, disease wounding, heat shock, UV-B radiation, etc., (Jiang & Joyce, 2003; Chmielowski *et al.*, 2008). However, in our study PAL activity after increasing initially did not differ from the controls at 8 days exposure. By contrast, TAL activity showed consistent increase over the controls up to 8 days of treatment. Interestingly, Khan *et al.*, (2003) demonstrated that both PAL and TAL activities increased up to 36 h following stress treatment and thereafter reached levels close to basal levels of controls. Nevertheless, both these enzymes convert their substrates (amino acids) to phenolic acids that are modified through phenylpropanoid metabolisms to precursors of secondary metabolites including lignin, flavonoids and phytoalexins that provide protection against various forms of stresses.

Conclusions

Exposure of hydrated seeds to UV-B radiation at 20-40 minutes significantly reduced germination though the speed of germination was increased. UV-B exposure not only suppressed root and shoot development but also caused curling and twisting of the seedlings. UV-B radiation suppressed the synthesis of photosynthetic pigments including chlorophyll a and b. Radiation absorbing pigments such as anthocyanins and flavonoids tend to accumulate in response to UV-B irradiation. Total soluble phenols also increase dramatically in response to UV-B exposure.

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