UV-B radiation mediated alterations in the nitrate assimilation pathway of crop plants 1. Kinetic characteristics of nitrate reductase

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Abstract

The kinetics and other characteristics of nitrate reductase (NR, EC 1.6.6.1) in cowpea [*Vigna unguiculata* (L.) Walp.] seedlings irradiated with biologically effective UV-B radiation (280-320 nm, 3.2 W m^{-2} s^{-1}) were recorded. The *in vivo* and *in vitro* NR activities were inhibited by 34 and 41 % under UV-B treatment, respectively. Both V_{max} and K_m for the substrate were enhanced by UV-B radiation. The K_m for nitrate increased from 1.2 to 1.7 mM after the UV-B irradiation. The change in K_m for NADH was from 0.12 to 0.17 mM. The increases in K_m indicate that UV-B radiation seriously changes the topology of NR, particularly with respect to the nitrate and NADH binding sites. The rate of NR turnover indicates the extent of damage inflicted by UV-B radiation on the nitrate metabolism. The half-life ($t_{1/2}$) of NR was reduced from 7 to 4 h in the UV-B treated seedlings. UV-B also inhibited the kinetics of nitrate uptake by plants: its K_m increased from 0.08 to 0.12 mM.

Additional key words: carotenoids; chlorophyll; fresh and dry mass; leaf area; root; shoot; *Vigna unguiculata*.

Introduction

Stratospheric ozone layer is being depleted as a result of contamination by man-made pollutants, namely chlorofluorocarbons (CFCs) (Molina and Rowland 1974, Manney *et al.* 1994). As a consequence, an increased level of ultraviolet-B radiation (UV-B,

280-320 nm) is currently penetrating the biosphere (Kerr and McElroy 1993). Exposure of plants to high UV-B levels can alter their growth and development, transpiration, and photosynthesis (Balakumar *et al.* 1993a,b, Tevini 1993, Teramura and Sullivan 1994), and induce DNA damage (Pang and Hayes 1991, Balakumar *et al.* 1997, Takeuchi *et al.* 1998) and alteration of several physiological processes. However, little is known about the changes brought by UV-B radiation on the nitrate assimilation pathway of crop plants. Therefore we studied changes in the kinetic characteristics of the enzyme nitrate reductase (NR, E.C.1.6.6.1) which catalyzes the rate-limiting step of the reduction of nitrate in the nitrate assimilation pathway of plants (Campbell 1999). This step is carried out in most plants by NADH:NR, which is the predominant isoform of NR in leaves of higher plants (Campbell 1999). Further, NR is the key indicator of metabolic and physiological status of plants and has often been used to indicate responses to stress or other physiological changes in plants, including the diurnal variations in metabolism (Srivastava 1992, Abrol *et al.* 1999, Campbell 1999).

Materials and methods

Healthy seeds of cowpea [*Vigna unguiculata* (L).Walp. cv. Co 4] were obtained from the Pulses Research Centre, Tamilnadu Agricultural University, Coimbatore 641 003, India. Seeds were surface-sterilized by immersion in 0.01 % (m/v) $HgCl_2$, soaked, and planted in plastic trays (30×20 cm) containing a mixture of garden soil and sand (1 : 1). Four seedlings of uniform size were retained in each tray and on the third day experimental treatments were started.

The source of UV-B irradiation was *FS-40* sunlamps (*Westinghouse*, Bloomfield, NJ, USA). For their spectral characteristics and the transmittance characteristics of the filters used see Fig. 1. Irradiation in the UV-B waveband (mainly 290-320 nm) was measured using a factory calibrated double-holographic-grating spectroradio-meter (model *742, Optronics*, Orlando, USA). The radiation filtered through the cellulose acetate filters supplied a weighted irradiance of 3.2 W m^{-2} s^{-1} of biologically effective UV-B generalized at 300 nm (Caldwell 1971). During the UV-B treatment, the control plants also were kept under the *FS-40* sunlamps wrapped with *Mylar* type *D* plastic films, which prevent transmission of any radiation below 320 nm. The treatments were given for 10 d.

Growth parameters: Plant height was measured, and leaf area was recorded using a *LiCOR 3100* leaf area meter. Fresh mass was estimated before the plants were dried at 100 °C to constant mass. Leaf mass per area (LMA) was derived using the formula of Pearce *et al.* (1968). Chlorophylls (Chls) were extracted with 80 % acetone and estimated according to Arnon (1949). The amount of carotenoids in the acetone extract was quantified using the absorbance at 480 nm after correction for Chl interference (Kirk and Allen 1965) using the extinction coefficient proposed by Ridley (1979).

Analysis of enzymes: *In vivo* NR activity in the leaf tissue was determined by adopting the method of Jaworski (1971). The *in vitro* enzyme assays were done using the crude enzyme extract of leaves. Leaves (200 mg) were cut into small pieces and homogenized with mortar and pestle at 4 °C in 50 mM Tris-HCl (pH 7.8), 10 mM $MgCl_2$, 10 mM 2-mercaptoethanol, and 0.25 mM EDTA. The homogenate was centrifuged at 2000×g for 5 min, and the clear yellowish-green supernatant was collected and used as the crude enzyme extract. This extract was adjusted to 50 % saturation with a chilled solution of ammonium sulfate in 100 mM Tris-HCl (pH 8.0), 5 mM EDTA, and 5 mM L-cysteine. After 15 min of stirring at 4 °C, the solution was centrifuged at 12 000×g for 15 min at 4 °C. The pellet was dissolved in 5 cm³ of the extraction medium and the solution was dialyzed overnight against the same buffer. During precipitation the pH was maintained at 8.0 by adding 4 M NH_4OH. Colorimetric determination of nitrate in plant tissue followed the method of Cataldo *et al.* (1975).
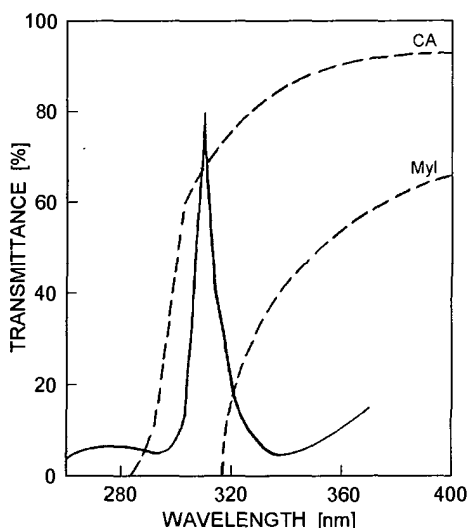

Fig. 1. Spectral irradiance at 25 cm from a *FS 40* fluorescent sunlamp and the transmittance properties of cellulose acetate, *CA* (0.13 mm thickness) and *Mylar* type *D* plastic filter, *Myl* (0.13 mm). The lamps were preburnt for 100 h and the spectral irradiance was measured with a double holographic grating spectroradiometer (model *742*, *Optronics*, Orlando, FL, USA). The transmittance spectra of the filters were obtained using a *Hitachi 2000* spectrophotometer.

Results

UV-B inhibited the growth parameters by 20 %, and reduced the biomass by 44 % (Table 1). However, there was a marginal increase in LMA, which was significant ($p = 0.05$). The photosynthetic pigments were also significantly ($p = 0.05$) decreased by UV-B (Table 1). Hence, cowpea is a system sensitive to UV-B radiation. Generally, the reduction in growth and photosynthetic parameters is correlated with the disturbance in nitrate metabolism. This metabolism, measured in terms of NR activity in the leaf tissue, showed severe inhibition under UV-B treatment (Table 2). While the *in vivo* activity exhibited a 34 % reduction in the UV-B treated seedlings, the extractable (*in vitro*) NR activity showed a 41 % inhibition, which indicates

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Table 1. Growth parameters and contents of chlorophyll and carotenoids of cowpea seedlings grown in the presence (daily exposure of 30 min at 3.2 W m^{-2} s^{-1}) and absence of UV-B radiation for 10 d. Means ± SE of ten replicates. Values in parentheses are % over control. Differences between control and treated seedlings are significant at $p = 0.05$.

Parameter	Control	UV-B treated
Shoot length [cm]	14.60 ± 0.78	11.30 ± 0.96 (78)*
Root length [cm]	3.40 ± 0.33	2.80 ± 0.29 (82)*
Leaf area [cm^2]	6.80 ± 0.52	3.80 ± 0.36 (56)*
Fresh mass [mg plant^{-1}]	820.00 ± 36.65	539.00 ± 28.82 (64)*
Dry mass [mg plant^{-1}]	89.00 ± 4.41	56.00 ± 4.16 (62)*
Leaf mass per area [g m^{-2}]	34.60 ± 3.24	38.20 ± 3.36 (110)*
Chlorophyll $(a+b)$ [g kg^{-1}(FM)]	2.60 ± 0.29	2.20 ± 0.18 (84)*
Chlorophyll a/b ratio	2.70 ± 0.33	2.60 ± 0.14
Carotenoids [mmol kg^{-1}(FM)]	0.58 ± 0.06	0.52 ± 0.06 (89)*

a greater sensitivity to UV-B radiation. Inasmuch as the synthesis of the NR enzyme is substrate inducible (Hageman and Flesher 1960, Srivastava 1992), we exposed etiolated cowpea seedlings to nitrate and checked the induction of NR synthesis with or without UV-B. UV-B treatment decreased the induction of NR synthesis by 44 %.
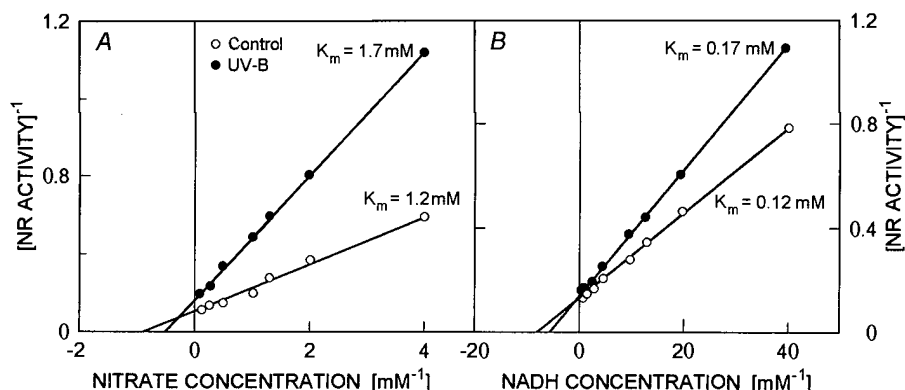

Fig. 2. Lineweaver-Burk plots of reaction rate of nitrate reductase (NR) with concentrations of nitrate (*A*) or NADH (*B*) in the control and UV-B treated cowpea seedlings. The seedlings growing on nutrient medium were fertilized daily with 15 mM KNO$_3$. The seedlings received no UV-B or a daily duration of 30 min of UV-B treatment (3.2 W m^{-2} s^{-1}). The NR in the leaves harvested from 10-d-old seedlings was extracted, fractionated with ammonium sulfate (to 50 % saturation), resuspended in the extraction buffer, and dialyzed overnight at 4 °C against the same buffer. The NR activity was assayed with 0.1 cm^3 aliquots at the indicated levels of nitrate or NADH.

The NR activity is regulated by the substrate, nitrate (Oaks *et al.* 1979). When we plotted the NR as a function of the nitrate concentration, the UV-B treatment shifted K_m for nitrate from 1.2 to 1.7 mM (Fig. 2*A*), and the K_m for NADH from 0.12 to 0.17 mM (Fig. 2*B*). Hence UV-B brought remarkable changes in the topology of the enzyme protein, particularly altering its nitrate and NADH binding sites.

Table 2. Nitrate reductase (NR) activity [μmol(NO$_2$ produced) s^{-1} kg^{-1}(FM)] in leaves of cowpea seedlings grown in the presence (daily exposure of 30 min at 3.2 W m^{-2} s^{-1}) and absence of UV-B radiation for 10 d. The seedlings were fertilized daily with 15 mM KNO$_3$. Means ± SE of four replicates. Values in parentheses are % over control. Differences between control and treated seedlings are significant at $p = 0.05$.

Treatment	NR *in vivo*	NR *in vitro*
Control	1.72 ± 0.09	5.94 ± 0.06
UV-B treated	1.14 ± 0.06 (66)*	3.50 ± 0.15 (59)*

The NR turnover is an index of damage inflicted by UV-B on nitrate metabolism. The half-life ($t_{1/2}$) of NR determined by the decay kinetics showed *in vitro* a shift from 7 to 4 h after the UV-B treatment (Fig. 3). Availability of nitrate at the
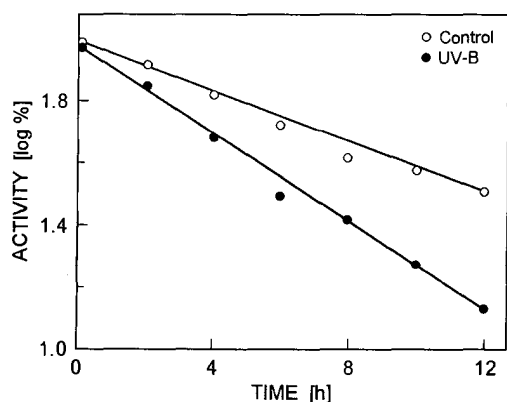

Fig. 3. Decay kinetics of nitrate reductase (NR) activity under UV-B treatment. The crude enzyme extracted from the leaves of 10-d-old control and UV-B irradiated plants (daily duration of 30 min at 3.2 W m^{-2} s^{-1}) fertilized daily with 15 mM KNO$_3$ was used for the assay of *in vitro* activity. The activity measured within 15 min of extraction was taken as the initial (100 %) activity: control = 4.5 and UV-B = 2.97 μmol(NO$_2$ produced) s^{-1} kg^{-1}(FM) and loss of activity over time at 4 °C was measured.

metabolic site depends on its uptake and transport (Jackson *et al.* 1986). To evaluate whether nitrate availability was affected by UV-B, we monitored the kinetics of nitrate uptake by the leaves as a function of nitrate concentration. The K_m registered a shift from 0.08 to 0.12 mM under UV-B treatment (Fig. 4).
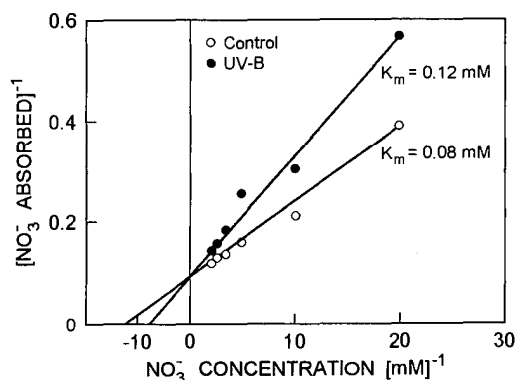

Fig. 4. Lineweaver-Burk plot of rate of nitrate uptake with concentration of nitrate. The seedlings were grown on a nitrate free nutrient medium for 10 d. The leaves were cut into uniform sized discs (3 mm diameter) and kept floating in varying concentrations of nitrate. Uptake of nitrate by leaf tissue was estimated after 4 h under visible (PAR, 640 μmol m^{-2} s^{-1}) and PAR + 3.2 W m^{-2} s^{-1} of UV-B radiation. Nitrate was determined by the method of Cataldo *et al.* (1975).

Discussion

Ultraviolet radiation is an environmental stress to plants for over a century (Caldwell *et al.* 1989). Within the 200–400 nm waveband of UV in the solar radiation, UV-B radiation (280-320 nm) inhibits growth and metabolism of plants (Caldwell *et al.* 1989, Stapleton 1992, Jansen *et al.* 1998, Takeuchi *et al.* 1998), their shoot and root length, leaf expansion (Tevini *et al.* 1981, Balakumar 1992, Balakumar *et al.* 1993a), and increases LMA. We found a similar effect of UV-B on leaf area and LMA. Increase in LMA is correlated with increase in leaf thickness in cowpea (Balakumar *et al.* 1993a). Chl content was reduced in treated plants similarly as in the work of Tevini *et al.* (1981); such reductions are paralleled by reductions in total biomass and net photosynthetic rate (Basiouny *et al.* 1978). However, in our study carotenoids were more stable under UV-B radiation than the Chls.

The effects of UV-B on nitrate metabolism confirmed the findings of Döhler (1988) done with marine diatoms. Our experiments with etiolated cowpea seedlings indicate that UV-B interferes at the level of nitrate reductase (NR) gene expression. NR is a substrate-inducible enzyme (Srivastava 1992, Balakumar *et al.* 1993b, Campbell 1999). According to Campbell (1999), nitrate is the initiator of events in NR synthesis starting from the appearance of NR mRNA until the detection of NR activity through the *de novo* synthesis of the enzyme. This information, together with that of Melzer *et al.* (1989), has elucidated that nitrate acts at the level of transcription of the NR gene.

The detection of 43 % lesser NR activity in the etiolated cowpea seedlings exposed to UV-B radiation than in control plants during NR induction confirms that the transcription of the NR gene could be a target site of UV-B. Though the molecular mechanism of NR gene expression by nitrate is not clearly understood, Campbell (1988) implicated the involvement of a protein mediator. Moreover, UV-B radiation is absorbed by DNA and proteins (Caldwell 1971, Setlow 1974). Therefore, the possible direct effect of UV-B on the repression of NR gene or its interference with the mediator protein leading to the inhibition of NR gene transcription also cannot be completely ruled out. Jordan *et al.* (1992) have shown that the major UV-B effect on ribulose-1,5-bisphosphate carboxylase/oxygenase synthesis in pea seedlings was also at the level of transcription. Vu *et al.* (1982) noted that the synthesis of photosynthetic carboxylases was also affected by UV-B. Thus UV-B interferes at the level of translation as well. However, we did not find any evidence for a similar effect of UV-B on the synthesis of NR protein. Further, the UV-B treated leaf tissue required higher concentration of nitrate to induce optimum NR activity as compared with the control. In etiolated seedlings of maize and barley supplied with nitrate, Oaks *et al.* (1979) observed that the appearance of NR protein and NR activity is closely associated with nitrate uptake from the medium and its subsequent accumulation. They hypothesize that the NR protein and NR activity are indeed induced by nitrate but there are significant differences in the timing and responses of these events to environmental cues. Our comparison with the results of Crawford *et al.* (1988) also substantiated the view of Oaks *et al.* (1979). Therefore, the increased

substrate concentration required for optimum induction under UV-B can be ascribed to the reduced uptake of nitrate from the medium and its subsequent mobilization to the active metabolic pool (Fig. 4). Alterations in the kinetics of nitrate uptake by the leaf tissue due to UV-B radiation would have resulted in the enhancement of nitrate concentration needed to saturate an induction. Saturation-type kinetics of NR induction was observed among many species (Wallace 1973).

While the ammonium sulfate fractionated NR enzyme in the control seedlings had a K_m for the substrate nitrate of 1.2 mM, in the UV-B stressed seedlings it was 1.7 mM (Fig. 2A). These K_m values were higher than those of Guerrero *et al.* (1981) who used for NR assays highly purified enzyme extracts, but we used only partially purified extracts. This is the probable reason for the higher values of K_m. The observed increase in the K_m for nitrate in the UV-B treated seedlings indicates the possibility of some stable conformational changes in the NR protein. These changes could have resulted in the altered substrate binding characteristics of NR due to UV-B irradiation (Giese 1976). Moreover, we speculate that in addition to the modifications in protein conformation, UV-B mediates changes in the NR activity modulators such as phosphorylation or adenylation, thus increasing the K_m.

UV-B treatment has also enhanced the K_m for NADH (Fig. 2B) suggesting modifications in the NADH binding site of the NR enzyme. Since NADH may protect the enzyme against inactivation of diaphorase activities (Solomonson and Barber 1990), the changes affecting the binding site of NADH may also alter the regulation of NR activity. Though our results do not explain the nature of alterations at the NADH binding site induced by UV-B, the NADH binding site might have been altered which would lower its affinity for NADH. The increase in K_m for nitrate and NADH indicates UV-B mediated changes in the topology of NR. The synthesis of NR enzyme was hampered by UV-B, and also the chemistry of the active enzyme protein was changed. The decay kinetics of NR activity revealed that in the UV-B irradiated seedlings, $t_{1/2}$ of NR activity was reduced (Fig. 3). This value is an index of rate of enzyme turnover (Aslam *et al.* 1976). Enzyme turnover (biosynthesis to degradation) is regulated by many physiological and environmental factors including irradiance, senescence, and nutrient status (Jordan *et al.* 1992). The $t_{1/2}$ of the *in vitro* NR activity was reduced from 7 to 4 h due to UV-B treatment. This reduction in $t_{1/2}$ can be ascribed to any one or a combination of factors such as the changes in the ratio of synthesis to degradation of the enzyme, the production of NR inactivating factors, and the modifications effected in the active enzyme *per se* (Remmler and Campbell 1986).

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