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UV-B radiation mediated alterations in the nitrate assimilation pathway of crop plants 2. Kinetic characteristics of nitrite reductase

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Abstract

Cowpea [*Vigna unguiculata* (L.) Walp. cv. Co 4] seedlings were subjected to a weighted irradiance of 3.2 W m^{-2} s^{-1} of biologically effective ultraviolet-B radiation (UV-B, 280-320 nm) and the changes in the kinetic and other characteristics of nitrite reductase (NiR) were recorded. The activity of NiR was hampered by 19 % under UV-B irradiation compared to the control. The UV-B treated plants required higher concentrations of nitrate for the induction of NiR synthesis than the controls. The NiR activity decay kinetics showed that the UV-B treatment significantly lowers the $t_{1/2}$ of the enzyme, thereby indicating a reduced rate of enzyme turnover. The comparison of kinetic characteristics of nitrate reductase (NR) and NiR under UV-B treatment showed that NiR was not so sensitive to UV-B radiation as NR. As shown by enzyme turnover rates, NiR extracted from plants irradiated by UV-B *in situ* was less sensitive to UV-B radiation than the enzyme extract subjected to *in vitro* UV-B irradiation. Though NiR was less damaged by UV-B treatment than NR, subtle changes occurred in its kinetic characteristics.

Additional keywords: nitrate reductase; nitrite reductase; enzyme induction and kinetics; *Vigna unguiculata*.

Introduction

Indiscriminate release of anthropogenic pollutants such as the chloroflurocarbons

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(CFCs) and other ozone antagonists into the atmosphere results in thinning of the stratospheric ozone layer (Blumthaler and Amback 1990, Gleason *et al.* 1992). Due to ozone depletion, ultraviolet-B radiation (UV-B, 280-320 nm) reaching the earth's surface is predicted to increase. Its potential impact on living organisms including the aquatic ones has been investigated by several research groups during the last three decades (Balakumar *et al.* 1993a, 1997, Weiler and Penhale 1994, Häder 1996). Generally, UV-B inflicts two kinds of damage to plants, *i.e.*, damage to DNA and to the vital physiological processes, namely photosynthesis, nitrogen metabolism, and synthesis of pigments (Döhler 1988, Renger *et al.* 1989, Balakumar 1992, Ambasht and Agrawal 1997, Wilhelm *et al.* 1997, Lingakumar and Kulandaivelu 1998). While the target sites of UV-B radiation in photosynthesis have been well documented (Renger *et al.* 1989), the impact of UV-B radiation in the nitrate assimilation pathway of plants has not yet been elucidated in detail.

Nitrate is the predominant form of N available in the soil and also the most preferred form for uptake of N by plants (Balakumar *et al.* 1993b, Campbell 1999). We report (Balakumar *et al.* 1999) the action sites of UV-B damage in nitrate reduction and the rate-limiting step of nitrate assimilation pathway, catalyzed by the cytosolic enzyme nitrate reductase (NR, EC 1.6.6.1). While three factors, namely the plastidic signal, nitrate availability, and irradiation, are suggested to operate in a hierarchy in the induction of both NR and NiR (Rajasekhar and Mohr 1986), nitrate alone has been proposed to play a precise role in the induction of NiR (EC 1.7.99.3) (Gupta *et al.* 1983). Since we previously found that UV-B radiation inflicts a severe damage on nitrate reduction (Balakumar *et al.* 1999), its impact might be reflected on the functional aspects of NiR, too. This formed the basis of our present investigation and here we report the changes mediated by UV-B in the kinetic characteristics of the NiR which catalyzes the sequential reaction to NR catalysis, the reduction of nitrite to ammonia.

Materials and methods

The methods of growing cowpea (*Vigna unguiculata* L. Walp. cv. Co 4) seedlings, UV-B radiation milieu, and measurements are described in Balakumar *et al.* (1999). Young, fully expanded, first unifoliate leaves were harvested from the cowpea seedlings exposed to light and nitrate at least 4 h before harvest.

Leaf material (1.0 g) was ground with a pre-chilled pestle and mortar at 4 °C in 4 cm^3 of freshly prepared ice-cold extraction medium containing 100 mM Tris-HCl (pH 8.0), 5 mM EDTA, and 5 mM L-cysteine. The extract was centrifuged at 25 000 $\times g$ for 15 min at 4 °C in a *Himag Hitachi* centrifuge (model *SCR7BA*; *Hitachi*, Tokyo, Japan). The clear, yellowish green supernatant was stored on ice before assaying the NiR activity or taken for fractionation with ammonium sulfate. The crude enzyme extract was adjusted to 50 % saturation with a chilled solution of ammonium sulfate in the extraction medium. After 15 min stirring at 4 °C, the solution was centrifuged at 12 000$\times g$ for 15 min at 4 °C. The pellet was dissolved in

5 cm^3 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 whenever needed. The activity of NiR was assayed at conditions such that the rate of reaction was proportional to time and to the amount of enzyme at 30 °C and pH 7.5. The reaction cocktail contained in a total volume of 1.9 cm^3 100 μmol of Tris-HCl (pH 7.5), 20 μmol of KNO_2, 1.0 μmol of reduced methyl viologen, and 0.2 cm^3 of enzyme. The reaction was started by adding 0.1 cm^3 of freshly prepared solution of 13 kg m^{-3} sodium dithionate in 95 mM sodium bicarbonate. After 15 min incubation at 30 °C, the reaction was stopped by vortexing. Aliquots of 0.1 cm^3 of the reaction mixture were suitably diluted and the residual nitrite content was estimated. Methyl viologen was omitted in the control tubes.

Results

UV-B irradiation has brought an about 19 % inhibition in the NiR activity compared to the control. To evaluate the changes in induction characteristics of NiR mediated by UV-B radiation, the leaves harvested from plants grown on a nitrate-free medium were kept floating in various concentrations of KNO_3 and allowed for induction. The control leaves showed optimum NiR activity at 15 mM KNO_3, while the UV-B treated leaves at 20 mM KNO_3 (Fig. 1). Hence the UV-B treated plants demand higher concentration of nitrate for induction of NiR than the control ones. These differences in the induction properties reveal that the protein of NiR has undergone alterations under UV-B. The kinetic characteristics of NiR under UV-B did not show

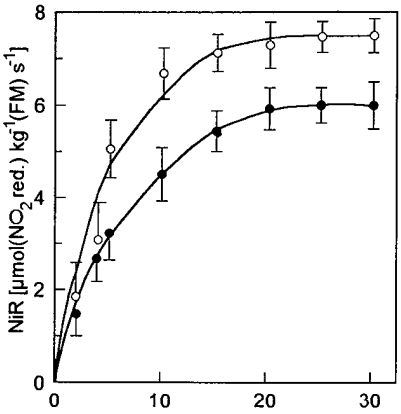

Fig. 1. Relationship between nitrate concentration and induction of nitrite reductase (NiR) activity in leaves of cowpea seedlings under UV-B treatment. Leaves harvested from 10-d-old cowpea seedlings raised on nitrate-free medium were cut into 1 cm^2 bits and kept floating in petri dishes containing various concentrations of KNO_3 and irradiated by 640 μmol m^{-2} s^{-1} (PAR, control, ○) or PAR + 3.2 W m^{-2} s^{-1} of UV-B (●). After 6 h of induction, *in vitro* NiR activity was determined using the crude enzyme extract.

any significant change. The K_m for nitrite in the control was 0.9 mM and in the UV-B treated plants 1.0 mM (Fig. 2). Thus the kinetic of NiR is not as sensitive to UV-B as NR (see Balakumar *et al.* 1999). The UV-B treatment lowered the $t_{1/2}$ of NiR from 3.7 to 3.0 h (Fig. 3*A*). The lowering of $t_{1/2}$ augments the interpretation that the NiR protein has been significantly altered in its conformation. The NiR extracted from the

plants grown under UV-B treatment had $t_{1/2}$ of 3.7 h, while the $t_{1/2}$ of the *in vitro* UV-B irradiated NiR was only 2.2 h (Fig. 3*B*). Hence the extracted NiR is appreciably more sensitive to UV-B than the enzyme *in situ*.

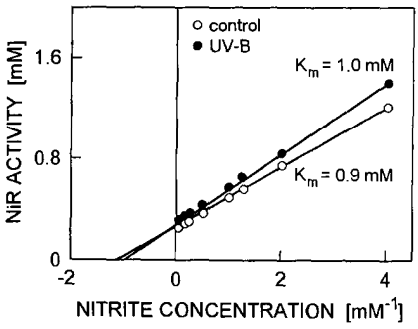

Fig. 2. Lineweaver-Burk plot for nitrite reductase (NiR) reaction rate with varied concentrations of nitrite. Cowpea seedlings growing on nutrient medium was fertilized daily with 15 mM KNO_3. The seedlings received either no UV-B (O) or a daily duration of 30 min of UV-B (3.2 W m^{-2} s^{-1}) treatment (●). The NiR in the leaves harvested from 10-d-old seedlings was harvested, fractionated with ammonium sulfate (to 50 % saturation), resuspended in the extraction buffer, and dialyzed overnight at 4 °C against the same buffer. NiR activity was assayed with 0.1 cm^3 of aliquots at the indicated concentrations of nitrite.

NR is remarkably more sensitive to UV-B radiation than NiR. Evaluation in terms of the *in vitro* activity, substrate concentration required for optimum induction, K_m for the respective substrates, and the enzyme turnover showed that under UV-B radiation NR underwent more drastic changes than NiR (Table 1). However, the trend of responses of both enzymes to inhibition was similar if they were extracted and irradiated *in vitro*. Thus the *in vitro* UV-B radiation elicited certain instantaneous NR and NiR inactivators which remain to be elucidated.

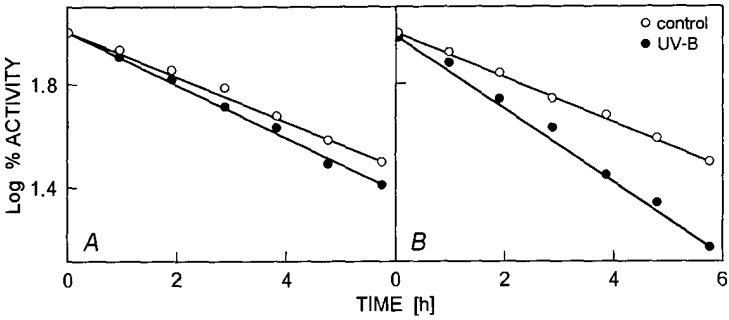

Fig. 3. Nitrite reductase (NiR) activity decay kinetics under UV-B treatment *in situ* (*A*) or *in vitro* (*B*). The crude enzyme extracted from the leaves of 10-d-old control or UV-B irradiated plants (daily duration of 30 min at 3.2 W m^{-2} s^{-1}) fertilized daily with 15 mM KNO_3 was used for the assay of *in vitro* activity at 4 °C. For the *in vitro* variant (*B*), a 0.5 mm thin film of the freshly extracted enzyme was taken in a petri dish. The control extract was covered with a *Schott* filter *WG 335* that removed all radiation below 335 nm. The activity measured within 15 min of extraction was taken as the initial (100 %) activity [control = 17.4 and UV-B treated = 14.1 μmol(NO_2^- reduced) s^{-1} kg^{-1}(FM)] and loss of activity over time at 4 °C was measured. The log % activity is presented.

Table 1. Comparative responses of nitrate reductase (NR) and nitrite reductase (NiR) in cowpea seedlings under UV-B treatment (daily exposure of 30 min at 3.2 W m^{-2} s^{-1} for 10 d). Means ± SE of three independent estimations. Differences between control and UV-B treatment significant ($p = 0.05$). Values in parentheses denote % over control.

Parameter	NR		NiR	
	control	UV-B	control	UV-B
Activity in seedlings [μmol(NO_2^-) kg^{-1}(FM) s^{-1}]	5.94 ± 0.34	3.50 ± 0.25 (59)	17.40 ± 1.01	14.14 ± 0.82 (81)
Substrate concentration for optimum induction [mM]	20.00 ± 1.02	25.00 ± 1.21	15.00 ± 0.92	20.00 ± 1.22
K_m for substrate [mM]	1.20 ± 0.14	1.70 ± 0.13	0.90 ± 0.08	1.00 ± 0.07
$t_{1/2}$ [h]	7.00 ± 0.48	4.00 ± 0.37	3.70 ± 0.28	3.00 ± 0.25
Activity after *in vitro* irradiation	16.20 ± 1.32	11.70 ± 1.04 (72)	59.60 ± 3.21	37.10 ± 2.27 (62)
$t_{1/2}$ for *in vitro* irradiation [h]	5.50 ± 0.38	4.20 ± 0.29	3.70 ± 0.23	2.20 ± 0.19

Discussion

Albeit in some of the plants the synthesis of NiR is induced either by nitrate or nitrite (Hewitt 1975, Guerrero *et al.* 1981, Srivastava 1992), nitrate is most often the inducer proper (Rao *et al.* 1981, Rajasekhar and Olemuller 1987, Campbell 1999). The activity of *in vitro* NiR has been hampered by 19 % due to UV-B radiation, and the analysis of the induction of NiR by nitrate revealed that the concentration of nitrate required for optimum induction was higher in the UV-B irradiated leaves than in the control (Fig. 1). The induction of NiR by nitrate is routed through a sequential induction of NR and NiR, which shows that NiR is induced probably by nitrite after its formation from nitrate by the action of NR (Ingle *et al.* 1966, Sluiters-Scholten 1973). Therefore, the reduction in NiR activity could be a consequence of the severe damage exerted by UV-B on the NR activity (Balakumar *et al.* 1999). Further, the nitrite formed in the cytosol through the activity of NR must be subsequently targetted for reduction by NiR into the chloroplast, where NiR is localized (Wallsgrove 1987, Abrol *et al.* 1999). Therefore, as the availability of nitrite from NR activity to attain saturation in induction is insufficient, the UV-B irradiated leaves may require higher concentrations of nitrate than the control ones.

In the control leaves, the NiR had only a marginally lower K_m for nitrite than in the UV-B treated leaves (Fig. 2). This shows that the kinetic characteristics of NiR are not significantly altered by UV-B. The plausible explanation for this may be that the enzyme protein does not undergo any serious conformational change at the substrate, nitrite binding site. Nevertheless, the lesser inhibition of NiR activity due to UV-B treatment could be attributed to certain changes that might have occurred at its regulatory site. This hypothesis was substantiated by the only marginal reduction in the leaf $t_{1/2}$ of NiR activity observed in the UV-B irradiated leaves (Fig. 3*A*). The higher resistance of NiR than NR to UV-B radiation can be ascribed largely to its

localization. As NiR is sequestered in the chloroplasts, its access to UV-B damage is limited. The chloroplast membranes could serve as effective additional barriers to minimize the transmittance of UV-B radiation into the inner NiR targets. The above proposal is confirmed by the observation that immediately after the *in vitro* irradiation for 30 min, NiR exhibits 38 % reduction in its activity. As the chloroplast membrane barriers were disrupted during homogenisation, the extracted enzyme was more sensitive than the enzyme *in situ* as it stands more exposed to UV-B. Therefore, sequestration within the chloroplast membranes only offers more resistance to the NiR enzyme *in situ* from UV-B radiation than to the extracted enzyme. Moreover, the *in vitro* irradiated enzyme showed also a sharp decline in its $t_{1/2}$ (Fig. 3*B*), indicating the likelihood of certain instantaneous changes in the conformation of the enzyme protein. Our results together with those in the paper of Balakumar *et al.* (1999) establish that UV-B radiation has several target sites in the nitrate assimilation pathway of crop plants which is sequentially catalyzed by the two metalloproteins, NR and NiR. Nevertheless, the comparison of responses of the two enzymes to UV-B (Table 1) reveals that NR is more sensitive to UV-B than NiR; this is based on their kinetic and other characteristics.

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