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REGULAR ARTICLE

# Existing antioxidant levels are more important in acclimation to supplemental UV-B irradiation than inducible ones: Studies with high light pretreated tobacco leaves

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**Abstract**

Greenhouse grown tobacco plants were exposed to supplemental ultraviolet irradiation (280-400 nm, UV-B centered) for 6 days and changes in their photosynthesis (gas exchange and electron transport) and general and specific antioxidant activities were measured. UV irradiation corresponded to 8.95 kJ m-2 d-1 biologically effective dose and was supplemented to below ambient (200 µmol m-2 s-1 photon flux density) photosynthetic photon flux density (PPFD, 400-700 nm). Two groups of plants, which were different in their leaf antioxidant capacities due to one of them having been acclimated to high irradiance (1000 µmol m-2 s-1 PPFD) before the UV treatment, responded differently. High light pretreated leaves lost approximately 25% of photosynthetic activity during the UV exposure and showed no change either in the amounts of UV-absorbing pigments or antioxidant levels. On the other hand, leaves which were exposed to UV irradiation without the preceding high light acclimation had 60% lower photosynthesis by the end of the treatment, and increased antioxidant activities. Our results emphasize the importance of base antioxidant levels over inducible pools in leaf responses to low doses of UV irradiation and may also contribute to hypotheses on acclimation under field conditions.

*Key words*: Ultraviolet radiation, Antioxidant capacities, UV-absorbing pigment, Photosynthesis

## Introduction

High energy ultraviolet (UV, 280-400 nm) radiation, especially the UV-B region (280-315 nm) affects photosynthesis in various ways, and can lead to severe damage when applied at high doses (reviewed by Teramura and Sullivan, 1994). Under such conditions, the inhibitory effect of UV on growth and $CO_2$-fixation is realized through the generation of reactive oxygen species (ROS), leading to oxidative stress (Hideg and Vass, 1996, Mackerness et al. 2001). Oxidative stress is caused by pro-oxidants as a result of an imbalance between the production and the neutralizing of these compounds (Mittler, 2002, Apel and Hirt, 2004). Plants protect themselves from the harmful effects of this radiation by alterations in pigment composition, including the production of compounds reflecting or absorbing UV radiation (e.g. flavonoids). In protection against pro-oxidants, the production of enzymatic and non-enzymatic components of the antioxidant system increases (e.g. ascorbate, phenols, for reviews see Jansen et al. 2008, Zhang and Björn, 2009). It should be noted that flavonoids have a role in both types of defense mechanisms as these compounds act not only as UV screens but are good antioxidants as well (Agati and Tattini, 2010).

On the other hand, UV radiation at lower doses has recently been conceived as a more complex signal, inducing changes in morphology, gene expression and plant metabolism, through the stimulation of the antioxidant machinery of cells and finally leading to acclimation (Frohnmeyer and Staiger, 2003 for reviews see Mackerness, 2000, Jordan, 2002, Kakani et al. 2003).

Several studies report enhanced protection against oxidative stress in plants with improved antioxidant capacities, many of which include transgenic plants altered at specific points of protection against pro-oxidants. Examples include plants overexpressing different antioxidant enzymes, such as chloroplast superoxide dismutase (Sen Gupta et al. 1993), peroxisomal ascorbate peroxidase (Wang et al., 1999), or these two enzymes together with dehydroascorbate reductase (Lee et al. 2007). Tolerance against UV-B radiation

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was also increased in tobacco leaves, where reactive oxygen scavenging capacity was enhanced by preceding mild drought (Hideg et al., 2003, Kubis and Rybus-Zajac, 2008).

The aim of the present work was to test whether acclimation to high intensity visible light resulted in plants more tolerant to subsequent supplemental UV. Similarly to UV-B, strong (excess) visible light can trigger oxidative stress, although via different mechanisms: visible light mainly induces triplet chlorophyll formation and ROS (singlet oxygen) production through acceptor side modifications of the photosystem II complex (Vass, 2011). Acclimation to non-destructive (non-photoinhibitory) light intensities can induce different components of the antioxidant system (Li et al., 2009, Takahashi and Badger, 2011). Our experiments were designed to address the question whether existing antioxidants (i.e. those present at the onset of UV irradiation) or antioxidants induced by exposure to UV-B are more important in providing tolerance to UV. Although in this work these exposures are applied sequentially (first high light without UV, then lower light supplemented with UV) and under greenhouse conditions, results are expected to promote our understanding of possible interactions between responses to the UV component and the high intensity visible component of sunlight in nature.

## Materials and methods

### Plant material and treatments

Tobacco (*Nicotiana tabacum* L. cv. Petite Havana SR1) seeds were sown in standard soil and plantlets were transferred into 16 cm diameter individual pots. Plants were grown in greenhouse conditions (until 5- to 6-leaves stage) at 25/20 $^{o}$C, at 12 h daily irradiation with 200 µmol $m^{-2}$ $s^{-1}$ photosynthetic photon flux density (PPFD) for four weeks before the treatments started. Plants were first divided into two pretreatment groups with different light conditions. Half of them were left at the same 200 µmol $m^{-2}$ $s^{-1}$ PPFD (referred to as "200"), while others were exposed to 1000 µmol $m^{-2}$ $s^{-1}$ PPFD for 5 days (referred to as "1000"). After this period plants were further divided into 2-2 groups: one of the groups was exposed to supplemental UV-B centered radiation for 6 days (referred to as "UV"), while the other represented the untreated group (referred to as "unt"). Therefore we had four different treatment conditions: (1) 200 µmol $m^{-2}$ $s^{-1}$ PPFD for 12 days ("200-unt"), (2) 5 days at 1000 µmol $m^{-2}$ $s^{-1}$ PPFD and 6 days at 200 µmol $m^{-2}$ $s^{-1}$ PPFD ("1000-unt"), (3) 5 days at 200 µmol $m^{-2}$ $s^{-1}$ PPFD and 6 days with supplemental UV-B radiation ("200-UV") and (4) 5 days at 1000 µmol $m^{-2}$ $s^{-1}$ PPFD and 6 days with supplemental UV-B radiation ("1000-UV"). Supplemental UV-B light was generated from Q-Panel UVB-313EL tubes for 8 hours daily. One layer of cellulose diacetate filter (Courtaulds Chemicals, Derby, UK) was used to exclude shorter wavelength (<280 nm) UV radiation. Integrated UV-B dose was 0.84 W $m^{-2}$ irradiance (Cole-Palmer radiometer, model 97503-00 with a broad range 312 nm centered sensor). The applied UV irradiance (280-400 nm) corresponded to 8.95 kJ $m^{-2}$ $d^{-1}$ biologically effective dose of which the UV-B part (280-315 nm) represented 8.04 kJ $m^{-2}$ $d^{-1}$, calculated using the Biological Spectral Weighting Function developed by Flint and Caldwell (2003). This UV-B dose is close to the ambient daily biologically effective UV-B at our latitude in the northern hemisphere in summer (Bassman et al., 2001). For further details on the spectral distribution of UV irradiance from the tube panel see Majer and Hideg, 2012.

Each treatment group included three plants and from each plant one fully-developed leaf was used for all the measurements, taken from the same level for excluding age effect and to ensure that the same UV and PPFD was experienced by the leaves.

### Photosynthesis and electron transport measurements

Photosynthesis ($CO_2$ uptake µmol $m^{-2}$ $s^{-1}$) was assessed on intact leaves at 200 µmol $m^{-2}$ $s^{-1}$ PPFD using LI-6400 Portable Photosynthesis System (LI-COR Environmental, Lincoln, Nebraska USA). Leaves were then cut off from the plants and kept in darkness for 30 min before chlorophyll fluorescence measurements with the MAXI-version of the Imaging-PAM (Heinz Walz GmbH, Effeltrich, Germany). After the dark adaptation period, minimum ($F_o$) and maximum ($F_m$) fluorescence yields were determined before and after a saturating pulse, respectively. This was followed by 30 sec long exposure to blue actinic light (160 µmol $m^{-2}$ $s^{-1}$ PPFD), and F and $F_m'$ values were obtained at each illumination step. Effective PS II quantum yields were calculated as $Y(II) = (F_m' - F) / F_m'$ and relative electron transport rates were determined following the standard formula $ETR = Y(II) \cdot PAR \cdot 0.5 \cdot 0.84$ (Genty et al., 1989).

### Determination of UV-B absorbing pigments

Two 0.6 cm discs were cut from each leaf and were extracted into acidified methanol and kept at 4$^{o}$C in darkness for 24 hours, then ground and centrifuged (3000 x g, 5 min, 4$^{o}$C). Supernatants were used for spectrophotometric determination of total UV-B absorption ($\sum OD_{280\text{-}315}$ $g^{-1}$ leaf fresh

weight) (Mirecki and Teramura, 1984), using a Shimadzu UV-1601 spectrophotometer.

**Leaf extraction**

For total and specific antioxidant capacity measurements (total phenolics content, FRAP and hydroxyl radical scavenging) twelve 0.6 cm leaf disks were cut, weighted and were first ground in liquid nitrogen, then in 1 mL phosphate buffer (50 mM, pH 7.0, 1 mM EDTA). Cell debris was first removed by a mild centrifugation (3000 x g, 5 min, 4$^{o}$C), then supernatants were re-centrifuged (30,000 x g, 25 min, 4$^{o}$C) and were stored at -80 $^{o}$C until use. The Folin-Ciocalteu reagent was purchased from Ferak Berlin GmbH (Berlin, Germany). All other chemicals were from Sigma-Aldrich (Sigma-Aldrich Kft Budapest, Hungary).

**Total phenolic content**

Total phenolic content was determined with the Folin-Ciocalteu method as described by Veliglu et al. (1998). For each sample, 80 µL plant extract was mixed with 500 µL Folin-Ciocalteu reagent (previously diluted 10-times with distilled water) and allowed to stand at room temperature for 5 min, then 500 µL $Na_2CO_3$ (60 g $L^{-1}$) was added to the mixture. After 90 min incubation at room temperature, absorbance at 725 nm was measured. Gallic acid (GA) was used for calibration and total phenolic contents were expressed in µmol GA equivalents $g^{-1}$ leaf fresh weight.

**Ferric reducing antioxidant power (FRAP)**

FRAP assay was carried out according to a modification of the original medicinal biochemical assay (Benzie and Strain, 1996) by Szőllősi and Szőllősi-Varga (2002). FRAP reagent was prepared by mixing sodium acetate buffer (300 mM, pH 3.6), tripyridyltriazine (TPTZ) solution (10 mM TPTZ in 40 mM HCl) and $FeCl_3$ (20 mM in water solution) in 10:1:1 ratio. For each sample, 80 µL plant extract was added to 1 mL freshly mixed FRAP reagent. After 30 min incubation time, the increase in 593 nm absorbance due to the formation of the blue-coloured ferrous form ($Fe^{2+}$-TPTZ complex) was measured. Ascorbic acid (AsA) was used for calibration and results were expressed as µmol AsA equivalents $g^{-1}$ leaf fresh weight.

**Hydroxyl radical scavenging capacity**

Specific hydroxyl radical ($^{\bullet}$OH) scavenging was determined based on the leaf extracts` ability to inhibit the formation of the strongly fluorescent 2-hydroxyterephthalate (HTPA) generated in a reaction between terephthalate (1,4-benzenedicarboxylic acid, TPA) and $^{\bullet}$OH (Šnyrychová and Hideg 2007). HTPA fluorescence was measured with a Quanta Master QM-1 spectrofluorometer (Photon Technology Inc., Birmingham, New Jersey, USA), using 315 nm excitation and 420 nm emission. $^{\bullet}$OH was produced in a reaction mixture containing 500 µM TPA, 10 µM EDTA, 10 µM $FeSO_4$, 100 µM AA and 100 µM $H_2O_2$ in a 50 mM Na-phosphate buffer (pH 7.2). $^{\bullet}$OH scavenging capacity of each leaf extract was characterized by its half-inhibitory concentration on HTPA formation as described earlier (Stoyanova et al., 2011). Ethanol, a strong $^{\bullet}$OH scavenger was used for calibrating the method, and specific $^{\bullet}$OH neutralizing capacities of leaf extracts were given as µM ethanol equivalent $g^{-1}$ leaf fresh weight.

**Ascorbate measurements**

Ascorbate content of the samples was determined according to Takahama and Oniki (1992), from the absorption of ascorbate at 265nm ($\varepsilon$=18$mM^{-1}cm^{-1}$). Ascorbate and dehydroascorbate were measured in 50mM potassium phosphate buffer (pH 6.0), in three different assay conditions: without addition, oxidised by 0.5 units $mL^{-1}$ ascorbate-oxidase or reduced by 2mM dithiothreitol. Samples were characterised by the amount of total ascorbate and by the ratio of oxidised to total ascorbate as described earlier (Hideg et al., 2006).

**Statistics**

Student's t-test was used to compare means of each two groups and to calculate P-values (GraphPad, GraphPad Software Inc., La Jolla, CA, USA). SigmaPlot (Systat Software Inc., San Jose, CA, USA) was used for creating graphs.

**Results and discussion**

Figure 1 illustrates the outline of the experiment and shows plant group identifiers. Data from high light pretreated leaves are labeled as "1000" and data from leaves without this pretreatment are marked with "200", referring to PPFD during the week preceding UV exposure. Plants which were not given the UV treatment and plants which were given the supplemental UV are labeled "unt" and "UV", respectively. Labels were doubled to indicate both pretreatment and UV irradiation, for example "200-UV" marks data from leaves which were exposed to supplemental UV without high-light acclimation and "1000-unt" was used for high-light acclimated leaves which were not exposed to UV afterwards (see Materials and methods section for details).

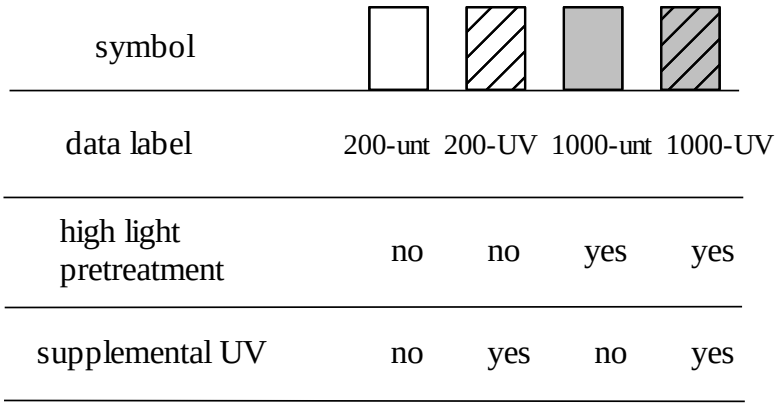


Figure 1. Outline of the experiment and group identifiers.

**Fig. 2.** (A) Photosynthesis and (B) photosynthetic electron transport of tobacco leaves belong to different treatment groups. Tables show P-values of Student's t-test in normal fonts ($p>0.1$), italics ($0.1>p>0.05$) or bold letters ($p<0.05$). For treatment group identifiers see Fig. 1.

The effect of the treatments on photosynthesis is displayed on Fig. 2. Fig. 2A and 2B show that high light acclimation had no effect on photosynthesis: both carbon-dioxide uptake and photosystem (PS) II electron transport were the same in 200-unt and in 1000-unt leaves. UV had smaller effect on $CO_2$-fixation ability in high light acclimated leaves: 1000-UV leaves retained 75% of the photosynthesis of 1000-unt ones, but 200-UV photosynthesis was only 40% of 200-unt (Fig. 2A). Electron transport was not lessened by UV irradiation, and was even slightly stimulated in 1000-UV leaves as compared to 1000-unt ones (Fig. 2B). In this way, the observed loss in $CO_2$-uptake was rather due to decreased stomata conductivity than to electron transport limitation. The same, but more pronounced UV-induced decrease pattern was observed in stomata conductivity as in photosynthesis: 85% decrease in non pre-treated tobacco leaves but only 30% decrease in 1000-UV plants, compared to their controls (data not shown). UV-B radiation has been shown to decrease photosynthetic $CO_2$-uptake, mainly via limiting stomata opening (Nogues et al., 1999, Jansen and van den Noort, 2000) but UV-inducible inhibition of electron transport (Renger et al., 1989, Vass et al., 1996) and Rubisco synthesis (Takeuchi et al., 2002, Choi and Roh, 2003) were also shown to be affected although the latter are usually reported in response to high UV doses.

Increased epidermal UV absorption is a known component of leaf responses to UV irradiation (Caldwell et al., 1983). In our experiment the production of pigments absorbing in the UV-B region (between 280 and 315 nm) was observed in the absence of UV treatment as well: high light pretreatment almost doubled the amount of these compounds (unt-1000 and unt-200 data in Fig. 3A). UV irradiation brought no significant changes in high light acclimated samples, but the amount of UV absorbing pigments increased slightly further in 200-UV leaves, although not to the amounts detected in 1000-UV ones. To interpret these results it is important to note that these are data from total leaf extracts, therefore the absorption of epidermal UV-B absorbers and of mesophyllic compounds can not be separated. As the difference between unt-1000 and unt-200 leaves is clearly due to the effect of high PPFD and thus can not be expected to originate in increased epidermal UV absorption, these data show that an increase in UV absorption may reflect increased antioxidant capacity and does not necessarily refer to increased epidermal screening. Typical UV absorbing antioxidant compounds are phenolic compounds and mostly flavonoids (Winkel-Shirley, 2002, Zhang and Björn, 2009). Flavonoids are considered to act primarily as epidermal UV screening compounds, but recent evidences support the antioxidant function of flavonoids localized deeper in plant tissues in protection against excess light induced photoinhibition (Agati and Tattini, 2010).

However, analysis of total phenolic compounds did not fully confirm this (Fig. 3B): unt-1000 leaves had only slightly elevated level of phenolic compounds compared to unt-200 ones. Exposure to UV irradiation increased this in 200-UV leaves to amounts characteristic to 1000-unt plants while data of 1000-UV samples were not different from their untreated pairs (Fig. 3B). This shows that the increase in UV absorption in response to high PPFD was not mainly due to the increase in phenolic compounds. As Levizou and Manetas (2002) showed, although total phenol content and UV-B screening pigment contents are strongly correlated in various plant species at given circumstances, but one has to keep in mind that not all UV-B absorbing pigments are phenolics and *vice versa*. In our experiment, although high light not, but UV radiation was capable of promoting the production of a large range of phenolic compounds in which UV absorbing ones are more responsive than others.

To characterize samples further in terms of antioxidants, two antioxidant parameters, one non-specific parameter measuring total antioxidant capacity (by means of ferric reducing antioxidant power, FRAP) and a selective ROS neutralizing parameter (hydroxyl radical scavenging) were also determined.

Regarding the ferric reducing ability, high light could not trigger this antioxidant power, but UV caused an almost 50% increase in 200 and a 35% increase in 1000 plants compared to 200-unt ones (Fig. 4A). This parameter indicates that under supplemental UV, leaves evoke protection against hydroxyl radical ($^{\bullet}$OH) production via Fenton chemistry (Halliwell and Gutteridge, 1999) by removing free iron, should it be released from damaged iron containing proteins under more severe stress conditions. To test whether this preventive mechanism is complemented by specific antioxidant capacity, $^{\bullet}$OH scavenging was also measured. 1000-unt leaves were 2-times richer in antioxidants that are capable of neutralizing $^{\bullet}$OH radicals than 200-unt leaves (Fig. 4B). This specific capacity were not different in 1000-UV and 1000-unt ones. UV treatment boosted the production of antioxidant responsive to $^{\bullet}$OH radicals in the leaves without pretreatment, while there were no further increase in 1000-UV plants compared to 1000-unt ones. These suggest that the increase in $^{\bullet}$OH radical scavenging capacity in response to high PPFD pretreatment could readily protect the leaves from additional damaging ROS effects deriving from exposure to UV.

Ascorbate is an important plant antioxidant and an increase in total ascorbate is frequently observed in leaves acclimated to stress conditions (Noctor and Foyer, 1998). Oxidation of leaf ascorbate beyond the capacity of its regeneration (i.e. an increase in concentration ratios of oxidized to reduced ascorbate) is considered as one of the many markers of oxidative stress (Heber et al. 1996, Hideg et al. 1997). While higher amounts of ROS reactive ascorbate contribute to total antioxidant capacity, ascorbate may also act as a pro-oxidant, promoting the generation of $^{\bullet}$OH radicals through the reduction of ferric molecules (Halliwell and Gutteridge, 1999). The high FRAP value in 200-UV plants (Fig. 4A) suggest an increased free iron level, which enhances the danger of ascorbate mediated ROS generation. In order to see whether UV irradiation imposed oxidative stress in our study, both amounts of total ascorbate and relative amounts of oxidized ascorbate were measured. Results in Figs. 5A and 5B show that the applied supplemental UV irradiation caused oxidative stress in 200-plants only, in which the ratio of oxidized ascorbate markedly increased. 200-UV leaves had significantly higher levels of ascorbate (1.7-times) than 200-unt ones (Fig. 5A), but in these samples regeneration of oxidized ascorbate was unable to keep up with oxidation and the ratio of oxidized ascorbate increased from 15% to 26% (Fig. 5B). High light pretreatment, on the other hand, caused no

increase in the ascorbate content (Fig. 5A), or in the degree of ascorbate oxidation (Fig. 5B). UV irradiation caused an increase in the ascorbate content of 1000-leaves, but these leaves were able to maintain a relatively low, 15% oxidized ascorbate ratio. Results of the above ascorbate measurements show that an important difference between 200-UV and 1000-UV leaves is that while the former suffer mild oxidative stress the latter were rather UV-acclimated than stressed. A possible interpretation of the above data is that the increase in ascorbate content in 200-UV plants compared to 200-unt may not be all beneficial if not accompanied by efficient regeneration of oxidized ascorbate which does not contribute to the leaf's antioxidant capacity.

**Conclusions**

Pretreatment under high PPFD protected tobacco leaves from ROS effects derived from consecutive exposure to supplemental UV irradiation. High light pretreated leaves were rather acclimated than stressed: although lost some $CO_2$ incorporating capacity, these maintained a more reduced ascorbate pool and better photosynthetic electron transport. The ability to acclimate to UV appears to be due to higher levels of UV-B absorbing and $^{\bullet}OH$ radical scavenging antioxidants in these leaves, which was maintained during the UV irradiation. Leaves which did not receive the antioxidant stimulating high light treatment increased protective pathways (total phenolics, FRAP, $^{\bullet}OH$ radical scavenging) during UV irradiation to levels found in high light pretreated plants. However, these induced lines of defence could not protect tobacco leaves from UV as efficiently as high levels of defensive antioxidants already present at the onset of UV. Our data show that acclimative responses to UV overlap at several points resulting in a cross tolerance effect. Moreover, the production of UV-B absorbing components was lower in response to UV treatment than to high light pretreatment. The same phenomenon was observed by Younis et al. (2010) with overlapping antioxidant responses for high light and UV in broad bean seedlings. Bolink et al. (2001) showed the reverse: growth under UV-B radiation increased photoprotection in high light situations in both pea and bean plants based on elevated thiol and UV-absorbing compound concentrations. This suggests the possibility of a synergy in high light and UV responses in plants exposed to sunlight, with acclimation to high light helping to cope with solar UV and *vice versa*. Compounds traditionally detected as UV-absorbing pigments are an example of this, as suggested by results of the laboratory experiments presented here. Due to the application of broad band UV irradiation centered in UV-B but also containing UV-A in the present work, it would take further experiments to study whether (and to which extent) UV-A is involved in this cross tolerance.

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