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Antioxidant defence in UV-irradiated tobacco leaves is centred on hydrogen-peroxide neutralization

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Highlights:

- Supplemental UV decreased photosynthesis by 30% and activated antioxidant defence.
- Defence against ROS focussed on H_2O_2 (peroxidases, especially APX).
- Chloroplast antioxidants APX and Fe-SOD were activated more than other pathways.
- Under low PAR/UV conditions acclimation to UV may not rely on H_2O_2 signals.

Abbreviations: ABTS, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid; APX, ascorbate peroxidase enzyme, EC 1.11.1.11; FRAP, ferric reducing antioxidant power; Fv/Fm, maximum PS II quantum yield; PAR, photosynthetically active radiation; PS, Photosystem; POD, peroxidase enzymes, EC 1.11.1.x; PPFD, photosynthetic photon flux density; SOD, superoxide dismutase enzymes, EC 1.15.1.1; UV, ultraviolet, 280-400 nm; Y(II), light acclimated effective PS II quantum yield;

Abstract

Greenhouse grown tobacco (*Nicotiana tabacum* L. cv. Petit Havana) plants were exposed to supplemental UV centred at 318 nm and corresponding to 13.6 kJ m^{-2} d^{-1} biologically effective UV-B (280-315 nm) radiation. After 6 days this treatment decreased photosynthesis by 30%. Leaves responded by a large increase in UV-absorbing pigment content and antioxidant capacities. UV-stimulated defence against ROS was strongest in chloroplasts, since activities of plastid enzymes FeSOD and APX had larger relative increases than other, non-plastid specific SODs or peroxidases. In addition, non-enzymatic defence against hydroxyl radicals was doubled in UV treated leaves as compared to controls. In UV treated leaves, the extent of activation of ROS neutralizing capacities followed a peroxidases > hydroxyl-radical neutralization > SOD order. These results suggest that highly effective hydrogen peroxide neutralization is the focal point of surviving UV-inducible oxidative stress and argue against a direct signalling role of hydrogen peroxide in maintaining adaptation to UV, at least in laboratory experiments.

1. Introduction

Recent research shows that at mid-latitudes of the Northern hemisphere ambient solar ultraviolet (280-400 nm) radiation is rather a developmental signal than a direct stressor for plants (Brosché and Strid, 2003; Jenkins, 2009; Ballaré et al., 2011; Hideg et al. 2013). However, the same UV wavelengths may cause reactive oxygen species (ROS) mediated oxidative stress when applied in controlled environments, such as growth cabinets or greenhouses where PAR to UV ratios are lower than in nature. Whether these treatments result in severe cell damage or acclimative responses depends on several factors including growth conditions preceding the UV treatment as well as doses and wavelength distribution of the applied artificial UV source. Metabolic responses include an increase in epidermal UV absorbing pigment content and in cellular antioxidants (Carletti et al., 2003; Yannarelli et al., 2006; Fini et al., 2011; Majer and Hideg, 2012a, 2012b). When applied at very high (20-40-times of ambient) intensities, 312 nm centred UV-B generated a variety of reactive oxygen species (ROS) in leaves including superoxide and hydroxyl radicals at concentrations detectable by EPR spin trapping (Hideg and Vass, 1996). UV irradiation of leaf segments pre-loaded with either superoxide radical or singlet oxygen selective fluorescent ROS probes showed that when UV was applied alone, without PAR, higher energy UV-B and lower energy UV-A generated different ROS (Barta et al., 2004). Since these methods are not sensitive enough to quantify ROS in leaf tissues exposed to lower, near-ambient UV intensities, the presence of ROS in such experiments is only assumed from increased antioxidant activities (Carletti et al., 2003; Fini et al., 2011; Majer and Hideg, 2012a). The aim of the present study was to explore acclimative responses of tobacco leaves

to supplementary UV radiation in a controlled environment experiment, in terms of ROS specific antioxidants. Daily UV-B doses applied in our experiment were approximately 1.8-times higher than ambient doses in the Northern hemisphere (latitude 46^{o}) in summer (Bassman et al., 2001) and were applied in combination with lower than ambient PAR, which aggravates the effect of UV. Consequently, our results cannot be directly related to naturally occurring UV but may help to further elucidate plant responses to these conditions.

2. Methods

2.1. Plant growing and UV treatment conditions

Tobacco (*Nicotiana tabacum* L. cv. Petit Havana SR1) plants were grown in growth chambers (Fitoclima D1200, Aralab, Portugal) at 25/20 ^{o}C, at 16 h daily irradiation with ca. 150 µmol m^{-2} s^{-1} photosynthetic photon flux density (PPFD). Four-week old plants were treated for 6 days afterwards, in two groups each containing three plants. The first group (UV plants) was exposed to low dose supplemental UV radiation from Q-Panel UVB-313EL tubes (Q-Lab Ltd., Bolton, UK) through a cellulose diacetate filter (Courtaulds Chemicals, Derby, UK) and the second group (control plants) were kept under PAR only. The applied UV was centred at 318 nm (Majer and Hideg, 2012a) and corresponded to 15.6 kJ m^{-2} d^{-1} global (280-400 nm) or 13.6 kJ m^{-2} d^{-1} UV-B (280-315 nm) biologically effective dose as calculated using the Biological Spectral Weighting Function developed by Flint and Caldwell (2003). PAR was 50-55 µmol m^{-2} s^{-1} PPFD for both UV and control plants. At the end of the 6-day treatment, the last fully-developed leaf (at the 3rd-4th node) was chosen from each plant for analysis. Photosynthesis and electron transport (section 2.2) were measured on intact plants, and the same leaves were sampled for pigment and antioxidant analyses (2.3-2.4). The whole experiment was repeated with newly grown plants using the same growth and treatment conditions.

2.2. Photosynthesis and variable chlorophyll fluorescence measurements

Photosynthesis was characterized by CO_2 uptake (µmol CO_2 m^{-2} s^{-1}) measured on intact leaves at 200 µmol photons m^{-2} s^{-1} PPFD using a LI-6400 Portable Photosynthesis System (LI-COR Environmental, Lincoln, Nebraska USA). Following this, plants were kept in darkness for 30 min before chlorophyll fluorescence measurements were made using the MAXI-version of the Imaging-PAM (Heinz Walz GmbH, Effeltrich, Germany). Maximal (Fv/Fm) and light acclimated effective PS II quantum yields (Y(II)) were determined according to Genty et al. (1989). Light acclimated Y(II) was measured at the end of a 5 min exposure to 55 µmol m^{-2} s^{-1} actinic light.

2.3. Pigment analysis

Two 1 cm diameter discs were cut from each leaf and soaked in either 80 % acetone or acidified methanol at 4^{o}C in darkness for 24 hours. Following this, leaf discs were ground in the same medium and centrifuged (3000 x g, 5 min, 4^{o}C). Supernatants made from acetone extracts were used

for photometric determination of chlorophyll and carotenoid contents, based on absorbances measured at 664.6, 646.6 and 440.5 nm (Yang et al., 1998). Supernatants of acidified methanol extracts were used for assessing total UV-B absorption (the area under the absorption curve integrated between 280-315 nm). Absorption measurements were carried out using a Shimadzu UV1601 photometer.

2.4. Antioxidant measurements

Twelve leaf discs (diameter=1 cm) were weighed and ground first in liquid nitrogen then in 0.8 mL Na-phosphate buffer (50 mM, pH 7.0, 1 mM EDTA). When processing leaf discs for ascorbate peroxidase activity measurements, the isolating buffer contained 5 mM ascorbate in addition to the above components. Cell debris was removed by low speed centrifugation (3000 x g, 5 min, 4^{o} C), then supernatants were re-centrifuged at higher speed (30,000 x g, 25 min, 4^{o}C). Protein contents of the extracts were determined using the standard Bradford assay (Bradford 1976) and samples were stored at -80 ^{o}C until performing antioxidant measurements.

2.4.1. Photometric antioxidant capacity measurements

Hydroxyl radical ($^{\bullet}$OH) scavenging was determined based on the ability of the leaf extracts to inhibit the formation of the $^{\bullet}$OH-mediated oxidation of low fluorescence terephthalate acid (1,4-benzenedicarboxylic acid, TPA) to high fluorescence 2-hydroxyterephthalate (HTPA). HTPA fluorescence was measured using a Quanta Master QM-1 spectrofluorometer (Photon Technology Inc., Birmingham, New Jersey, USA), and $^{\bullet}$OH antioxidant capacities of leaf extracts were characterized by their half-inhibitory concentration on HTPA formation as described earlier (Stoyanova et al., 2011). Ethanol was used for calibration and $^{\bullet}$OH antioxidant capacities of leaf extracts were given as µM ethanol equivalent g^{-1} leaf fresh weight.

Peroxidase (EC 1.11.1.7) activity was tested using the ABTS method (Childs and Bardsley, 1975). The reagent solution contained 10% ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) in 50 mM citrate buffer (pH 5.0) and 360 µM H_2O_2. The POD activity of the samples was tested against the activity of standard horseradish peroxidase (Sigma) and was expressed as unit POD mg^{-1} protein.

SOD activity was measured as inhibition of superoxide-induced reduction of nitro blue tetrazolium (NBT) to formazan (Giannopolitis and Ries, 1977) as described earlier (Majer et al., 2010). The reaction mixture contained 0.015 U xanthine-oxidase in 50 mM Na-phosphate buffer (pH 7.2) with 0.3 mM EDTA, 0.2 mM xanthine, and formazan production was measured as absorption change at 560 nm. Results were expressed as unit SOD mg^{-1} protein.

FRAP (ferric reducing antioxidant power) assay was carried out according to a modification of the original medicinal biochemical assay (Benzie and Strain, 1996) as detailed in Majer and Hideg

(2012b). Ascorbic acid (AsA) was used for calibration and FRAP values were expressed as µmol AsA equivalents g^{-1} leaf fresh weight.

2.4.2. SOD and APX activity measurements using native PAGE

To determine enzyme activities, samples were first separated on SDS free native 12% PAGE. Gels for APX activity contained 4 mM ascorbate. After separation, gels were rinsed either in distilled water (SOD gels) or in a 50 mM Na-phosphate buffer (pH 7.0) containing 4 mM ascorbate (APX gels). This was followed by staining procedures which were carried out at room temperature.

SOD activities were determined as described by Song et al. (2007). First gels were incubated in darkness for 30 min in a 50 mM Na-phosphate buffer (pH 7.8) containing 1 mM EDTA, 0.05 mM riboflavin, 0.1 mM NBT and 0.3% N,N,N′,N′-tetramethylethylenediamine (TEMED). Following this, gels were rinsed in water and illuminated for 15 min to make the colourless bands with SOD activities in the purple-stained gel visible. To separate various SOD isoforms, either 2 mM KCN (a Cu/Zn-SOD inhibitor) or 2 mM H_2O_2 (inhibitor of FeSOD and Cu/Zn-SOD) was added to the staining mixture.

APX activity was determined according to Mittler and Zilinkas (1993). Gels were first incubated in a Na-phosphate buffer (pH 7.8) containing 8 mM ascorbate and 4 mM H_2O_2, then rinsed with buffer and stained with a mixture containing 2.45 mM nitroblue tetrazolium (NBT) and 28 mM TEMED in 50 mM Na-phosphate buffer (pH 7.8). APX activity was visualized as colourless bands on the greyish-blue gel, where the colouration was caused by TEMED-formazan, formed in a reaction between TEMED-NBT and ascorbate.

Gels were analysed with ImageJ software (Schneider et al., 2012) to quantify relative activities. Changes in SOD and APX activities brought about by the preceding UV treatment of leaves were determined as percentages of control leaf values.

2.5. Statistics

With the exception of native PAGE based enzyme activity measurements, all parameters were measured six-times, using six different leaves representing two biological repetitions and 3-3 parallels of UV or control samples in each repetition. Samples were pooled for SOD and APX activities in gels and these were measured twice, representing the two biological repetitions. Student's t-test was used to compare means and to calculate P-values, and differences were considered significant at $P<0.05$.

3. Results and Discussion

3.1. Photosynthetic responses to supplemental UV

Figure 1 illustrates changes induced by the 6-day supplemental UV treatment in tobacco leaves. Photosynthesis measured as CO_2 uptake at 200 µmol photons m^{-2} s^{-1} was 30% lower than in controls (Fig.1A). Both potential (maximum, Fv/Fm) quantum yields and effective (Y(II)) PSII quantum yields were lower in UV treated leaves than in controls. Y(II) was only 18% lower when measured at 55 µmol photons m^{-2} s^{-1} (which was the PAR applied during the UV treatment) and the difference between UV treated and control leaves was even smaller, 8-12%, when measured at 200 µmol photons m^{-2} s^{-1} (data not shown). High doses of UV-B were shown to have a direct effect on stomata (Nogues et al. 1999), in addition to changes in mesophyll photosynthesis including a decrease in both the amount and the activity of Rubisco (Strid et al., 1990; Allen et al., 1997). Supplemental UV did not result in a significant change in stomata conductance and caused a larger decrease in photosynthetic CO_2 uptake than in photochemical yield, suggesting that a partial inactivation of dark reactions contributes to the lower photosynthesis in UV treated leaves. This implies that the applied supplemental UV resulted in stress, although a major part of photosynthesis was maintained during the treatment allowing acclimatory responses to occur.

3.2. Pigment responses to supplemental UV

The taxing nature of supplemental UV is also shown by a significant, 12 and 34% loss of leaf chlorophyll and carotenoid contents respectively, by the end of the 6-day treatment (Fig.1A). On the other hand, a large, 80% increase in UV-absorbing pigment content supports the occurrence of acclimatory processes. Whole leaf extracts contain both epidermal UV screening pigments and various other UV absorbing flavonoids with primarily antioxidant functions (Caldwell et al., 1983; Middleton and Teramura, 1993; Zhang and Björn, 2009). An increase in UV absorbing pigment content is a common response when UV is applied to greenhouse grown plants (Liu et al., 1995; Carletti et al., 2003; Garcia Macias et al., 2007). Several plant metabolites, for example various flavonoids feature both UV absorbing and antioxidant characteristics (Agati and Tattini 2010; Majer et al., 2014). In our experiment, the observed strong increase in the UV absorbing capacity of leaf extracts suggested an increase in non-enzymatic antioxidants, which were assessed as total antioxidant capacity.

3.3. Antioxidant responses to supplemental UV

Extracts from UV treated leaves had more than twice (236%) the total antioxidant capacity (measured as FRAP) compared to untreated leaves (Fig.1B). In addition to this total capacity, specific ROS neutralizing capacities were also measured. The applied UV treatment had no significant effect on either total superoxide scavenging capacity (data not shown) or Cu/Zn-SOD, but increased the activity of chloroplast-located Fe-SOD by 65 % (Fig.1B). Chloroplastic Cu/Zn-SOD in tobacco is only

present in detectable amounts in immature leaves, and the abundant isoform in chloroplasts is Fe-SOD which is present at a relatively constant level in photosynthetic tissues of various ages (Van Camp et al., 1997). The observed large increase in Fe-SOD in UV-treated leaves suggests a plastid response. Our Fe-SOD activity data are in agreement with the result of Kliebenstein et al. (1998) who reported increased gene expression and protein levels of Fe-SOD in Arabidopsis in response to 15 kJ m^{-2} d^{-1} UV-B, a condition very similar to the one applied in our experiment. Increased superoxide neutralization leads to higher H_2O_2 concentrations; thus a successful acclimation to UV also requires effective H_2O_2 antioxidants. In our experiment, both total peroxidase and plastid APX activities increased to much larger extents (by 170 and 340%, respectively) than Fe-SOD (Fig.1B). These results differ from those found by Fini et al. (2011) in wild privet (*Ligustrum vulgare*) leaves exposed to higher supplemental UV doses (803 kJ m^{-2} UV-A + 38.8 kJ m^{-2} UV-B). In their experiment both SOD and APX increased by approximately 30-40% by the 8^{th} day of treatment but decreased afterwards to or even below activities measured in control leaves (Fini et al., 2011). The authors attributed the observed steep decline in APX activity to an acclimative response, assuming that higher plastid H_2O_2 concentrations prompted signalling to increase flavonoid biosynthesis (Fini et al., 2011). In another study, Yannarelli et al. (2006) found that sunflower plants acclimatized to 15 or 30 kJ m^{-2} biologically effective UV-B through the induction of various peroxidases, but not of APX which remained unaltered. Although differences in UV sources, UV dose and plant species make direct comparisons with these studies difficult, our data clearly contradict observations of decreased or unaltered APX activities in response to UV-B. In our experiment, the marked increase in peroxidase defence, especially in APX, suggests that increased H_2O_2 concentrations in UV exposed leaves are hazardous rather than beneficial. It is important to note that although tobacco leaves reportedly contain catalase forms which also possess peroxidatic activity (Havir and McHale 1987) the assay applied in our study may underestimate total H_2O_2 neutralizing activities due to its insensitivity to monofunctional forms. The importance of efficient defence against H_2O_2 may be explained by the possibility of UV-B inducible photo-cleavage of H_2O_2 yielding highly oxidizing hydroxyl radicals (Czégény et al. 2014). This is supported by the observation that protection against $^{\bullet}OH$ was doubled in UV-B exposed leaves (Fig.1B). In addition, ferric reducing capacities were also enhanced protecting against an UV-B independent, Fenton-type $H_2O_2 \rightarrow {}^{\bullet}OH$ reaction, although to a smaller extent than that of peroxidase defence (Fig.1B).

4. Conclusions

In leaves H_2O_2 is part of the complex signalling network that may induce acclimatory defence responses as well as cell death (Neill et al., 2002; Apel and Hirt, 2004). ROS concentrations during

acclimative responses should be optimized to fulfil signalling roles while avoiding oxidative damage. It was recently suggested that not only concentrations *per se*, but ratios of different ROS, determine the activation of the defence network or programmed cell death. According to Sabater and Martin (2013) a high ($^1O_2$ + $O_2^{-\bullet}$)/ H_2O_2 concentration ratio could trigger a transition from defence to senescence responses. It follows from this model that when relatively low PAR results in lower photooxidative pressure which is less likely to lead to chloroplastic $^1O_2$ production it takes less H_2O_2 to keep ($^1O_2$ + $O_2^{-\bullet}$)/ H_2O_2 low. Accordingly, supplemental UV-B treatment in our experiment resulted in augmented H_2O_2 neutralization allowing high chloroplastic peroxidase activity to protect from possible UV-B induced hydroxyl radical production (Czégény et al. 2014) without risking an increase in ($^1O_2$ + $O_2^{-\bullet}$)/ H_2O_2. This situation is different from experiments where high intensity PAR or sunlight is supplemented with UV radiation, which reportedly results in partial suppression of leaf peroxidase activities (Fini et al., 2011).

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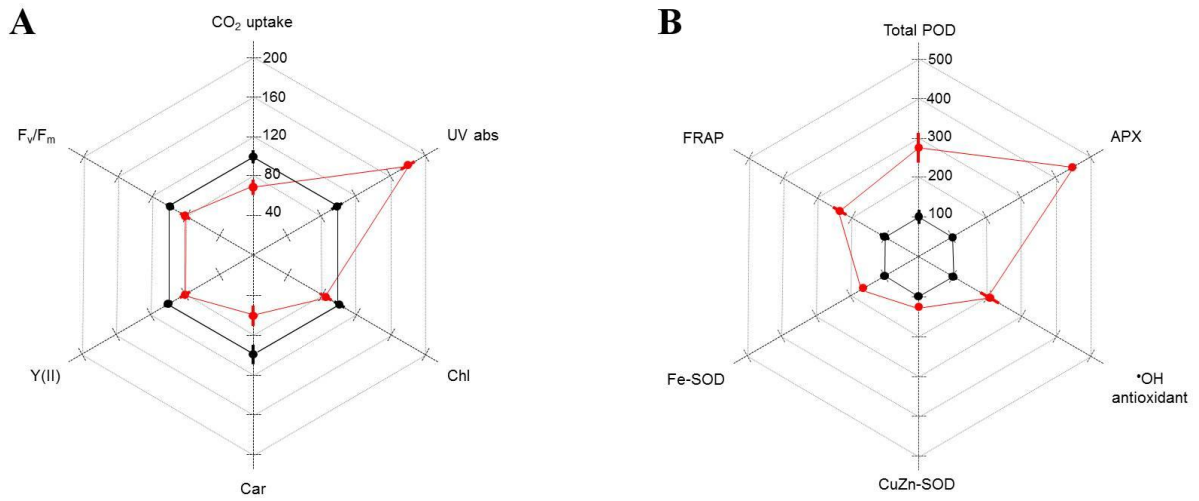

Figure 1

Figure caption

Figure 1

Tobacco leaf responses to 6-day supplementary UV treatment

Black and red symbols correspond to untreated (PAR only) and UV-B treated (PAR+UV-B) leaves, respectively.

Changes in (A) leaf photosynthesis, photochemical quantum yields, pigment content and (B) antioxidant capacities are shown as % of corresponding values in untreated leaves. Data points represent averages and error bars correspond to standard deviations (n=3 for Fe-SOD, Cu/Zn-SOD and APX, n=6 for all other samples).

100% values are: Photosynthesis, 6.73±1.14 µmol CO_2 m^{-2} s^{-1} uptake; Maximum PSII quantum yield Fv/Fm, 0.783±0.005; Effective PSII quantum yield at 55 µmol photons m^{-2} s^{-1} Y(II), 0.607±0.022; Total carotenoid content (Car) 1.834±0.701 µg g^{-1} FW; Total chlorophyll content (Chl) 23.978±2.916 µg g^{-1} FW; Total UV-B absorbing pigment content (UV abs) 35.435±52.116 OD nm; Total peroxidase activity (POD) 295.875±56.606 Unit mg^{-1} protein; FRAP 1.596±0.131 AsA equivalents g^{-1} FW; •OH antioxidant capacity, 89.366±3.013 µmol ethanol equivalents g^{-1} FW. Fe-SOD, Cu/Zn-SOD and APX activity data were evaluated using native gel images and activities were not quantified as enzyme units.

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