

Petra Majer^{1, #}, Gyula Czégény^{1, 2}, Györgyi Sándor¹, Philip J Dix³, Éva Hideg^{2, *}

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

¹Institute of Plant Biology, Biological Research Centre, Hungarian Academy of Sciences, Szeged, Hungary

²Department of Plant Biology, Institute of Biology, University of Pécs, Pécs, Hungary

³Biology Department, National University of Ireland Maynooth, Maynooth, Co. Kildare, Ireland

* corresponding author: ÉH, University of Pécs, address: Ifjúság u. 6, H-7624 Pécs, Hungary; email: ehideg@gamma.ttk.pte.hu; telephone: +3672 503600

permanent address: Corvinus University of Budapest, Department of Viticulture, H-1118 Budapest, Villányi út 29-43. Hungary

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Highlights:

- Supplemental UV decreased photosynthesis by 30% and activated antioxidant defence.
- Defence against ROS focussed on H₂O₂ (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 H₂O₂ 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;

1 **Abstract**

2 Greenhouse grown tobacco (*Nicotiana tabacum* L. cv. Petit Havana) plants were exposed to
3 supplemental UV centred at 318 nm and corresponding to $13.6 \text{ kJ m}^{-2} \text{ d}^{-1}$ biologically effective UV-B
4 (280-315 nm) radiation. After 6 days this treatment decreased photosynthesis by 30%. Leaves
5 responded by a large increase in UV-absorbing pigment content and antioxidant capacities. UV-
6 stimulated defence against ROS was strongest in chloroplasts, since activities of plastid enzymes
7 FeSOD and APX had larger relative increases than other, non-plastid specific SODs or peroxidases. In
8 addition, non-enzymatic defence against hydroxyl radicals was doubled in UV treated leaves as
9 compared to controls. In UV treated leaves, the extent of activation of ROS neutralizing capacities
10 followed a peroxidases > hydroxyl-radical neutralization > SOD order. These results suggest that
11 highly effective hydrogen peroxide neutralization is the focal point of surviving UV-inducible
12 oxidative stress and argue against a direct signalling role of hydrogen peroxide in maintaining
13 adaptation to UV, at least in laboratory experiments.

14

15 **1. Introduction**

16 Recent research shows that at mid-latitudes of the Northern hemisphere ambient solar
17 ultraviolet (280-400 nm) radiation is rather a developmental signal than a direct stressor for plants
18 (Brosché and Strid, 2003; Jenkins, 2009; Ballaré et al., 2011; Hideg et al. 2013). However, the same
19 UV wavelengths may cause reactive oxygen species (ROS) mediated oxidative stress when applied in
20 controlled environments, such as growth cabinets or greenhouses where PAR to UV ratios are lower
21 than in nature. Whether these treatments result in severe cell damage or acclimative responses
22 depends on several factors including growth conditions preceding the UV treatment as well as doses
23 and wavelength distribution of the applied artificial UV source. Metabolic responses include an
24 increase in epidermal UV absorbing pigment content and in cellular antioxidants (Carletti et al.,
25 2003; Yannarelli et al., 2006; Fini et al., 2011; Majer and Hideg, 2012a, 2012b). When applied at very
26 high (20-40-times of ambient) intensities, 312 nm centred UV-B generated a variety of reactive
27 oxygen species (ROS) in leaves including superoxide and hydroxyl radicals at concentrations
28 detectable by EPR spin trapping (Hideg and Vass, 1996). UV irradiation of leaf segments pre-loaded
29 with either superoxide radical or singlet oxygen selective fluorescent ROS probes showed that when
30 UV was applied alone, without PAR, higher energy UV-B and lower energy UV-A generated different
31 ROS (Barta et al., 2004). Since these methods are not sensitive enough to quantify ROS in leaf tissues
32 exposed to lower, near-ambient UV intensities, the presence of ROS in such experiments is only
33 assumed from increased antioxidant activities (Carletti et al., 2003; Fini et al., 2011; Majer and
34 Hideg, 2012a). The aim of the present study was to explore acclimative responses of tobacco leaves

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

7 **2. Methods**

8 **2.1. Plant growing and UV treatment conditions**

9 Tobacco (*Nicotiana tabacum* L. cv. Petit Havana SR1) plants were grown in growth chambers
10 (Fitoclima D1200, Aralab, Portugal) at 25/20 °C, at 16 h daily irradiation with ca. 150 $\mu\text{mol m}^{-2} \text{s}^{-1}$
11 photosynthetic photon flux density (PPFD). Four-week old plants were treated for 6 days afterwards,
12 in two groups each containing three plants. The first group (UV plants) was exposed to low dose
13 supplemental UV radiation from Q-Panel UVB-313EL tubes (Q-Lab Ltd., Bolton, UK) through a
14 cellulose diacetate filter (Courtaulds Chemicals, Derby, UK) and the second group (control plants)
15 were kept under PAR only. The applied UV was centred at 318 nm (Majer and Hideg, 2012a) and
16 corresponded to 15.6 $\text{kJ m}^{-2} \text{d}^{-1}$ global (280-400 nm) or 13.6 $\text{kJ m}^{-2} \text{d}^{-1}$ UV-B (280-315 nm) biologically
17 effective dose as calculated using the Biological Spectral Weighting Function developed by Flint and
18 Caldwell (2003). PAR was 50-55 $\mu\text{mol m}^{-2} \text{s}^{-1}$ PPFD for both UV and control plants. At the end of the
19 6-day treatment, the last fully-developed leaf (at the 3rd-4th node) was chosen from each plant for
20 analysis. Photosynthesis and electron transport (section 2.2) were measured on intact plants, and
21 the same leaves were sampled for pigment and antioxidant analyses (2.3-2.4). The whole
22 experiment was repeated with newly grown plants using the same growth and treatment conditions.

23 **2.2. Photosynthesis and variable chlorophyll fluorescence measurements**

24 Photosynthesis was characterized by CO_2 uptake ($\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$) measured on intact
25 leaves at 200 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ PPFD using a LI-6400 Portable Photosynthesis System (LI-COR
26 Environmental, Lincoln, Nebraska USA). Following this, plants were kept in darkness for 30 min
27 before chlorophyll fluorescence measurements were made using the MAXI-version of the Imaging-
28 PAM (Heinz Walz GmbH, Effeltrich, Germany). Maximal (F_v/F_m) and light acclimated effective PS II
29 quantum yields ($Y(II)$) were determined according to Genty et al. (1989). Light acclimated $Y(II)$ was
30 measured at the end of a 5 min exposure to 55 $\mu\text{mol m}^{-2} \text{ s}^{-1}$ actinic light.

31 **2.3. Pigment analysis**

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

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

6 **2.4. Antioxidant measurements**

7 Twelve leaf discs (diameter=1 cm) were weighed and ground first in liquid nitrogen then in
8 0.8 mL Na-phosphate buffer (50 mM, pH 7.0, 1 mM EDTA). When processing leaf discs for ascorbate
9 peroxidase activity measurements, the isolating buffer contained 5 mM ascorbate in addition to the
10 above components. Cell debris was removed by low speed centrifugation (3000 x g, 5 min, 4° C),
11 then supernatants were re-centrifuged at higher speed (30,000 x g, 25 min, 4°C). Protein contents of
12 the extracts were determined using the standard Bradford assay (Bradford 1976) and samples were
13 stored at -80 °C until performing antioxidant measurements.

14 **2.4.1. Photometric antioxidant capacity measurements**

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

23 Peroxidase (EC 1.11.1.7) activity was tested using the ABTS method (Childs and Bardsley,
24 1975). The reagent solution contained 10% ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic
25 acid) in 50 mM citrate buffer (pH 5.0) and 360 μM H_2O_2 . The POD activity of the samples was tested
26 against the activity of standard horseradish peroxidase (Sigma) and was expressed as unit POD mg^{-1}
27 protein.

28 SOD activity was measured as inhibition of superoxide-induced reduction of nitro blue
29 tetrazolium (NBT) to formazan (Giannopolitis and Ries, 1977) as described earlier (Majer et al.,
30 2010). The reaction mixture contained 0.015 U xanthine-oxidase in 50 mM Na-phosphate buffer (pH
31 7.2) with 0.3 mM EDTA, 0.2 mM xanthine, and formazan production was measured as absorption
32 change at 560 nm. Results were expressed as unit SOD mg^{-1} protein.

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

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

3 **2.4.2. SOD and APX activity measurements using native PAGE**

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

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

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

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

24 **2.5. Statistics**

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

31

32 **3. Results and Discussion**

33 **3.1. Photosynthetic responses to supplemental UV**

1 Figure 1 illustrates changes induced by the 6-day supplemental UV treatment in tobacco leaves.
2 Photosynthesis measured as CO₂ uptake at 200 μmol photons m⁻² s⁻¹ was 30% lower than in controls
3 (Fig.1A). Both potential (maximum, Fv/Fm) quantum yields and effective (Y(II)) PSII quantum yields
4 were lower in UV treated leaves than in controls. Y(II) was only 18% lower when measured at 55
5 μmol photons m⁻² s⁻¹ (which was the PAR applied during the UV treatment) and the difference
6 between UV treated and control leaves was even smaller, 8-12%, when measured at 200 μmol
7 photons m⁻² s⁻¹ (data not shown). High doses of UV-B were shown to have a direct effect on stomata
8 (Nogues et al. 1999), in addition to changes in mesophyll photosynthesis including a decrease in
9 both the amount and the activity of Rubisco (Strid et al., 1990; Allen et al., 1997). Supplemental UV
10 did not result in a significant change in stomata conductance and caused a larger decrease in
11 photosynthetic CO₂ uptake than in photochemical yield, suggesting that a partial inactivation of dark
12 reactions contributes to the lower photosynthesis in UV treated leaves. This implies that the applied
13 supplemental UV resulted in stress, although a major part of photosynthesis was maintained during
14 the treatment allowing acclimatory responses to occur.

15 **3.2. Pigment responses to supplemental UV**

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

28

29 **3.3. Antioxidant responses to supplemental UV**

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

1 present in detectable amounts in immature leaves, and the abundant isoform in chloroplasts is Fe-
2 SOD which is present at a relatively constant level in photosynthetic tissues of various ages (Van
3 Camp et al., 1997). The observed large increase in Fe-SOD in UV-treated leaves suggests a plastid
4 response. Our Fe-SOD activity data are in agreement with the result of Kliebenstein et al. (1998) who
5 reported increased gene expression and protein levels of Fe-SOD in Arabidopsis in response to 15 kJ
6 m⁻² d⁻¹ UV-B, a condition very similar to the one applied in our experiment. Increased superoxide
7 neutralization leads to higher H₂O₂ concentrations; thus a successful acclimation to UV also requires
8 effective H₂O₂ antioxidants. In our experiment, both total peroxidase and plastid APX activities
9 increased to much larger extents (by 170 and 340%, respectively) than Fe-SOD (Fig.1B). These results
10 differ from those found by Fini et al. (2011) in wild privet (*Ligustrum vulgare*) leaves exposed to
11 higher supplemental UV doses (803 kJ m⁻² UV-A + 38.8 kJ m⁻² UV-B). In their experiment both SOD
12 and APX increased by approximately 30-40% by the 8th day of treatment but decreased afterwards
13 to or even below activities measured in control leaves (Fini et al., 2011). The authors attributed the
14 observed steep decline in APX activity to an acclimative response, assuming that higher plastid H₂O₂
15 concentrations prompted signalling to increase flavonoid biosynthesis (Fini et al., 2011). In another
16 study, Yannarelli et al. (2006) found that sunflower plants acclimatized to 15 or 30 kJ m⁻² biologically
17 effective UV-B through the induction of various peroxidases, but not of APX which remained
18 unaltered. Although differences in UV sources, UV dose and plant species make direct comparisons
19 with these studies difficult, our data clearly contradict observations of decreased or unaltered APX
20 activities in response to UV-B. In our experiment, the marked increase in peroxidase defence,
21 especially in APX, suggests that increased H₂O₂ concentrations in UV exposed leaves are hazardous
22 rather than beneficial. It is important to note that although tobacco leaves reportedly contain
23 catalase forms which also possess peroxidatic activity (Havir and McHale 1987) the assay applied in
24 our study may underestimate total H₂O₂ neutralizing activities due to its insensitivity to
25 monofunctional forms. The importance of efficient defence against H₂O₂ may be explained by the
26 possibility of UV-B inducible photo-cleavage of H₂O₂ yielding highly oxidizing hydroxyl radicals
27 (Czégény et al. 2014). This is supported by the observation that protection against •OH was doubled
28 in UV-B exposed leaves (Fig.1B). In addition, ferric reducing capacities were also enhanced
29 protecting against an UV-B independent, Fenton-type H₂O₂ → •OH reaction, although to a smaller
30 extent than that of peroxidase defence (Fig.1B).

31

32 **4. Conclusions**

33 In leaves H₂O₂ is part of the complex signalling network that may induce acclimatory defence
34 responses as well as cell death (Neill et al., 2002; Apel and Hirt, 2004). ROS concentrations during

1 acclimative responses should be optimized to fulfil signalling roles while avoiding oxidative damage.
2 It was recently suggested that not only concentrations *per se*, but ratios of different ROS, determine
3 the activation of the defence network or programmed cell death. According to Sabater and Martin
4 (2013) a high ($^1\text{O}_2 + \text{O}_2^{\bullet-}$)/ H_2O_2 concentration ratio could trigger a transition from defence to
5 senescence responses. It follows from this model that when relatively low PAR results in lower
6 photooxidative pressure which is less likely to lead to chloroplastic $^1\text{O}_2$ production it takes less H_2O_2
7 to keep ($^1\text{O}_2 + \text{O}_2^{\bullet-}$)/ H_2O_2 low. Accordingly, supplemental UV-B treatment in our experiment resulted
8 in augmented H_2O_2 neutralization allowing high chloroplastic peroxidase activity to protect from
9 possible UV-B induced hydroxyl radical production (Czégény et al. 2014) without risking an increase
10 in ($^1\text{O}_2 + \text{O}_2^{\bullet-}$)/ H_2O_2 . This situation is different from experiments where high intensity PAR or
11 sunlight is supplemented with UV radiation, which reportedly results in partial suppression of leaf
12 peroxidase activities (Fini et al., 2011).

13

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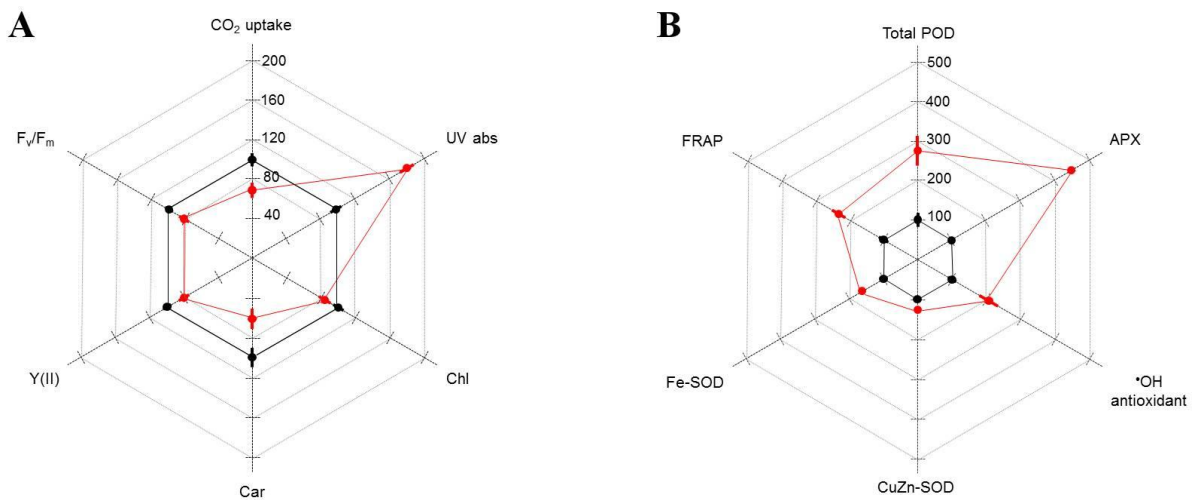


Figure 1

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, $6.73 \pm 1.14 \mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ uptake; Maximum PSII quantum yield F_v/F_m, 0.783 ± 0.005 ; Effective PSII quantum yield at $55 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ Y(II), 0.607 ± 0.022 ; Total carotenoid content (Car) $1.834 \pm 0.701 \mu\text{g g}^{-1}$ FW; Total chlorophyll content (Chl) $23.978 \pm 2.916 \mu\text{g g}^{-1}$ 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, $89.366 \pm 3.013 \mu\text{mol ethanol equivalents g}^{-1}$ 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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