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Ultraviolet-B acclimation is supported by functionally heterogeneous phenolic peroxidases

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Tobacco plants were grown in plant chambers for four weeks, then exposed to one of the following treatments for 4 days: (1) daily supplementary UV-B radiation corresponding to $6.9 \text{ kJ m}^{-2} \text{ d}^{-1}$ biologically effective dose (UV-B), (2) daily irrigation with 0.1 mM hydrogen peroxide, or (3) a parallel application of the two treatments (UV-B + H_2O_2). Neither the H_2O_2 nor the UV-B treatments were found to be damaging to leaf photosynthesis. Both single factor treatments increased leaf H_2O_2 contents but had distinct effects on various H_2O_2 neutralising mechanisms. Non-enzymatic H_2O_2 antioxidant capacities were increased by direct H_2O_2 treatment only, but not by UV-B. In contrast, enzymatic H_2O_2 neutralisation was mostly increased by UV-B, the responses showing an interesting diversity. When class-III peroxidase (POD) activity was assayed using an artificial substrate (ABTS, 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)), both treatments appeared to have a positive effect. However, only UV-B-treated leaves showed higher POD activities when phenolic compounds naturally occurring in tobacco leaves (chlorogenic acid or quercetin) were used as substrates. These results demonstrate a substrate-dependent, functional heterogeneity in POD and further suggest that the selective activation of specific isoforms in UV-B acclimated leaves is not triggered by excess H_2O_2 in these leaves.

Abbreviations

ABTS	2,2'-Azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)
ABTS-POD	Peroxidase activity measured with ABTS as substrate
APX	Ascorbate peroxidase, EC 1.11.1.11
CAA	Caffeic acid
CAT	Catalase, EC 1.11.1.6
CGA	Chlorogenic acid
Fv/Fm	Maximum quantum efficiency of PS II
GPX	Glutathione peroxidase, EC 1.11.1.9
guaiacol	2-Methoxyphenol
Gua-POD	Peroxidase activity measured with guaiacol as substrate
HRP	Horseradish peroxidase
PAR	Photosynthetically active radiation
QUE	Quercetin
QUE-POD	Peroxidase activity measured with QUE as substrate
POD	Peroxidase, EC 1.11.1.7
RUT	Quercetin-3-O-rutinoside, rutin
RUT-POD	Peroxidase activity measured with RUT as substrate
SOD	Superoxide dismutase, EC 1.15.1.1
TLC	Thin-layer chromatography
UV-B	280–315 nm ultraviolet radiation
Y(II)	Quantum efficiency of light acclimated PS II

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Y(NO)	Non-regulated non-photochemical quenching of PS II
Y(NPQ)	Regulated non-photochemical quenching of PS II

Hydrogen peroxide is produced in plants in a variety of metabolic and stress-inducible pathways¹. The electron transport of chloroplasts and mitochondria as well as various peroxisomal and plasma-membrane localised oxidases produce superoxide anion radicals ($O_2^{\cdot-}$), which are converted into H_2O_2 by the superoxide-dismutase enzyme (SOD)^{2–4}. Because H_2O_2 is relatively stable in biological systems with a half-life of milliseconds to seconds⁵, it may act as a second messenger molecule or enzyme substrate^{4–6} in addition to being a damaging oxidising agent when present in higher concentrations⁷. Pathogens and various abiotic factors were shown to increase H_2O_2 production, although stress responses generally involve the activation of various H_2O_2 neutralising enzymes as well. Elevated H_2O_2 concentrations in plant leaves were documented in response to UV-B irradiation⁸, excess photosynthetically active radiation (PAR)⁹, high temperature^{10–12}, drought¹³, or heavy metal stress¹⁴. Although H_2O_2 was considered capable of diffusing across membranes by itself¹⁵, its transport between intracellular compartments and between cells is mainly through aquaporins¹⁶. Such mobility facilitates the molecule's messenger function but also requires the antioxidant control of local concentrations further away from H_2O_2 production sites.

Ultraviolet-exposed plants especially need to maintain an effective H_2O_2 regulating system^{17–22} since the UV-B (280–315 nm) component of sunlight not only elevates H_2O_2 concentrations *in planta* but may also photoconvert H_2O_2 to more hazardous hydroxyl radicals ($\cdot OH$)⁸. Earlier studies have shown that the proper activation of class-III plant peroxidase (POD) enzymes is a key factor in the successful acclimation to UV-B both in model plants exposed to supplemental UV radiation in growth chambers²⁰ and in sun leaves outdoors²³. Our recent work with tobacco plants also showed that leaf acclimation to supplementary UV-B is realised through a selective activation of POD isoforms²².

There are numerous POD isoenzymes in a plant tissue, mainly in cell walls and vacuoles²⁴, but phenolic peroxidases were also found in chloroplasts²⁵. The common view is that POD enzymes are not substrate-selective but rather use a wide range of phenolic compounds as electron donors^{26,27} depending on the availability of these secondary metabolites²⁸. At least three distinct pathways have been identified to facilitate flavonoid transport among cellular locations^{29,30}, and phenolic compounds were found in a variety of cell compartments including the cytosol, vacuole, ER, as well as chloroplast and nucleus^{31,32}.

Acclimative responses to UV-B include an increase in leaf phenolic contents^{33,34} and the biosynthesis of these secondary metabolites occurs under the regulation of UVR8, the UV-B photoreceptor³⁵. Little is known about the molecular mechanism of UV-inducible peroxidase upregulation. UV-B-induced UVR8-regulated genes include a glutathione peroxidase in *Arabidopsis thaliana*³⁶. However, to the best of our knowledge, UVR8-regulated POD genes have not been identified so far. The possibility of indirect ROS-mediated upregulation has been suggested as the mode of action of UV-B on genes encoding antioxidant enzymes³⁷. Such signalling has been demonstrated to occur as an upregulating UV-B effect on the multi-function defence genes PR1 and PDF1.2³⁸. This model is supported by the overlap between antioxidant responses to UV-B and several other abiotic factors⁷. Given the well-established role of H_2O_2 as a signal molecule, a plausible assumption is that UV-B stimulates leaf antioxidants through the increased production of ROS. In order to test this hypothesis, here, we compare the antioxidant responses of tobacco (*Nicotiana tabacum*) leaves to supplementary UV radiation and to direct H_2O_2 treatment. The latter was achieved as irrigation with a water solution of H_2O_2 , which has already been shown to increase H_2O_2 concentrations in an experiment performed with rice seedlings³⁹. The main phenolics in tobacco leaves include chlorogenic acid, rutin, and caffeic acid, but the presence of vanillic acid, ferulic acid, or quercetin has also been reported^{40–45}.

The second aim of our work was to study whether UV-inducible phenolic compounds contribute to modulating leaf H_2O_2 concentrations as POD substrates or as direct ROS scavengers. The latter function is feasible because several phenolic compounds are highly reactive to H_2O_2 *in vitro*⁴⁶. Regardless of their mode of action, phenolic compounds are oxidised when acting as antioxidants. This yields a wide range of products, which include phenoxyl and semiquinone radicals^{47,48}. The chemistry of these reactions has been studied extensively in nutrition science⁴⁹ and results may also be relevant to reactions assumed to occur *in planta*. However, the threat of antioxidant phenolic compounds turning pro-oxidant is less likely in plant than in animal tissues. Experiments using a specific class-III plant peroxidase, horseradish peroxidase (HRP), demonstrated that phenolic antioxidants can be regenerated from their radical forms by ascorbate (ASA)⁵⁰, glutathione (GSH)⁵¹, or by monodehydroascorbate reductase⁵².

As exposure to UV-B radiation affects the way plants respond to changes in other environmental factors⁵³, our experimental setup also provided an opportunity to test the following hypothesis: Do responses to exogenous H_2O_2 and endogenous, UV-B triggered H_2O_2 overlap? To this end, a two-factor treatment, H_2O_2 irrigation under supplemental UV-B, was also added.

Results

Tobacco leaves were analysed from plants in four treatment groups: (1) kept under PAR only and irrigated with water (untreated control, C), (2) kept under supplementary UV-B and irrigated with water (UV-B), (3) kept under PAR only and irrigated with H_2O_2 solution (+ H_2O_2), or (iv) kept under supplementary UV-B and irrigated with H_2O_2 solution (UV-B + H_2O_2). Photochemical yield measurements showed that neither UV-B nor the application of H_2O_2 damaged photosynthetic electron transport (Supplementary Figure S1). In fact, a slight (6–8%) increase in yield was detected in treated plants compared to untreated controls. This observation shows that leaves were not damaged by but are rather acclimated to the applied treatments.

UV-B irradiation and H₂O₂ treatment induce distinct antioxidant responses. Leaf H₂O₂ contents were significantly increased by both treatments, and the effects of these were interactive: one factor had a stronger positive effect in the presence of the other (Fig. 1A). SOD activity was lower both in UV-B-exposed and in H₂O₂-treated plants than in untreated controls, and the interaction of the two factors was negative (Fig. 1B). APX activity was increased by ca. 50% in UV-B treated leaves but was unaffected by the H₂O₂ treatment either in the presence or absence of UV-B (Fig. 1C). When GPX was assayed using H₂O₂ as substrate, the enzyme activity showed only a non-significant marginal ($p=0.084$) increase in UV-B treated leaves. Plant H₂O₂ treatment had no effect on enzyme activity (Fig. 1D). Using an organic hydroperoxide as GPX substrate revealed no differences in the activities as well (data not shown). The effects of UV-B and H₂O₂ on CAT activity were opposed: UV-B had a positive (ca. 47%) but H₂O₂ treatment had a negative (ca. -87%) effect. Despite its negative effect as a single factor, the H₂O₂ treatment had no effect on how CAT activity reacted to UV-B (Fig. 1E). Non-enzymatic H₂O₂ neutralising capacities showed relatively small changes in response to the applied treatments. H₂O₂ treatment as a single factor resulted in a ca. 20% higher antioxidant capacity and UV-B alone had no significant effect. However, the two factors interacted and the H₂O₂ treatment resulted in a larger ca. 35% increase when UV-B was also applied (Fig. 1F).

Before examining POD responses, changes in leaf phenolics were also assessed. Dualex measurements indicated a strong (120–130%) increase in the adaxial phenolic index in response to the UV-treatment, both with and without the exogenous H₂O₂ treatment. The application of H₂O₂ alone had no significant effect (data not shown). Because the Dualex technique is based on 375 nm absorption in leaf tissue⁵⁴, it is expected to reflect an increase in flavonoids to a larger extent than those in phenolic acid content due to differences between the UV absorption of these two compound groups⁴⁶. TLC separation of methanolic leaf extracts was attempted to illustrate changes in phenolic compounds (Supplementary Figure S2). However, base levels in untreated leaves were too low for detection. As expected from Dualex measurements, the H₂O₂ treatment alone did not amend this situation. Extracts from UV-B exposed leaves, however, showed a large increase in chlorogenic acid (CGA) and the flavonol quercetin-rutinoside (RUT) contents. Since the TLC technique does not allow quantitative comparisons, our results only indicate that this marked change in phenolic composition was a common characteristic of UV-B and UV-B + H₂O₂ treated leaves (Supplementary Figure S2).

UV-B responsive phenolic compounds support diverse defence functions. In order to investigate the possible contributions of the two major UV-B responsive phenolic compounds RUT and CGA to the non-enzymatic H₂O₂ neutralising capacity of leaves, we used pure test compounds. This is an extension of a previous study, which reported the non-enzymatic H₂O₂ neutralising capacities of 36 different phenolic compounds including RUT and QUE but not CGA⁴⁶. In addition to phenolic compounds used as POD substrates in the present study, two non-phenolic antioxidants, ASA and GSH, were also added due to their potential to re-reduce oxidised phenolics^{50,51}. The results are shown in Table 1, relative to the non-enzymatic H₂O₂ neutralising capacity of ASA. The two phenolic acids, CGA and caffeic acid (CAA), were weaker antioxidants than ASA and much weaker than the two flavonols; their H₂O₂ neutralising capacities were similar to those of GSH in this in vitro model. Following this comparison of direct H₂O₂ reactivities, we compared phenolic compounds as POD substrates.

Using ABTS or guaiacol as substrates is a common practice when POD activities are assayed. We complemented these two methods by using four different phenolic compounds that occur in tobacco leaves. CGA and RUT were chosen because these were present in higher amounts in UV-B treated leaves (Supplementary Figure S2). The choice of the aglycone form of RUT (quercetin, QUE) was based on the earlier use of this substrate to assess POD in tobacco leaves²². CAA was included in the study as a reported major phenolic component of tobacco leaves⁵⁵. In the following, POD activities are discussed according to the substrate used in the assay; for example, RUT-POD refers to POD activity measured using RUT as substrate. Figure 2 shows that the results were strongly affected by the choice of substrate, both in activity (enzyme units) and in terms of responses to treatments. POD enzyme units measured in untreated leaves using various substrates followed a RUT-POD > CGA-POD > ABTS-POD > CAA-POD > Gua-POD > QUE-POD > order. Activities measured in untreated leaves with these substrates are given in legends to Figs. 1 and 2 in enzyme units. CAA-POD and RUT-POD showed no change in response to either treatment (Fig. 2D,F, respectively). ABTS-POD increased in response to UV-B and H₂O₂ treatment by ca. 120% and 30%, respectively, compared to untreated leaves. The positive effect of UV-B was maintained in H₂O₂ treated plants, although the two factors interacted and the H₂O₂ treatment lessened the extent of the positive UV-B effect (Fig. 2A). Gua-POD and CGA-POD was increased by UV-B regardless of the application of H₂O₂ treatment, which was not a significant factor. Interestingly, the two treatments had opposite effects on QUE-POD: the UV-B treatment increased the enzyme activity, while the H₂O₂ treatment decreased it. These effects were maintained as significant in the two factor treatment without interaction, and the positive effect of UV-B was smaller without the H₂O₂ treatment (Fig. 2C).

In the following section, we studied the effects of ASA or GSH on the oxidation rates of QUE, RUT, CAA, or CGA as POD substrates. In this experiment, various amounts of ASA or GSH were added and the kinetics of phenolic substrate oxidation by POD enzymes contained in the leaf extract was followed photometrically at the indicated wavelengths. ASA and GSH were used in the 1.4 to 140 μ M concentration range and the ability of the reactivity of these antioxidants to restore oxidised phenolic substrates was illustrated by a time delay in the consumption of these compounds. In this experiment, we used a pooled sample of UV-B treated leaves because these had the highest relative POD activities (Fig. 2). The oxidation rate of RUT was not affected by the presence of either ASA or GSH up to 140 μ M concentrations (data not shown). Figure 3 shows that GSH was most reactive to oxidised CGA (Fig. 3A), less reactive to oxidised CAA (Fig. 3B), and did not restore oxidised QUE, even

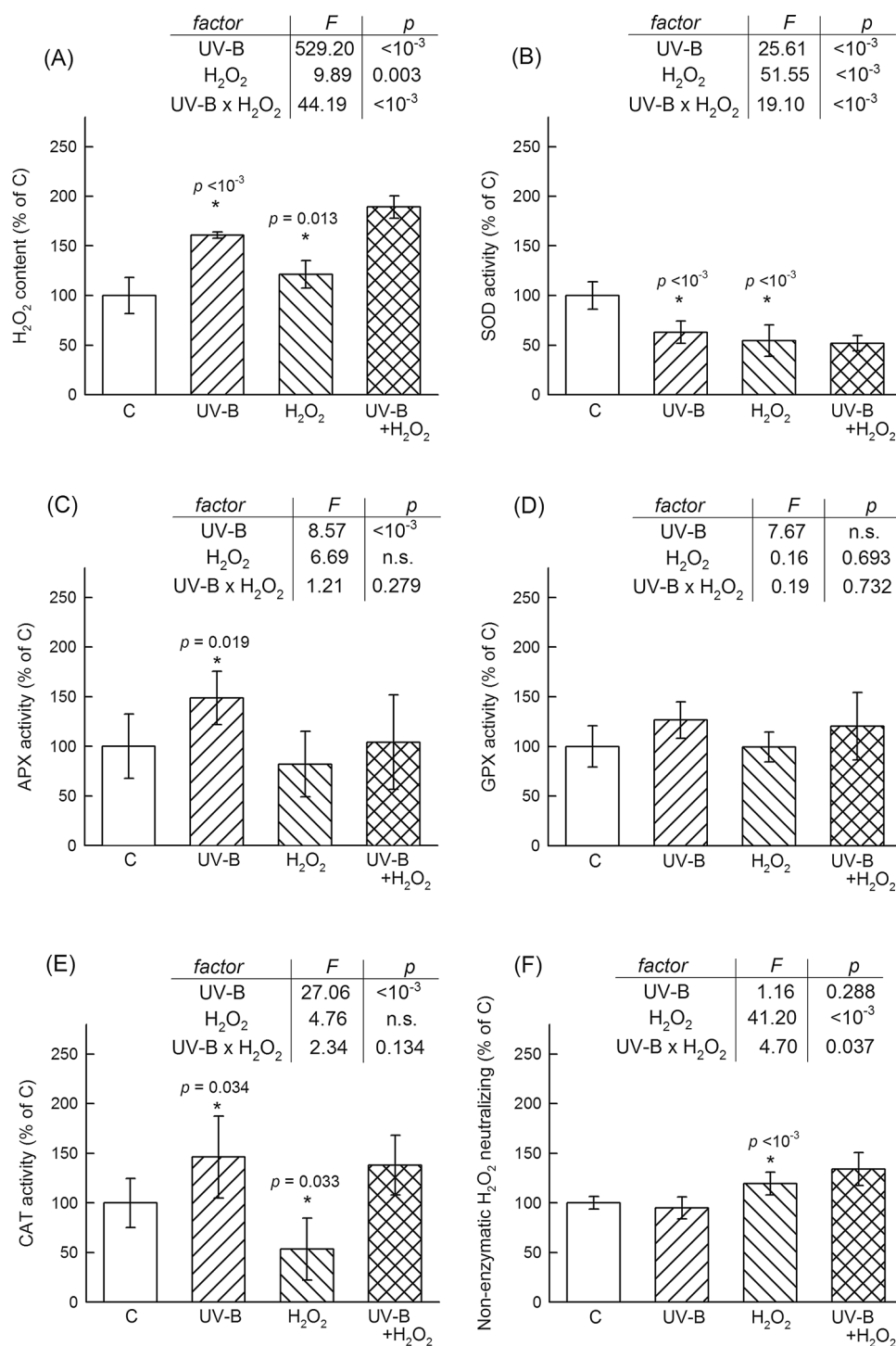


Figure 1. Comparison of leaf H₂O₂ content (A); activities of superoxide dismutase (B), ascorbate peroxidase (C), glutathione peroxidase (D), and catalase (E) enzymes; and non-enzymatic H₂O₂ neutralizing capacity (F) in four treatment groups: untreated control, C; UV-B treated, UV-B; H₂O₂ treated, H₂O₂; and treated with both UV-B and H₂O₂; UV-B + H₂O₂. Column heights and error bars represent means and standard deviations, respectively. 100% leaf H₂O₂ content = 21.49 μM mg⁻¹ leaf FW, 100% SOD activity = 257.30 U mg⁻¹ protein, 100% APX activity = 347.29 mU mg⁻¹ protein, 100% GPX activity = 66.70 mU mg⁻¹ protein, 100% CAT activity = 20.61 mU mg⁻¹ protein, 100% non-enzymatic H₂O₂ neutralizing = 1.55 μM ASA equivalent mg⁻¹ leaf FW. Columns and error bars correspond to means and standard deviations, respectively, n = 10. Significant single factor effects, such as UV-B vs. C or H₂O₂ vs. C, are indicated with an asterisk and *p* value above the corresponding column. Results of two-factor ANOVA are shown in insets as *F* and *p* values. Replacing a *p* value with n.s. indicates that although the corresponding *F* value was higher than *F*_{crit} (1,36) = 4.11 but factor significance was not confirmed in Tukey's post hoc test.

Compound	H ₂ O ₂ neutralizing capacity (μM compound in μM ascorbic acid equiv.)
Caffeic acid (CAA)	0.55
Chlorogenic acid (CGA)	0.69
Glutathione (GSH)	0.57
Quercetin (QUE)	3.50
Quercetin-rutinoside (RUT)	1.65
Ascorbic acid (ASA)	1.00

Table 1. Non-enzymatic H₂O₂ neutralizing capacities.

at the highest applied concentration (Fig. 3C). The efficiency of the same concentration (20 μM) ASA to retard phenolic substrate oxidation followed an opposite QUE > CAA > CGA order (Fig. 3).

Discussion

Using model plants in growth chambers, the present work shows that UV-B irradiation selectively enhances a subset of the antioxidant network. Analysing data from publications, which reported UV-induced changes in antioxidant enzyme activities, we have already shown that the stronger activation of peroxidases than SOD is a special characteristic of acclimative plant responses to UV-B²⁰. The role of this response is to avoid high leaf H₂O₂ levels prone to UV-B photocleavage into hydroxyl radicals⁸. The present study supports this model. Moreover, it shows that successful acclimation (avoided loss in leaf photochemical yield) may also be realised with a decrease in SOD activity combined with increased APX, POD, and CAT activities (Figs. 1 and 2). In the present experiment, GPX activities were not affected by the applied UV-B treatment (Fig. 1D), contrary to earlier reports on UV-B regulated GPX expression in Arabidopsis⁵⁶. This may be due to differences in the UV-B fluence rates applied and in the levels of detection (gene vs. enzyme activity). CAT activities were significantly lower (20.6 and 30.1 mU mg protein⁻¹ control and in UV-B acclimated leaves, respectively) than those of APX (347 and 516 mU mg protein⁻¹) or POD (1–100 U mg protein⁻¹, depending on substrate and treatment), which is probably due to the low photorespiration in leaves grown under relatively low PAR in this experiment. POD responses to UV-B showed an interesting heterogeneity and suggest that only specific isoforms contribute to the acclimation. Our earlier study has already demonstrated that two POD assays using traditional substrates, ABTS and guaiacol, registered different extents of POD activation in UV-B-treated tobacco leaves²². Using four phenolic compounds naturally occurring in tobacco leaves (CAA, CGA, RUT, QUE), we show here that although these can be oxidised as POD substrates, only specific POD isoforms contribute to acclimation to supplemental UV-B (Fig. 2). This conclusion is similar, but not identical, to that of Jansen et al.⁵⁷. Studying the UV-susceptibility of transgenic tobacco lines over-expressing phenol-oxidising peroxidases, the authors put forward a model in which isoenzyme diversity resulted in the polymerisation and/or crosslinking of specific phenolic compounds, and which increased the protection of plants from UV-radiation⁵⁷. While not debating the validity of their model, we offer an alternative, which can be coexisting in leaves. Our hypothesis is based on the assumptions that (1) phenolic compounds protect leaves from radiation not only as UV screening compounds but also as antioxidants, (2) phenolics oxidatively modified by either POD or in a direct reaction with H₂O₂ can be re-generated, and (3) the assignment of individual compounds to defence functions depends not only on their antioxidant capacities but also on the metabolic economy of their regeneration.

Several authors have already shown that the phenolic compounds included in our study act as electron donors to a specific POD form: HRP^{58,59}. Our data show that these are also capable of supporting other POD, such as the ones in tobacco leaves, although the biochemical properties of the tobacco enzyme were found to be distinct from those of HRP⁶⁰. First, we discuss the possible roles of the two phenolic components abundant in UV acclimated leaves, CGA and RUT. Even in the absence of the UV treatment, both compounds were efficient POD substrates, conferring 15 to 90-times higher POD activities than CAA or QUE in untreated leaves. The complexity of the phenolic defence response is supported by the result that substrates favoured by UV-responsive POD isoforms (CGA and QUE, Fig. 2) do not fully correspond to compounds accumulated in high amounts in UV-treated leaves (CGA and RUT, Supplementary Fig. 2). However, while CGA-POD was ca. 50% more active in UV-B exposed leaves than in controls, there was no significant change in RUT-POD (Fig. 2E,D, respectively). On the other hand, RUT is a strong direct H₂O₂ neutralising antioxidant, with 1.65-times higher reactivity to ROS than ASA (Table 1). Therefore, the explanation for the substantial increase in leaf RUT content in UV-exposed leaves is not the increased need for this compound as electron donor to POD but rather as a direct antioxidant. Contrary to RUT, CGA is a relatively poor non-enzymatic antioxidant (Table 1) but an efficient POD substrate. POD-oxidised CGA was efficiently recovered by ASA or GSH. The former finding is in agreement with earlier reports using HRP^{58,61}, and the latter is a novel one. We found no such recovery in the case of POD-oxidised RUT. This difference also supports the participation of CGA-POD, but not RUT-POD, in the observed UV response. CGA biosynthesis is reportedly induced by a variety of stress conditions in addition to UV⁶², and our results suggest that its main role is lessening damage as a POD substrate.

Considering the other two phenolic leaf components, CAA and QUE, the amounts of these were either unaffected by the UV exposure or the increase was minor and below the detection threshold of the applied TLC method. CAA is a relatively weak direct antioxidant (Table 1) and the relatively low POD activity it conferred (6% of CGA-POD) did not change upon UV exposure (Fig. 2F). These observations suggest that the role of CAA in the UV response is negligible. QUE, an aglycone flavonol, is of more interest. First, flavonoid aglycones are

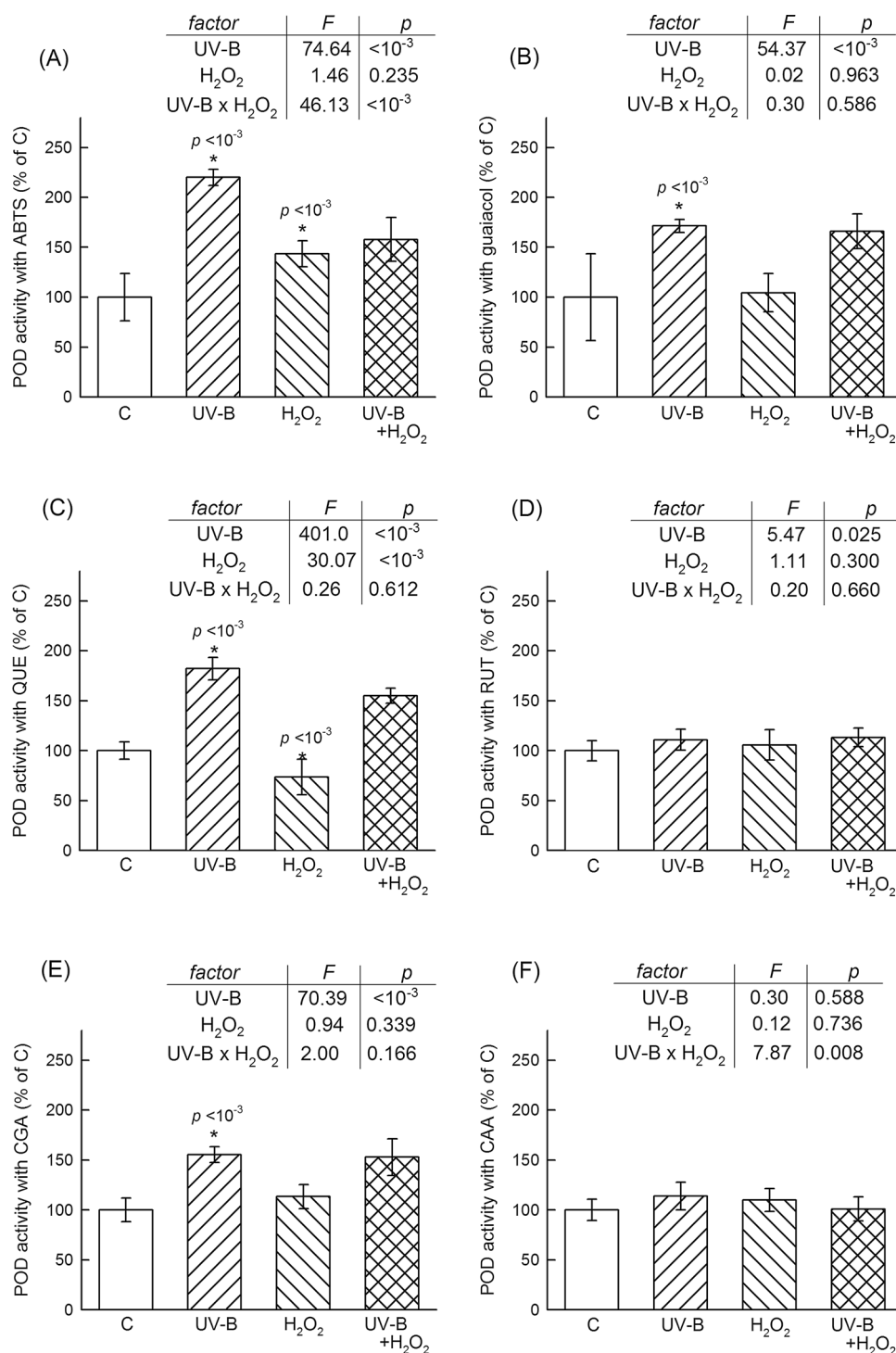


Figure 2. Comparison of class-II peroxidase enzyme activities assayed with various substrates, ABTS (A), guaiacol (B), quercetin (C), quercetin-rutinoside (D), chlorogenic acid (E) or caffeic acid (F) in four treatment groups: untreated control, C; UV-B treated, UV-B; H₂O₂ treated, H₂O₂; and treated with both UV-B and H₂O₂; UV-B + H₂O₂. Column heights and error bars represent means and standard deviations, respectively. All values are shown as percentage of the mean of values measured in untreated plants. 100% POD activities as U mg⁻¹ protein were the following: 5.97 with ABTS, 1.77 with guaiacol (Gua), 0.93 with quercetin (QUE), 88.46 with quercetin-3-O-rutinoside (RUT), 43.51 with chlorogenic acid (CGA), and 2.66 with caffeic acid (CAA). Columns and error bars correspond to means and standard deviations, respectively, n = 10. Significant single factor effects, such as UV-B vs. C or H₂O₂ vs. C, are indicated with an asterisk and p value above the corresponding column. Results of two-factor ANOVA are shown in insets as F and p values.

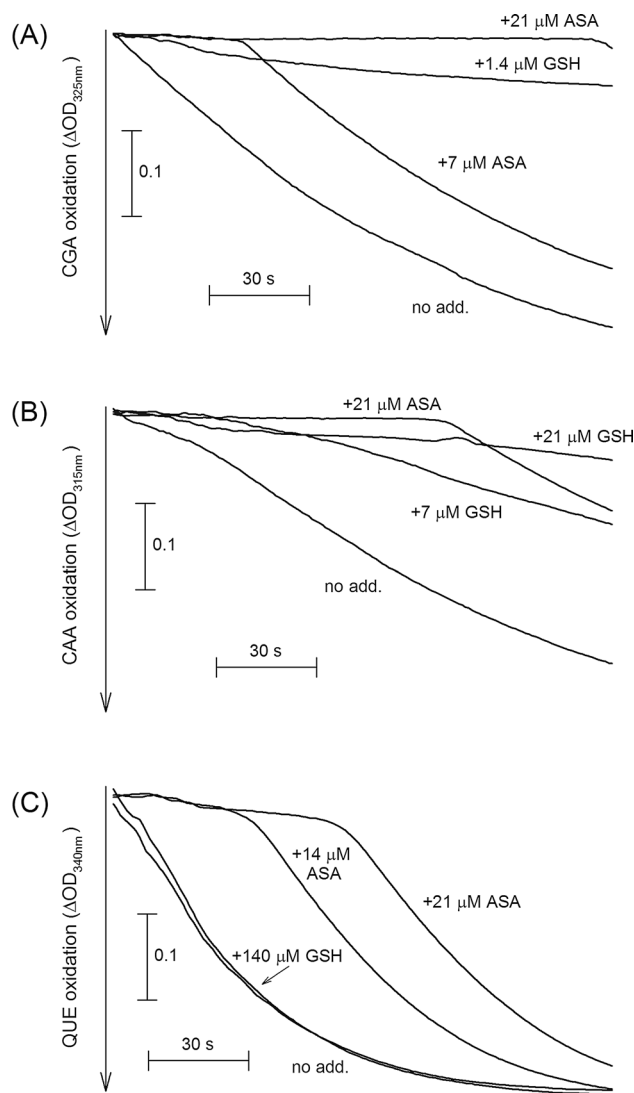


Figure 3. The effect of ascorbate (ASA) or glutathione (GSH) on the oxidation of phenolic compounds (A) chlorogenic acid, CGA, (B) caffeic acid, CAA, and (C) quercetin, QUE as POD substrates. All substrates were used at 3 mM concentrations. Using molar extinction coefficients from Table 2, $\Delta OD = 0.1$ corresponds to the oxidation of 83 μM CGA (A), 62 μM CAA (B) or 387 μM QUE (C).

usually present in leaves in much lower amounts than their glycosylated forms, such as RUT⁶³. Thus, the low activity of QUE-POD (ca. 90-times lower than RUT-POD and 45-times lower than CGA-POD in untreated leaves) may be explained by a relatively low amount of POD isoforms preferring a substrate in short supply. However, the significance of QUE in the UV response should not be dismissed. QUE is a very efficient direct H_2O_2 antioxidant, 3.5-times stronger than ASA. Also, the relative activity of QUE-POD may be low, but it nearly doubled in response to UV-B. On the other hand, the regeneration of QUE from its oxidised form is the least 'economical' among the phenolic compounds in this study in the sense that it required more ASA than the restoration of CGA or CAA, and it was not recovered by GSH (Fig. 3). This result also suggests that the QUE form yielded by leaf QUE-POD is a phenoxyl radical rather than a GSH-reactive semiquinone, similar to the form identified in animal cells⁵¹.

In summary, the antioxidant aspect of tobacco leaf UV acclimation was realised mainly through lowered SOD and increased peroxidase activities; the latter involving isoforms that use CGA and, to a smaller extent, QUE as electron donors. Although the increased RUT pool indicates the potential of more efficient non-enzymatic H_2O_2 neutralisation, there was no significant change in this function in UV exposed leaves (Fig. 1F), indicating that the contribution of RUT to this pathway was minor.

Nevertheless, H_2O_2 levels were higher in these UV-B-acclimated leaves than in controls (Fig. 1A). A UV-inducible increase in leaf H_2O_2 concentrations has already been reported in stressed plants, where the irradiation resulted in a decrease in photosynthetic performance⁸ but not yet in well-acclimated ones. Hydrogen peroxide is a well-established secondary messenger¹ and it is plausible that controlled low levels of this ROS participate in the induction of the antioxidant response to UV-B as well. Whether acclimative UV-responses are triggered

by UV-B directly, through the UVR8 photoreceptor³⁵ or by UV-B induced elevated H₂O₂ levels, is still an open question. The former model would result in a large number of specifically UV-B responsive peroxidase genes, but so far, the only example is the GPX encoding Arabidopsis AT4G35870^{35,36,56,64}. In this study, we compared antioxidant responses to UV-B and exogenous H₂O₂ at functional (activity) levels. The manner in which irrigation with H₂O₂ increases the leaf concentrations of this ROS is unknown so far, but cross membrane transport¹⁶ and NADPH oxidase activation⁷ are among possible candidates. Wan and Liu³⁹ found that 0.6–15 mM H₂O₂ root application resulted in a 50–200% increase in rice leaf H₂O₂ content and caused oxidative membrane damage, a strong decline in photosynthesis and APX down-regulation. In our experiment, a lower concentration (0.1 mM) was used for soil irrigation, and the treatment resulted in a 20% increase in leaf H₂O₂ levels (Fig. 1) but did not cause any loss in leaf photochemical yield. APX activity was unaffected, and the only positive effect on POD was detectable as ABTS-POD. None of the phenolic-substrate-using POD isoforms were stimulated by the H₂O₂ (Fig. 2), confirming that the synthetic compound ABTS as electron donor assesses a different subset of leaf POD than natural compounds. Contrary to the UV-B treatment, which enhanced the enzymatic but not the non-enzymatic neutralisation of H₂O₂, the direct ROS treatment applied increased the latter defence pathway but had only a minor positive effect on enzymatic defence. Moreover, QUE-POD and CAT activities were lower (by 25% and 50%, respectively) in H₂O₂ treated leaves than in controls. The only common response to the two different treatments was a decrease in SOD activity. This suggests that the source of neither UV-B-induced nor H₂O₂-irrigation-induced excess H₂O₂ is an increased enzymatic conversion of superoxide radicals. Because exogenous H₂O₂ resulted in an increase in non-enzymatic antioxidant capacity without a marked increase in phenolic content, the contribution of these compounds as direct H₂O₂ scavengers was most likely minor in acclimating to this treatment.

Factor interactions between UV-B and H₂O₂ treatments were explored further using two-way ANOVA. Statistically verified interactions, which do not grant but only imply the possibility of crosstalk between the two factors, require two conditions: One is that single factors are significant in the two-factor experiment, for example, UV-B increasing the studied effect (e.g. enzyme activity) both in the absence and in the presence of exogenous H₂O₂ ($p < 0.05$ in the first row of the inset tables in Figs. 1 and 2) and vice versa ($p < 0.05$ in the second row). The second condition is a $p < 0.05$ interaction (the third row in these tables). These two conditions are met only in the case of leaf H₂O₂ content (Fig. 1A) and SOD (Fig. 1B). The effect of treatments was positive in the former and negative in the latter case. Lowering SOD-mediated production is possibly a common acclimative response to an increase in cellular H₂O₂ levels, regardless of the nature of the external stimulus. A parallel application of UV-B and H₂O₂ resulted in an additive effect on H₂O₂ content in the sense that the simultaneous presence of the two factors led to an effect that was equal to the sum of the effects caused by the two factors applied separately⁵³.

The major differences in antioxidant responses to H₂O₂ and UV-B, as well as the very limited interaction between the two factors when applied in parallel, as discussed above, suggest that UV acclimation is unlikely to have been brought about by the UV-induced increase in leaf H₂O₂ content. A difference in production H₂O₂ sites in response to the two treatments may argue against this assumption, but the relatively long life-time and ability of this ROS to spread in tissues¹⁶ diminishes the importance of this aspect. In the absence of evidence for UVR8-initiated activation of acclimative antioxidant signalling, one can only speculate on potential routes. If this pathway involves direct UV perception, then candidates include a UV-B photoreceptor distinct from UVR8⁶⁵ or a contribution of UV-A photoreceptors⁶⁴ as the broad-band UV source applied in our experiment contained UV-A as well. A metabolite initiated pathway may include oxidised ascorbate, which has already been implicated in responses to stressors other than UV radiation⁶⁶ or possibly, oxidised phenolic compounds.

Increased phenolic peroxidase activity has been widely reported as a general, non-specific defence response. Our present study, however, shows the existence of inducement-specific, phenolic substrate-dependent POD responses in UV-treated leaves and suggests a further investigation of the heterogeneity of POD responses under different abiotic stress conditions. Further, the present study also draws attention to the possibility of the novel yet unexplored complexity of POD responses to other stress conditions as well. As illustrated by the example of the UV-induced changes, an increase in a certain phenolic component in the leaf does not necessarily correspond to its increased use as POD substrate; thus, the latter cannot be fully explained by its increased availability. A more plausible model is the selective upregulation of POD isoforms using phenolic substrates, which can be recovered from their oxidised form by relatively low amounts of other antioxidants, such as ascorbate or glutathione. This hypothesis is supported by our data but must be verified further through a quantitative analysis of antioxidant metabolites and correlations between changes in their levels during UV acclimation.

Methods

Chemicals, plant material, and treatments. Pure phenolic compounds quercetin, rutin, caffeic acid, and chlorogenic acid were purchased from Extrasynthese S.A.S. (Genay, Rhone, France). Hydrogen peroxide was obtained from VWR International (Debrecen, Hungary). Other chemicals were purchased from Sigma-Aldrich Kft (Budapest, Hungary). Tobacco (*Nicotiana tabacum* cv. Xanthi) plants were grown in growth chambers (Fitotron, SGC 120 Plant Growth Chamber, Weiss Technik UK, Loughborough, UK) under 150 $\mu\text{mol m}^{-2} \text{s}^{-1}$ photosynthetically active radiation (PAR) using long day conditions (16/8 h, 25/20 °C). Four weeks after emergence, the plants were divided into four groups, each containing 4 plants: (1) treated with exogenous H₂O₂, (2) exposed to supplemental UV radiation, (3) H₂O₂ + UV, and (4) untreated controls. Plants in the two H₂O₂ treatment groups were irrigated with 100 mL 100 μM H₂O₂ daily and plants in the two other groups received equal volumes of water. UV radiation was provided by Q-Panel UVB-313EL tubes (Q-Lab Ltd., Bolton, UK) wrapped in a single layer of cellulose diacetate filter (Courtaulds Chemicals, Derby, UK), and it was measured in the growth chamber with a spectroradiometer (Flame, Ocean Optics, Largo FL, USA). The spectrum was centred at 311 nm (Supplementary Figure S3), and irradiation for 4 h (between 10 a.m. and 2 p.m. daily) provided 6.9 $\text{kJ m}^{-2} \text{d}^{-1}$

Substrate	Concentration (mM)	Absorbance wavelengths (nm)	Molar extinction coefficient (ϵ , $\text{mM}^{-1} \text{cm}^{-1}$)	Reference
ABTS	183	765	11.23	72
GUA	2	450	5.98	73
CAA	3	315	0.62	This study
CGA	3	325	0.83	This study
QUE	3	340	3.87	*58
RUT	3	265	0.03	This study

Table 2. Characterization of POD enzyme substrates used in the present study. *Due to the fast oxidation of quercetin a substrate, the reaction was followed by the loss of ascorbate due to recovering oxidized quercetin at 295 nm, using $\epsilon_{295 \text{ nm}} = 1.47 \text{ mM}^{-1} \text{ cm}^{-1}$.

biologically effective UV-B (280–315 nm) calculated using the biological spectral weighting function developed for plants⁶⁷. At the end of the treatment period, non-invasive leaf measurements were performed; thereafter, detached leaves were frozen in liquid N₂ and stored at –80 °C until used for either chromatography analysis or antioxidant capacity assays. Each measurement was carried out using the fully expanded leaf of the 4th node, in order to exclude effects of age-related heterogeneity of the UV response²².

Chlorophyll fluorescence measurements. Plants were kept in darkness for 30 min before photochemical yields and non-photochemical quenching were characterised by chlorophyll fluorescence-derived parameters using the MAXI-version of the Imaging PAM (Heinz Walz GmbH, Effeltrich, Germany). At the end of the dark adaptation period, a saturating pulse was applied in order to measure the minimum and maximum fluorescence yields (F_0 : before the pulse and F_m : after the pulse). Following this, the leaf was illuminated with blue actinic light corresponding to 110 $\mu\text{mol m}^{-2} \text{ s}^{-1}$ PAR applied for 4 min, then the F' and F'_m fluorescence yields were measured before and after a saturating pulse. The maximum and effective Photosystem (PS) II quantum yields were calculated from these data as $F_v/F_m = (F_m - F_0)/F_m$, and $Y(\text{II}) = (F'_m - F')/F'_m$, respectively⁶⁸. Non-regulated and regulated non-photochemical energy dissipation processes were characterised by $Y(\text{NO}) = F'/F_m$ and $Y(\text{NPQ}) = F'/F'_m - F'/F_m$, respectively⁶⁹.

Assessments of leaf phenolic contents. Leaf flavonoid content was estimated using a non-invasive optical method on both adaxial and abaxial sides with a Dualex Scientific optical sensor (ForceA, Orsay, France). This method is based on the absorption in leaf tissue at 375 nm⁵⁴. In addition, phenolic compounds were analytically separated with thin-layer chromatography (TLC) performed on silica gel-coated aluminium sheets (60 F₂₅₄, 12 × 20 cm; Merck KGaA, Darmstadt, Germany). Frozen leaves were powdered in liquid N₂ using a pestle and mortar and extracted in 70% methanol. Leaf extracts (0.3 mg leaf FW mL⁻¹) and test compounds (1 mg mL⁻¹) were applied on the TLC plate at 5 μL volumes. The developing buffer was a 30 mL mixture of ethyl acetate, formic acid, acetic acid, and water (100:11:11:27, V:V:V:V). The plate was first air-dried, then dried in a desiccator for 20 min. Phenolic compounds were detected under UV light centred at 365 nm (VL215.L, Vilbert Lormat, France) after spraying the plate with a NaturStoff solution⁷⁰.

Enzyme activity measurements. Frozen leaves were powdered in liquid N₂ using a pestle and mortar and extracted in an ice cold sodium-phosphate buffer (50 mM, pH 7.0) containing 1 mM EDTA. Leaf homogenates were centrifuged (24,400 × g for 30 min at 4 °C, Hettich Rotina 380 R, Andreas Hettich GmbH, Tuttlingen, Germany) and supernatants were kept at –20 °C until use. Protein contents were determined using the standard Bradford assay⁷¹.

POD (EC 1.11.1.7) activities were measured in acidic reaction mixtures (50 mM phosphate citrate buffer, pH 5.0) using six different substrates. The reactions also contained 400 μM H₂O₂ in all cases, and one of the following substrates: (1) ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)), (2) guaiacol (2-methoxyphenol), (3) caffeic acid ((2E)-3-(3,4-Dihydroxyphenyl)prop-2-enoic acid), (4) chlorogenic acid ((1S,3R,4R,5R)-3-[[[(2E)-3-(3,4-dihydroxyphenyl)prop-2-enoyl]oxy]-1,4,5-trihydroxycyclohexanecarboxylic acid]), (5) quercetin (2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-1-benzopyran-4-one) + 14 mM ascorbate, or (6) rutin (2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-3-[α -L-rhamnopyranosyl-(1 → 6)- β -D-glucopyranosyloxy]-4H-chromen-4-one). Enzyme activities were quantified following the oxidation of the corresponding substrate as absorption change using a spectrophotometer (Shimadzu UV1800, Shimadzu Corp., Kyoto, Japan). Substrate concentrations in the reaction mixture and absorbance wavelengths are summarised in Table 2 along with the molar extinction coefficients used to calculate enzyme activities as mU activity mg⁻¹ protein, where 1 U = 1 mM substrate min⁻¹. References describing the details of original methods are also listed in Table 2. When indicated, the reaction mixture contained either ascorbate (7–14 μM) or GSH (1.4–140 μM) in addition to one of the phenolic compounds.

Superoxide dismutase (SOD, EC 1.15.1.1) activity measurements were carried out according to Sun et al.⁷⁴, based on the inhibition of 0.1 mM nitroblue tetrazolium (NBT) reduction by xanthine–xanthine-oxidase (2 mM and 25 mU, respectively), which generated superoxide anions, and activity was determined as U SOD mg⁻¹ protein.

Ascorbate peroxidase (APX, EC 1.11.1.11) activity was measured according to Nakano and Asada⁷⁵ by following the oxidation of ascorbate at 295 nm in a sodium phosphate buffer (50 mM, pH 7.0) containing 1 mM

EDTA, 0.5 mM ascorbate, and 1 mM H₂O₂. The results were corrected for APX-independent H₂O₂ reduction, which was typically less than 10% of enzymatic rates. Enzyme activities were calculated using the molar extinction coefficient of ascorbate ($\epsilon_{295\text{ nm}} = 1.47\text{ mM}^{-1}\text{ cm}^{-1}$) as mU APX mg⁻¹ protein.

Glutathione peroxidase (GPX, EC 1.11.1.9) activity was measured by following the NADPH oxidation at 340 nm according to Lawrence and Burk⁷⁶. The reaction mixture contained 1 mM EDTA, 0.2 mM NADPH, 1 mM NaN₃, 1 mM reduced glutathione, and 1 U mL⁻¹ glutathione reductase in 50 mM potassium phosphate buffer (pH 7.0) and either 0.25 mM H₂O₂ or 0.25 mM cumene hydroperoxide. NADPH oxidation was followed at 340 nm ($\epsilon = 6.42\text{ mM}^{-1}\text{ cm}^{-1}$) and enzyme activities were determined as mU GPX mg⁻¹ protein.

Catalase (CAT, EC 1.11.1.6) activity was determined as described by Aebi et al.⁷⁷ by following the decrease in H₂O₂ concentration as 240 nm absorbance in a reaction mixture containing 18.6 mM H₂O₂ and 1 mM EDTA in a 50 mM sodium-phosphate buffer (pH 7.0). The reaction was started by adding 60 μ L leaf sample (corresponding to 1.3–4.1 μ g soluble protein) and CAT activities were given as mU mg⁻¹ protein.

Non-enzymatic H₂O₂ antioxidant capacity measurement. Hydrogen peroxide neutralising antioxidant capacities were evaluated through the photometric detection of iodine (I₂) yielded in the reaction between H₂O₂ and potassium iodide (KI), and the ability of H₂O₂ reactive compounds to lessen the amount of this product⁷⁸. For this experiment frozen leaves were powdered in liquid N₂ using a pestle and mortar and extracted in 70% (v/v) ethanol. The reaction mixture contained 25 μ M H₂O₂, 595 μ M KI in potassium-phosphate buffer (pH 7.0) and either leaf extracts (corresponding to 300 μ g leaf FW) or one of the pure test compounds (0.42–3.2 mM). The final concentration of ethanol in the reaction mixture was always 7.5% (v/v). Absorption at 405 nm was measured twice, immediately and 3 min after mixing assay components using a Multiskan FC plate reader (Thermo Fischer Scientific, Shanghai, China). Non-enzymatic H₂O₂ antioxidant capacities were given as μ M ascorbic acid (ASA) equivalents.

Hydrogen peroxide content measurement. Leaf H₂O₂ levels were estimated using a photometric assay⁷⁹ based on the H₂O₂-induced absorption change of 125 μ M xylenol orange in 6% (v/v) trichloroacetic acid (TCA). For this assay, samples were collected from plants within the growth chamber under light conditions corresponding to treatment groups i.e. PAR only or PAR plus UV-B. Three leaf disks corresponding to 26–56 mg FW were homogenised in 6% TCA immediately after cutting, centrifuged (15,000 \times g, 10 min, 4 °C, Heraeus Fresco 17 Centrifuge, Thermo Fisher Scientific, Waltham, USA), and the supernatants were incubated for 30 min before detecting 560 nm absorptions. Leaf H₂O₂ contents were given in nM mg⁻¹ FW units using calibration curves in the 0–10 nM H₂O₂ range.

Statistical analysis. Each treatment group contained four plants. One leaf from each plant was chosen for the analyses, and all measurements were performed in 3–4 repetitions. Results are presented as means \pm standard deviations. The combined and single factor effects of UV-B and H₂O₂ were analysed with a two-way ANOVA. Three null hypotheses were tested: (1) the H₂O₂ treatment had no effect, (2) the absence/presence of UV-B over the PAR background had no effect, and (3) there was no interaction between the two factors. Tukey HSD was used as post-hoc test and verified rejections of the ANOVA null hypotheses were characterised with *p* values. Statistical analyses were performed using the PAST software⁸⁰.

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Author contributions

É.H. and A.R. conceived the research plan with contributions of Gy.Cz.; É.H. and A.R. designed the experiments with contributions of Gy.Cz. and K.Cs.; A.R. performed the experiments and evaluated data with contributions of Gy.Cz. (calculations), K.Cs. (TLC and non-enzymatic antioxidant capacities) and É.H. (statistics); the article was written with contributions of all the authors.

Competing interests

The authors declare no competing interests.

Additional information

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