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Oxidation of polyphenols and inhibition of photosystem II under acute photooxidative stress

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Abstract

Main conclusion We observed a close correlation between the inhibition of photosystem II and the oxidation of polyphenols during an acute oxidative stress in sunflower leaf discs.

To assess the physiological significance of polyphenols as antioxidants *in planta*, we compared the kinetics of polyphenols oxidation with the inhibition of the photosynthetic apparatus in sunflower leaf discs exposed to an acute photooxidative stress. Illumination of leaf discs in presence of methyl viologen induced a rapid and large non-photochemical quenching of chlorophyll-*a* fluorescence, which was reversed after four hours of treatment as indicated by the $\approx 30\%$ increases of the steady-state (F_s) and maximal (F_m') levels of chlorophyll-*a* fluorescence relative to the first hour of treatment. This event coincided with the accelerated decreases of the maximum (F_v/F_m) and effective ($\Delta F/F_m'$) quantum yields of photosystem II, and also with the beginning of polyphenols oxidation, estimated by the UV absorbance of methanolic leaf extracts, and supported by the Folin-Ciocalteu method and cyclic voltammetry. The decreases of F_v/F_m and the concentrations of reducing polyphenols were highly correlated ($R^2 = 0.877$) during the experiment. Coherent with the decrease of UV absorbance of methanolic extracts, polyphenols oxidation resulted in a marked decrease of UV-absorbance of leaf epidermis. Also, polymerization of oxidized polyphenols caused the accumulation of brown pigments in the MV-treated leaf discs, decreasing leaf reflectance, especially at 550 and 740 nm. Fluorescence intensities were also decreased during the MV-treatment. Interestingly, the emission fluorescence ratio F^{740}/F^{684} (excitation at 550 nm) decreased similarly to F_v/F_m ($R^2 = 0.981$) due to the brown pigments. Moreover, the excitation fluorescence ratio F_{484}/F_{680} (emission at 740 nm) was linearly correlated ($R^2 = 0.957$) to $\Delta F/F_m'$, indicating a decrease of efficiency of energy transfer between the antenna pigments to the photosystem II reaction center during the oxidative stress. These results support the view that polyphenols can be effective antioxidants protecting the plants against reactive oxygen species.

Key words: Antioxidants, Brown pigments, Chlorophyll Fluorescence, Excitation spectra, Reflectance spectra, *Helianthus annuus*, Photosynthesis, Polyphenols.

Introduction

Polyphenols (PPs) are specialized metabolites in plants that are essential for their successful interactions with the environment. By their structural diversity and different localizations, PPs fulfil multiple functions such as deterrent against herbivores, barriers against pathogens, aid in pollen and seed dispersal, and molecule signals for symbiotic relations with microorganisms (Treutter 2005; Ghasemzadeh and Ghasemzadeh 2011; Mandal et al. 2010). PPs also contribute to plant tolerance against a wide range of abiotic stresses, like UV, high light intensities, drought, salinity, heavy metals, air pollution, mechanical wounding, and nitrogen limitation (Dixon and Paiva 1995; Nakabayashi and Saito 2015; Treutter 2005). Besides their specific targets, all these stresses have in common to accelerate the production of reactive oxygen species (ROS) leading to oxidative stress.

Widely accepted but not fully demonstrated, the role of PPs as antioxidant *in planta* is inferred from several observations. PPs show antioxidant capacity *in vitro*, and their concentrations increase in plants exposed to the above-mentioned stresses (Dixon and Paiva 1995). Among the stress-induced PPs, there is a preferential accumulation of strong antioxidants notably quercetin, with its o-dihydroxy B-ring compared to the monohydroxy B-ring kaempferol (Agati et al. 2012; Edreva 2005). High flavonoid content was related to higher viability in senescent *Vitis vinifera* suspension cell cultures by quenching ROS, hence limiting the signalling cascade leading to programmed cell death (Bertolini et al. 2016). Moreover, the antioxidative role *in planta* of PPs is supported by *Arabidopsis* mutants that overaccumulate flavonoids with strong antioxidant capacity. Compared to the wild-type Col-0, these mutants showed higher tolerance to drought and methyl viologen-induced oxidative stress (Nakabayashi et al. 2014). Similar observations can be made with salicylic acid. This plant growth regulator improves plant tolerance to a wide range of abiotic stresses (Hayat et al. 2010), and it was shown to enhance PPs concentrations in plants (Kováčik et al. 2009), plant cell cultures (Dong et al. 2010) and algal cultures (Kováčik et al. 2010).

Under intense and transient stress conditions and under high light intensities, excess of H₂O₂ generated in the chloroplasts that is not scavenged by the ascorbate-glutathione cycle diffuses to the vacuoles. There, class-III peroxidases can catalyse H₂O₂ reduction to H₂O by using PPs, mostly flavonoids and hydroxycinnamates, as electron donors. The resulting phenoxyl radicals are normally re-reduced by ascorbate, the latter being regenerated in the cytosol by glutathione and NADPH. This peroxidase/polyphenol/ascorbate system is efficient as long as the cytosolic ascorbate pool is not depleted: at this point, the phenoxyl radicals accumulate and react with other radicals, amino acids or proteins, to polymerize into complex brown pigments (Pérez et al. 2002; Pourcel et al. 2007; Takahama 2004; Yamasaki et al. 1997).

One of the missing evidences required to confirm the physiological significance of PPs as antioxidants is the presence of a spatio-temporal correlation between the extent of oxidative stress and the oxidation of PPs (Agati et al. 2012; Hernández et al. 2009). Oxidation of PPs *in planta* and the accumulation of brown pigments is common during plant development, causing for instances the browning of seed coats, onion scales, heartwood (Pourcel et al. 2007; Takahama 2004), and aging tobacco leaves (Takahama 2004). But during oxidative stress, there are only few

reports of PPs oxidation in leaves (Agati et al. 2007; Hernández et al. 2006), in contrast with the numerous studies mentioned above showing their accumulation during plant acclimation to chronic environmental stress. Simultaneous decreases of the levels of phenolics and chlorophylls were observed during foliar senescence in *Kalanchoe pinnata* (Jaiswal et al. 2012). Meir et al. (1995) made similar observations during senescence of leaf discs from various edible herbs, i.e. similar decreases of chlorophyll contents and “aqueous reducing compounds”, which phenolic acids and flavonoids were considered as the main components. In another study, *Arabidopsis* leaves wild type and mutants with different anthocyanin contents were illuminated in presence of methyl viologen (Shao et al. 2008). Photosynthetic reactions estimated from chlorophyll fluorescence parameters decreased in a triphasic pattern, to be nearly abolished after 3 to 4.5 hours of illumination depending on the anthocyanin contents. In contrast, total phenolics and flavonoids, estimated from UV absorbance of methanolic extracts, decreased more or less linearly by 10 to 25 % only after 12 hours of treatments. In that experiment, vacuum infiltration of methyl viologen into the leaves may have lowered O₂ concentrations, and the brown pigments resulting from the oxidation products of leaf phenolics were not monitored.

In this context, there is a need to further characterize the relation between the oxidation of PPs in leaves with the physiological damages occurring during an acute oxidative stress. To this end, we illuminated sunflower leaf discs in presence of methyl viologen and correlated the inhibition of photosynthetic electron transport with the oxidation of PPs estimated from leaf methanolic extracts by three independent methods, notably cyclic voltammetry. This method is frequently used as a direct indicator of reducing capacity of PPs in food and beverage research, especially on wine and teas (Kilmartin and Hsu 2003; Kilmartin et al. 2001), but has not been used previously to characterise PPs in leaf extracts in relation to photosynthetic activity and photooxidative stress. We also monitored by reflectance and fluorescence spectroscopy the impacts of accumulating brown pigments on leaf optical properties. The results show a close temporal correlation between the oxidation of PPs and the loss of integrity of the photosynthetic electron chain.

Material and Methods

Plant material

Dwarf sunflower plants (*Helianthus annuus* L., var. Sunspot) were grown during eight weeks in a growth chamber. The photoperiod was 16h/8h and the day light intensity (PFD) was 150 $\mu\text{moles photons m}^{-2}\text{s}^{-1}$. The day/night temperatures were 25°/17°C. Plants received tap water as required and appropriate amount of NPK 20-20-20 fertilizer once a week.

Just before the experiments, discs (2.2 cm diam.) were cut with a cork borer from non-shaded and horizontally oriented areas of the 4th to 6th leaves, and avoiding the midribs. Discs were kept between moist paper towels until the beginning of the treatments. PPs contents in leaf discs were estimated just before the experiments with a Dualex Scientific (Force-A, Orsay,

France) leaf-clip sensor. To minimize the variations due to leaf development, only leaf discs with Dualex Flv values between 0.6 and 0.8 were used for the experiments.

Treatments

Leaf discs were floated with their abaxial side in contact with distilled water containing 0 or 0.4 mM methyl viologen (MV), and maintained in the growth chamber under the same PFD and temperature as the growth conditions. During the experiment, six discs were taken after 0, 3, and 7 hours for the control, and after 1, 2, 4, 6, and 8 hours for the MV treatment. At each hour, the following measurements described below were sequentially made within 30-40 minutes: modulated chlorophyll-a fluorescence, Multiplex 330 fluorescence, reflectance spectra, and fluorescence spectra. Then, the discs were stored at -18°C for less than two weeks for pigments and PPs analysis. The whole experiment was repeated twice.

Modulated chlorophyll-a fluorescence

Pulse-amplitude modulated chlorophyll-a fluorescence was measured from leaf discs with a MINI-PAM fluorometer (Walz, Germany). For each disc, the stationary fluorescence level F_s was measured under the same ambient light intensity ($150 \mu\text{moles photons m}^{-2}\text{s}^{-1}$) and then the maximum F_m' was induced by a 1 s saturating flash. From the F_s and F_m' , the effective quantum yield of photochemistry of Photosystem II (PSII) $\Delta F/F_m'$ was calculated, where $\Delta F = F_m' - F_s$ (Klughammer and Schreiber 2008).

To not interfere with the time course of the MV-induced photooxidative stress, a separate experiment was done to measure the changes of the maximum quantum yield of photochemistry of PSII during the experiment. At each hour during the treatments, leaf discs were placed in darkness for 40 minutes before the minimum F_o level was measured and then, the maximum F_m was induced by a 1 s saturating flash. The maximum quantum yield of PSII photochemistry F_v/F_m was calculated, where $F_v = F_m - F_o$.

Reflectance

To follow the accumulation of brown pigments resulting from the oxidation of PPs, reflectance spectra from 360 nm to 740 nm (10 nm intervals) were measured on the adaxial side of leaf discs with a portable spectrophotometer CM-2600d (Konica Minolta, Roissy, France).

In a separate experiment, reflectance spectra were measured as described above on depigmented leaf discs, which had been exposed 8 hours with 0 or 0.4 mM MV, and then incubated in methanol at 4°C for 24 hours.

178

179 **Fluorescence spectroscopy**

180 The consequences of PPs oxidation on leaf optical properties were also monitored during the
181 experiment by recording the excitation and emission chlorophyll-*a* fluorescence spectra with a
182 spectrofluorometer Cary Eclipse (Varian, Les Ulis, France). The discs were placed in a leaf
183 holder positioned at 60° from the excitation source and 30° from the detector side. Fluorescence
184 emission and excitation spectra were recorded with a bandwidth of 10 nm on both the excitation
185 and the emission side, and a scan rate of 120 nm min⁻¹. Fluorescence emission was recorded from
186 650 to 800 nm, excited at 550 nm, with the additional short-pass and long-pass protection filters,
187 SP600 (Edmund Scientific) and RG630 (Schott), respectively. Fluorescence excitation spectra
188 from 390 to 680 nm, emitted at 740 nm, were recorded with the additional filters SP700 (Schott)
189 and RG715 (Schott), on the excitation and emission side, respectively. Fluorescence excitation
190 spectra from 300 to 390 nm, emitted at 740 nm, were recorded with the DUG11 (Schott) and
191 RG715 (Schott) filters, on the excitation and emission side, respectively. All spectra were
192 corrected for the response of detectors and transmission of monochromators and filters as
193 described in Louis et al. (2006). Throughout the manuscript fluorescence excitation wavelengths
194 are indicated by subscripts and fluorescence emission wavelengths, by superscripts.

195 The Multiplex 330 (FORCE-A, Orsay, France) proximal sensor was also used to measure
196 instantaneously fluorescence from the whole surface of leaf discs. This photodiode- and filter-
197 based sensor records chlorophyll-*a* fluorescence like the MINI-PAM, but simultaneously at two
198 emission bands, centered at 684 and at 740 nm, with LED-excitation centered at 330, 450 and 630
199 nm.

200

201 **Measurements of pigment and polyphenol concentrations**

202 Leaf discs were cut into small pieces with a scalpel and incubated overnight in pure methanol at
203 4°C in the dark. The extracts were filtered and the absorbance spectra from 220 to 800 nm were
204 recorded with an Agilent/HP 8453 spectrophotometer (Hewlett Packard, Les Ulis, France).
205 Chlorophyll *a+b* concentrations were calculated according to Lichtenthaler (1987).

206 The levels of soluble PPs were first estimated by the UV absorbance of the methanolic
207 extracts after subtraction of chlorophylls contribution, calculated from spectra of pure
208 chlorophyll-*a* and chlorophyll-*b* as described by Ounis et al. (2001). PPs concentrations were also
209 estimated by the Folin-Ciocalteu method (Prior et al. 2005). In brief, 1 ml of the methanolic
210 extract was added to 2.25 ml of diluted Folin-Ciocalteu reagent (0.2 N). After six minutes, 1.75
211 ml of saturated Na₂CO₃ was added, and the mixture was incubated for two hours, and then
212 centrifuged. The absorbance of the supernatants was read at 760 nm. A calibration curve
213 ($R^2=0.998$) was made using the same procedure with nine concentrations of gallic acid (from 0.25
214 to 50 mg L⁻¹).

215

216 **Cyclic voltammetry**

217 Cyclic voltammetry was used to clearly illustrate the oxidation of leaf PPs. For these
218 measurements, higher PPs concentrations in leaf methanolic extracts were required. So, a distinct
219 experiment was done where discs were treated identically as described above in presence or
220 absence of MV for eight hours. For both treatments, 3 groups of eight leaf discs were made, and
221 each group was incubated in 40 mL of methanol at 65°C for 20 minutes, followed by a second
222 incubation in 15 minutes in 20 mL methanol. For each group, the two extracts were pooled, and
223 concentrated in a roto-evaporator to a final volume of 5 mL.

224 Methanolic extracts (1 mL) were introduced in a three-electrodes cell containing 1 ml of 0.2
225 M potassium phosphate buffer (final pH of 7.4). A glassy carbon disk (BAS MF-2012, 3 mm
226 diameter), an Ag/AgCl electrode, and a glassy carbon plate were used as working, reference and
227 counter electrodes respectively. Argon was bubbled for several minutes in the solution to remove
228 dissolved air. Cyclic voltammograms were recorded using a Voltalab PGZ 100 potentiostat, at a
229 scanning rate of 50 mV/s, over the potential window from -100 to 500 mV vs. Ag/AgCl. All
230 electrode potentials were read against the reference electrode of Ag/AgCl. Before each
231 measurement, the glassy carbon working electrode was gently polished with an aluminum powder
232 solution on an abrasive surface for 2 min and then sonicated also for 2 min. The cell and the
233 electrodes were washed with deionized water (milliQ grade, Millipore) between each experiment.
234 Reducing equivalent contents expressed in microcoulomb (μC) were determined by first
235 calculating the area under the curve “current versus voltage”, and then dividing this surface area
236 (in $\mu\text{A}\cdot\text{mV}$ or $\mu\text{C}\cdot\text{s}^{-1}\cdot\text{mV}$) by the constant scanning rate (here 50 mV s^{-1}) (Kilmartin and Hsu
237 2003).

238

239 **Statistical analysis**

240 For each dependent variable measured, descriptive statistics (means and standard errors of the
241 mean) and analyses of variance were calculated with the SYSTAT software (SPSS, Chicago IL.).
242 The factors (independent variables) were “hours” (i.e. with or without MV) and “experiments”.
243 For all dependent variables tested, highly significant effects ($p < 0.01$) of MV (hours) were
244 observed except for chlorophylls and carotenoids contents ($p > 0.05$). Also, the interactions
245 “hours * experiments” were non-significant for all dependent variables. This means that the same
246 effects of MV on the different variables were observed in the two experiments.

247 Two measurements made with the Multiplex were considered as outliers by the SYSTAT
248 software and discarded, since their difference with the mean was more than three times larger
249 than the standard deviation.

250

251

252 **Results**

Characterization of methyl viologen effects on photosynthetic electron transport and photosynthetic pigments

Exposure of sunflower leaf discs to MV 0.4 mM under a white light of $150 \mu\text{mol m}^{-2} \text{s}^{-1}$ for 8 hours inhibited the photosynthetic electron transport in 3 distinct phases. Within the first hour of treatment, $\Delta F/F_m'$ decreased from about 0.7 to 0.3 and then remained stable for 2 hours, before it further decreased close to 0 between 4 and 8 hours of treatment (Fig. 1a). In contrast, the decrease of F_v/F_m due to the presence of MV was biphasic: it remained little affected during the first two hours, and decreased afterward down to 0.2 (Fig. 1b).

The decrease of $\Delta F/F_m'$ in the first half of the treatment could be related to the formation of a large non-photochemical quenching (NPQ), as F_m' was strongly quenched (Fig. 1c) while F_s remained similar to the control values (Fig. 1d). The F_s and F_m' values transiently increased at 4 hours, indicating the collapse of the NPQ and the subsequent loss of integrity of the photosynthetic electron transport system.

Fig. 2 shows that despite its strong effect on photochemical reactions, MV did not affect significantly the concentrations of chlorophyll *a+b* and of carotenoids throughout the experiment.

Polyphenol oxidation during MV-induced oxidative stress estimated from leaf extracts

To determine if the losses of PSII photochemical efficiency were correlated to the oxidation of PPs in MV-treated leaf discs, we analysed the spectra of methanolic extracts from which chlorophylls *a* and *b* absorbance was subtracted as in Ounis et al. (2001) (Fig. 3a). The control spectrum shows the typical absorbance of carotenoids in the blue region, and the UV absorbance peak near 328 nm is characteristic of hydroxycinnamates (Stelzner et al. 2019). The MV treatment caused a decrease of the UV absorbance accompanied by a large increase in the violet region. These opposite changes are typical of phenolic oxidation (Ramešová et al. 2015; Sokolová et al. 2012). Also, the increase of absorbance at 420 nm is a common indicator of phenolic oxidation causing the browning of plant tissues and processed plant products (Takahama et al. 1999; Tomás-Barberán et al. 1997).

The absorbance at 328 nm decreased in a biphasic manner during the MV-treatment: A_{328} decreased slightly in the first two hours, before a more pronounced decline between 4 and 8 hours. After eight hours of MV-treatment, A_{328} accounted for 56% of the control values (Fig. 3b). The increase of absorbance near 420-440 nm is prominent but the spectra are noisy due to the large absorbance of chlorophylls in that region, close to the sensitivity limit of the spectrophotometer (see original spectra in Fig S1).

The oxidation of PPs monitored by the decrease of A_{328} was supported by the contents of reducing PPs (gallic acid equivalents, GAE) estimated with the Folin-Ciocalteu method: we observed a small decrease of GAE in the first two hours, followed by a larger decline after 4

hours (Fig. 3c). At the end of the treatment, the GAE in MV-treated discs represented 67% of the control values. There is a close correlation ($R^2=0.934$) between the estimations of PPs contents by the Folin-Ciocalteu method and the absorbance at 328 nm.

The Folin-Ciocalteu method estimates the overall capacity of PPs (and other antioxidants) to reduce the molybdo-tungsto-phosphoric heteropolyanion (Prior et al. 2005). So, we used cyclic voltammetry to provide a more detailed illustration of the loss of the reducing capacity of PPs in extracts from MV-treated leaf discs. Cyclic voltammograms shown in Fig. 4 are plots of the cell current as a function of the potential (in mV vs. Ag/AgCl) of the working electrode, measured on the methanolic extracts from control and MV-treated leaf-discs. Peak currents (y-axis) are proportional to the concentration of reducing-active components in the extracts. Peak potentials (along the x-axis) indicate the potential at which the species donate their electrons. The curve of MV-treated leaf-discs is markedly lower than the control curve above 100 mV. The antioxidant capacity of the samples can be estimated by the area under the curve, from which we calculated the number of charges passed during the oxidative scanning from -100 to 500 mV. A value of 1285 μC was obtained for the control, and a value of 778 μC for leaf discs treated with MV during eight hours, i.e. 60% of the control value.

Polyphenol oxidation monitored *in situ* by alterations of leaf discs optical properties

Reflectance

The treatment of leaf discs with MV caused their browning that became visually apparent in the second half of the experiment. To characterize these changes, reflectance spectra of leaf discs were measured throughout the experiment. The most noticeable effect of the MV-treatment is the flattening of the spectra, with decreased reflectance that is more evident in the far-red and green regions, i.e. where chlorophylls absorb less (Fig. 5a). The red-edge, i.e. the position of the sharp reflectance increase, remained unchanged. Reflectance at 740 nm was little affected until the 4th hour of treatments, and then it decreased to 52% of the control value (Fig. 5b). In the blue and red regions of the spectra, reflectance decreased between 0 and 4 hours, but then increased slightly.

Reflectance spectra of leaf discs depigmented after their incubation in methanol were also measured (Fig. 6). Much higher reflectance values are seen in control than MV-treated depigmented leaf discs, showing that the browning of MV-treated leaf discs is caused by the accumulation of insoluble brown pigments following the polymerization of oxidised PPs (Takahama 2004; Yamasaki et al. 1997).

Fluorescence emission spectra

Considering the loss of non-photochemical quenching, the decrease of UV-absorbing PPs and the accumulation of brown pigments during MV-treatment, fluorescence spectroscopy can be a powerful tool to detect oxidative stress in leaves. So we firstly measured emission spectra of chlorophyll-*a* fluorescence excited at 550 nm (Fig. 7a). In the first two hours, the intensity of the fluorescence peak at 740 nm was slightly decreased by the MV-treatment (Fig. 7b). Then, the F^{740}

intensity increased at 4 hours, reminiscent of the enhanced F_s level observed at that time (Fig. 1c), before it decreased by more than 50% after 6 and 8 hours.

Not only the intensity but also the shape of the spectra was affected by the MV-treatment, as seen by the fluorescence ratio F^{740}/F^{684} (Fig. 7c). The MV effect on the F^{740}/F^{684} ratio was biphasic: it remained unaffected during the first two hours, and then decreased from 4.30 to a value of 2.28, 53% relative to the control. Interestingly, the changes of F_v/F_m (Fig. 1c) and F^{740}/F^{684} during the MV treatment were highly correlated ($R^2=0.981$).

Fluorescence excitation spectra

Fluorescence excitation spectra can provide useful information about light absorption and energy transfer by the antenna pigments, processes that could be affected by the PPs oxidation and the losses of PSII photochemical efficiency during the MV-oxidative stress. The excitation spectrum of control leaf discs shows two pronounced peaks, one at 420 that can be assigned to excitation of Chl-*a*, and the other at 484 nm, associated to the energy transfer from the accessory pigments chlorophyll-*b* and xanthophylls to chlorophyll-*a* (Zeng et al. 2016) (Fig. 8a). There are also minor Qx bands visible between 500 and 600 nm. In spectra from leaf discs exposed MV, the averaged fluorescence intensity F^{740} excited over 390 and 680 nm decreased similarly to F_s and F^{740} : a small decrease in the first two hours, then an increase at the fourth hour before a pronounced decrease beyond that time. More interestingly, the shape of the F^{740} excitation spectra was significantly altered: larger decreases occurred in the blue, especially the peaks at 420 and 484 nm, compared to the red region of the spectrum. The Qx bands between 500 and 600 nm were strongly suppressed in MV-treated leaf discs.

Since the fluorescence peak at 484 nm can be related to the energy transfer between the antenna pigments (Zeng et al. 2016), we calculated the ratio of the fluorescence intensities at 484 nm over 680 nm as a potential indicator of the efficiency of energy transfer from the antenna pigments to the reaction center of PSII. The results show that during the MV-treatment, the F_{484}/F_{680} decreased strongly during the first hour, less between one and four hours, and then more rapidly after the fourth hour (Fig. 8b). Interestingly, the changes of F_{484}/F_{680} are closely correlated ($R^2=0.957$) to the values of $\Delta F/F_m'$ measured after different times of treatment with MV (Fig. 8c).

Compared to the chlorophyll-*a* fluorescence (F^{740}) excited by visible light, the intensity of UV-excited chlorophyll-*a* fluorescence was relatively weak (Fig. 8a). But in a marked contrast, its intensity increased during the MV-treatment (Fig. 8d) presumably due to a loss of epidermal PPs.

Multiplex 330 fluorescence measurements

To better document the increase of UV-induced chlorophyll-*a* fluorescence during the MV-treatment, far-red fluorescence (FRF) intensities were measured from control and MV-treated leaf discs with a portable fluorometer Multiplex 330. This sensor has an additional excitation wavelength 330 nm close to the absorption maximum of hydroxycinnamates. The intensities of the FRF induced by blue excitation (Fig. 9a) followed the same patterns during the experiment as

for the F_s (Fig. 1c) and the F^{740} excited at 550 nm (Fig. 7b) measurements made with different fluorometers. Also, the Multiplex measurements of FRF induced by UV (330 nm) markedly increased during the MV-treatment (Fig. 9b), strongly supporting the measurements made with the Cary spectrofluorometer (Fig. 8d).

The logarithm of the ratio of the FRF intensities induced by blue and UV excitations ($\log(\text{FRF}_{\text{BLUE}}/\text{FRF}_{\text{UV330}})$ or LogFER) is a good estimate of the epidermal UV-absorbance due to the presence of soluble PPs (Cerovic et al. 2002). The LogFER values remained stable during the first four hours, and then declined to 53% of the control value after eight hours of MV-treatment (Fig. 9c).

Discussion

The role of PPs as antioxidants *in planta* is inferred from several circumstantial evidences, but direct observations of their oxidation co-occurring with physiological damages during oxidative stress are still lacking. The difficulty to demonstrate such correlation arises in part from the variations of reducing PPs concentrations during normal plant development (Louis et al. 2012; Louis et al. 2009) and during plant acclimation to chronic environmental stress (Dixon and Paiva 1995; Treutter 2005). So we induced an acute oxidative stress in sunflower leaf discs that were exposed to MV (0.4 mM) under their growth light intensity for up to eight hours. Near the fourth hour of the MV-treatment, there was a collapse of the non-photochemical quenching (NPQ) and an accelerated decline of the photochemical efficiencies of PSII. This loss of integrity of the photosynthetic reactions concurred with the beginning of PPs oxidation monitored by different methods in leaf methanolic extracts, and supported *in situ* by changes in leaf optical properties.

Under our experimental conditions, the effects of MV on photosynthetic electron transport in sunflower leaf discs can be described in three distinct phases. First, MV caused a rapid decline of the effective PSII quantum yield (occurring within five minutes, results not shown), followed by a second phase where $\Delta F/F_m'$ remained stable or even slightly increased for up to two hours. This second phase is characterized by the presence of a large NPQ as seen by the marked decrease of F_m' , which could recover after a dark period of 40 minutes (F_v/F_m remained unaffected). This large NPQ would result from the acidification of the thylakoid lumen (Derks et al. 2015). Indeed, lower production of NADPH relative ATP due to the diversion by MV of electrons from PSI to O_2 restricts the Calvin Cycle, decreases ADP+Pi concentrations due to lower ATP utilization, and thereby impedes photophosphorylation. Then the third phase of the MV effects began around the fourth hour by transient increases of F_s and F_m' , followed by their quenching below their initial values. These transient increases suggest the collapse of the NPQ as if the thylakoid membranes became suddenly permeable to H^+ due to oxidative damages. This effect accelerated the declines of the PSII quantum yields F_v/F_m and $\Delta F/F_m'$. It is noteworthy that the inhibition of the photosynthetic electron transport during the eight hours of MV-induced oxidative stress occurred before any significant decrease in the concentrations of the photosynthetic pigments.

Similar triphasic patterns of MV effects on photosynthetic electron transport were reported previously by Shao et al. (2008). They observed, however, in some phenotypes of *Arabidopsis* leaves a large recovery of $\Delta F/F_m'$ during the second phase of the MV effects. The origin of this difference is unknown but such increase could be explained notably by the actinic PFD during the MV-treatment in their experiments, which was 6 times higher than the growth PFD, as well as the absorption of MV into the leaves by vacuum infiltration.

The turning point of the MV-treatment in sunflower leaf discs could be the loss of integrity of the photosynthetic apparatus at the fourth hour, indicated by the collapse of NPQ. This collapse coincides with the accelerating rates of PPs oxidation in sunflower leaf discs. Indeed, the values of F_v/F_m (Fig. 1b) and of the A328 (Fig. 3b) values during the MV-treatment are highly correlated ($R^2 = 0.877$). The 4-hour delay before the acceleration of PPs oxidation is coherent with the model where H_2O_2 generated in chloroplasts, mitochondria or peroxisomes diffuses into the vacuoles, even those of epidermal cells, to be scavenged in a reaction catalysed by class-III peroxidases using PPs as electron donors. The oxidized PPs would be re-reduced by ascorbate, as long as ascorbate is regenerated from dehydroascorbate in the cytosol by glutathione and NADPH (Pérez et al. 2002; Takahama 2004). According to this model, it appears that under our experimental conditions, this H_2O_2 peroxidase/polyphenol/ascorbate scavenging cycle could operate during four hours before the reducing capacity to regenerate ascorbate become exhausted, overwhelmed by the high rate of ROS production, and then the oxidized PPs started to accumulate.

In the third phase of the MV-induced oxidative stress, about 40% of the soluble pool of PPs found in methanolic extracts of sunflower leaf discs was oxidized. This oxidation was characterized by a concomitant decrease of the absorbance that was maximal near 328 nm, and a large increase of absorbance near 420 nm, changes that are typical of phenolic oxidation (Ramešová et al. 2015; Sokolová et al. 2012). Specifically, the maximum near 328 nm is characteristic for hydroxycinnamic acids and their ester chlorogenic acid, which are the dominant type of PPs in sunflower leaves (Stelzner et al. 2019). The estimations of polyphenol oxidation by A328 decrease were corroborated by their close correlation with the reducing PPs contents estimated by the Folin-Ciocalteu method.

Cyclic voltammetry is frequently used to estimate the antioxidant capacity of plant extracts (Neill et al. 2002) and beverages such as wines and teas (Kilmartin and Hsu 2003; Kilmartin et al. 2001), but has not been used yet to characterise PPs in leaf extracts in a physiological context related to photosynthesis and photooxidative stress. Here, we used this method to obtain a descriptive illustration of the loss of reducing PPs in methanolic extracts from MV-treated leaf discs. Compared to the control leaf discs, the main effect of the MV-treatment is the loss of the oxidative wave near 240 mV. Different phenolic compounds such as gallic acid, caffeic acid, quercetin, and catechin show similar voltammograms, with the same main oxidation wave near 240 mV measured at pH 7.0 (Hernanz-Vila et al. 2017). Voltammograms from green and black teas also show a main oxidation wave near 230 mV (pH 7.0), which was lower for the black tea, consistent with the oxidation of black tea leaves (Kilmartin and Hsu 2003; Sava et al. 2001).

The presence of oxidized PPs was detected by the absorbance increase near 420 nm in methanolic extracts of leaf discs treated with for four to eight hours (Fig. 3a). However, most of the oxidized PPs may have polymerized *in situ* or react with proteins to form insoluble brown pigments, or melanin-brown pigments (Pourcel et al. 2007; Takahama 2004; Yamasaki et al. 1997). Their presence affected leaf optical properties, notably by decreased reflectance in the green and far-red regions, less dominated by chlorophylls. The decrease was significant after 6 hours of treatment, i.e. two hours later than the absorbance increase at 420 nm in the methanolic extracts was detected. This suggests that concentration of oxidized PPs must reach some level to initiate their polymerization leading to the accumulation of brown pigments in significant amount to affect leaf reflectance. The presence of insoluble brown pigments in MV-treated leaves was confirmed by measuring reflectance spectra of depigmented leaf discs after incubation in methanol: the reflectance was much lower for MV-treated leaf discs than control leaf-discs.

Fluorescence spectroscopy provides a comprehensive view of the effects induced by the MV-treatment described above, i.e. the transient changes of the fluorescence levels F_s , the losses of PSII photochemical efficiencies, the accumulation of brown pigments, and the decrease of UV-absorbing compounds. Variations of the F_s levels are the results of two opposite effects. A closure of PSII reactions centers causes F_s to increase, whereas an opposite effect on F_s results from the presence of a non-photochemical quenching, either due to the presence of the light-induced acidification of the thylakoid lumen (leading to the formation of zeaxanthin) or to the presence of damaged PSII (Klughammer and Schreiber 2008). So, the F_s increase seen at the fourth hour indicates a higher proportion of closed PSII, likely due to the loss of the regulated NPQ. This transient F_s increase is followed by F_s quenching below the initial values, indicating damages PSII at six and eight hours. This F_s pattern is evident in the averaged intensity of the chlorophyll-*a* emission spectrum of fluorescence excited at 550 nm (Fig. 7), of the excitation spectrum of fluorescence emitted at 740 nm (Fig. 8), and also in the intensities of the far-red fluorescence induced by blue excitation measured by the Multiplex 330 portable fluorometer (Fig. 9a).

Besides the variations of the overall intensity of the fluorescence emission spectrum, it is interesting to note the preferential decrease of the F^{740} relative to F^{684} , so the F^{740}/F^{684} ratio decreased closely to F_v/F_m ($R^2=0.981$) during the MV-treatment. This correlation does not indicate a causal relation between the two variables. Indeed, specific inhibition of PSII due to photoinhibition or other stress (heat, chilling...) decreases preferentially the F^{684} band (Agati et al. 1995). Thereby, the F^{740}/F^{684} ratio should increase during the MV-treatment rather than decrease. However, the chlorophyll-*a* fluorescence emission spectra from our sunflower leaf discs are characterized by a low F^{684} relative to F^{740} intensities. This is due to the large chlorophylls concentrations in sunflower leaves and the excitation at 550 nm, two factors causing the preferential re-absorption of red fluorescence and then high F^{740}/F^{684} (Agati et al. 1995; Agati et al. 1996; Buschmann and Lichtenthaler 1998; Gitelson et al. 1999). Here, the low F^{684} intensity relative to F^{740} makes the F^{740}/F^{684} less sensitive to lower PSII quantum yield. The observed correlation F^{740}/F^{684} and F_v/F_m rather indicates that both variables are similarly affected by a common factor, here the oxidative stress, causing the decrease of F_v/F_m , and the accumulation of brown pigments. The stronger decrease of reflectance at 740 nm than at 684 nm seen in MV-

treated leaf discs (see Fig. 5a) would cause preferential absorption of F^{740} , and therefore the decrease of the F^{740}/F^{684} ratio.

Complementary information about the MV-induced oxidative damaged in sunflower leaf discs was provided by the excitation spectra of the chlorophyll-*a* fluorescence emitted at 740 nm (the emission band not reabsorbed by chlorophyll). Besides the averaged fluorescence intensity that varied similarly to Fs (a transient increase at four hours before declining afterward), there is a specific decrease of the main fluorescence-excitation peak at 484 nm during the MV-treatment. Zeng et al. (2016) showed that illumination of isolated chloroplasts caused a preferential decrease of fluorescence excitation near 477 nm, which was interpreted as a decrease of energy transfer between the antenna pigments, both chlorophyll-*b* and carotenoids. In contrast, Nichelmann et al. (2016) observed that a strong illumination ($1200 \mu\text{moles m}^{-2} \text{s}^{-1}$ for 15 minutes) barely decreased the fluorescence excitation ratio F_{470}/F_{660} measured in *Arabidopsis thaliana* leaves. They rather demonstrated the presence of a good correlation between this F_{470}/F_{660} ratio and the violaxanthin cycle pool size in *A. thaliana* leaves acclimated to different growth irradiances. Since during our experiment the total carotenoid contents remained constant, the similar decreases of F_{484}/F_{680} and $\Delta F/F_m'$ therefore indicates a loss of energy transfer between the antenna pigments and the reaction center of PSII caused by the MV-treatment. It will be interesting to determine the relative importance of energy transfer between the antenna pigments and xanthophylls pool size on the F_{484}/F_{680} ratio in different species and conditions, as the chlorophyll-*a* fluorescence intensities induced at these two excitation wavelengths could potentially provide an estimate of photosynthetic efficiency in proximal fluorosensing.

Concerning the second main excitation peak, at 422 nm, its larger attenuation relative to the red part of the spectrum can be attributed to the accumulation of brown pigments, which absorb maximally at 420 nm (Takahama et al. 1999; see Fig. 3a).

In contrast of the decreases of averaged fluorescence intensities mentioned above, excitation spectra showed higher UV-excited chlorophyll-*a* fluorescence intensities in MV-treated leaf discs compared to control discs. Intensities of UV-excited chlorophyll-*a* fluorescence are naturally weak due to the lower absorbance of chlorophylls in that spectral region, and also due to the blocking of UV-excitation by the presence of hydroxycinnamates in the epidermis of sunflower leaves (Cerovic et al. 2002; Stelzner et al. 2019). These increases of UV-excited fluorescence in MV-treated leaf discs are coherent with the oxidation of PPs seen by the decrease of UV-absorbance of the methanolic leaf extracts. They are also well supported by the measurements made with the portable fluorometer Multiplex 330. Whereas blue-excited far-red fluorescence followed the same patterns as Fs, UV-excited (330 nm) far-red fluorescence markedly increased after four hours of MV-treatment. This corresponds to a 53% decrease of LogFER, that estimates leaf epidermal UV-absorbance, coherent with the loss of UV-absorbing compounds with an absorbance maximum near 328 nm (Fig. 3b).

Globally, our study provide one of the missing evidences required to confirm that PPs act as effective antioxidants protecting the plants against ROS, by showing the presence of a temporal correlation between the inhibition of Fv/Fm and the oxidation of PPs during an acute oxidative stress in sunflower leaf discs. We showed that fluorescence and reflectance

spectroscopy as well as cyclic voltammetry could be useful in future studies to test the hypothesis that leaves with higher PPs contents, Fv/Fm could either persists longer or declines slower during acute oxidative stress.

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Compliance with ethical standards

Conflict of interest ZGC declares a link to the company FORCE-A as one of the co-authors of the Multiplex patent that the company exploits. Other authors have no competing interests.

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678 Figure legends

679 **Fig. 1** Time course of the photooxidative damage to the photosynthetic electron transport in
680 sunflower leaf discs monitored by the inhibition of the effective $\Delta F/F_m'$ (a) and maximum
681 (Fv/Fm) (b) quantum yields of photosystem II, and the changes of the maximum Fm' (c)
682 and the stationary Fs (d) fluorescence levels. Leaf discs were exposed to 150 μmoles
683 photons $\text{m}^{-2}\text{s}^{-1}$ in absence ($\circ$) or in presence of 0.4 mM methyl viologen ($\bullet$).

684 **Fig. 2** Concentrations of the chlorophyll *a+b* and carotenoids in sunflower leaf discs during
685 exposure to 150 μmoles photons $\text{m}^{-2}\text{s}^{-1}$ in absence ($\circ$) or in presence of 0.4 mM methyl
686 viologen ($\bullet$).

687 **Fig. 3** Polyphenol oxidation estimated from leaf methanolic extracts. a) Chlorophyll-less
688 absorbance spectra of extracts from leaf treated or not with methyl viologen for different
689 times. b) Absorbance values at 328 nm of the methanolic extracts from control ($\circ$) and
690 MV-treated ($\bullet$) leaf discs, c) Concentrations of reducing polyphenols in the methanolic
691 extracts by the Folin-Ciocalteu method, expressed in gallic acid equivalents.

692 **Fig. 4** Cyclic voltammograms of methanolic extracts from leaf discs exposed to 150 μmoles
693 photons $\text{m}^{-2}\text{s}^{-1}$ in absence or in presence of 0.4 mM methyl viologen during 8 hours.
694 Voltammograms were measured with a 3 mm glassy carbon electrode at 50 mV s^{-1} in pH
695 7.4 in a phosphate buffer.

696 **Fig. 5** a) Reflectance spectra of sunflower leaf discs treated or not with methyl viologen for
697 different times. b) Reflectance values at 740 nm of control ($\circ$) and methyl viologen-treated
698 ($\bullet$) leaf discs,

699 **Fig. 6** Reflectance spectra of depigmented sunflower leaf discs treated or not with methyl
700 viologen for 8 hours. After the treatments, intact discs were incubated in methanol at 4°C
701 for 24 hours.

702 **Fig. 7** a) Emission spectra of chlorophyll fluorescence excited at 550 nm measured from leaf
703 discs treated or not with methyl viologen for different times. b) Fluorescence intensities
704 emitted at 740 nm and c) Ratio of fluorescence intensities emitted at 740 and 684 nm from
705 control ($\circ$) and methyl viologen-treated ($\bullet$) leaf discs during the experiment.

706 **Fig. 8** a) Excitation spectra of chlorophyll fluorescence emitted at 740 nm measured from leaf
707 discs treated or not with methyl viologen for different times. b) Ratio of fluorescence
708 intensities excited at 484 and 680 nm measured from control ($\circ$) and MV-treated ($\bullet$) leaf
709 discs during the experiment. c) Correlation between the F_{484}/F_{680} excitation ratio and the
710 quantum yield of photosystem II $\Delta F/F_m'$ measured. d) Intensities of fluorescence at 740
711 nm excited at 350 nm during the 8-hour experiment in control ($\circ$) and methyl viologen-
712 treated ($\bullet$) leaf discs.

713 **Fig. 9** Blue-excited (a) and UV-excited (b) far-red fluorescence measured with a Multiplex
714 portable fluorometer from control ($\circ$) and methyl viologen-treated ($\bullet$) leaf discs during the
715 8-hour experiment. c) LogFER ($\log(\text{FRF}_{\text{BLUE}}/\text{FRF}_{\text{UV}})$) as an estimation of the epidermal

UV-absorbance of control (○) and methyl viologen-treated (●) leaf discs during the experiment.

Fig. 1S Fig. 3 Polyphenol oxidation estimated from leaf methanolic extracts. Absorbance spectra of extracts from leaf treated or not with methyl viologen for different times.

Figure 1a

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746 Figure 1b

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Figure 1c

Figure 1d

Figure 2a

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849 Fig. 4

Figure 5a

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Fig. 7b

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Fig. 8a

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965 Fig. 8c

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