Journal of Experimental Botany, Vol. 65, No. 14, pp. 3845–3857, 2014
doi:10.1093/jxb/eru086 Advance Access publication 28 March, 2014

Journal of Experimental Botany
www.jxb.oxfordjournals.org

REVIEW PAPER

Photo-oxidative stress markers as a measure of abiotic stress-induced leaf senescence: advantages and limitations

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Received 31 October 2013; Revised 13 January 2014; Accepted 3 February 2014

Abstract

Inside chloroplasts, several abiotic stresses (including drought, high light, salinity, or extreme temperatures) induce a reduction in CO_2 assimilation rates with a consequent increase in reactive oxygen species (ROS) production, ultimately leading to leaf senescence and yield loss. Photo-oxidation processes should therefore be mitigated to prevent leaf senescence, and plants have evolved several mechanisms to either prevent the formation of ROS or eliminate them. Technology evolution during the past decade has brought faster and more precise methodologies to quantify ROS production effects and damage, and the capacities of plants to withstand oxidative stress. Nevertheless, it is very difficult to disentangle photo-oxidative processes that bring leaf defence and acclimation, from those leading to leaf senescence (and consequently death). It is important to avoid the mistake of discussing results on leaf extracts as being equivalent to chloroplast extracts without taking into account that other organelles, such as peroxisomes, mitochondria, or the apoplast also significantly contribute to the overall ROS production within the cell. Another important aspect is that studies on abiotic stress-induced leaf senescence in crops do not always include a time-course evolution of studied processes, which limits our knowledge about what photo-oxidative stress processes are required to irreversibly induce the senescence programme. This review will summarize the current technologies used to evaluate the extent of photo-oxidative stress in plants, and discuss their advantages and limitations in characterizing abiotic stress-induced leaf senescence in crops.

Key words: Abiotic stress, antioxidants, chloroplasts, crops, oxidative stress, photoinhibition, reactive oxygen species, senescence.

Photo-oxidative stress in abiotic stress tolerance and leaf senescence

Since pioneering observations by Darwin (1859) of the environmental stresses that provide the selective pressures underpinning evolutionary change, many plant physiologists have focused their research on abiotic stress responses. Furthermore, the tremendous development of biochemical and molecular tools over the last three decades has led to an excellent opportunity to get an in-depth understanding of abiotic stress tolerance in crops. Currently, the central aim is to fully understand plant stress responses from agro-ecological aspects to gene expression, including physiological aspects at the whole-plant level and the underlying cellular, biochemical, and molecular biology. With this purpose, it is essential to identify reliable markers of stress tolerance at different levels of organization, from the ecosystem to the gene-expression level.

One of the most affected compartments during abiotic stress and senescence is the chloroplast. Stressful conditions lead to suboptimal CO_2 assimilation rates, and then light absorption can exceed the demand for photosynthesis (Takahashi and

Abbreviations: AsA, ascorbic acid; ^{1}Chl*, singlet chlorophyll; ^{3}Chl*, triplet chlorophyll; H_2O_2, hydrogen peroxide; HPLC, high-performance liquid chromatography; MDA, malondialdehyde; NPQ, non-photochemical quenching; $^1O_2$, singlet oxygen; $O_2^{\cdot-}$, superoxide radicals; OH·, reactive hydroxyl radical; PS, photosystem; PUFAs, polyunsaturated fatty acids; RCS, reactive carbonyl species; RES, reactive electrophilic species; ROS, reactive oxygen species; SAG, senescence-associated gene; Toc, tocopherol; TQ, tocopherol quinone.

Badger, 2011). Extra energy on the electron transport chain can lead to the accumulation of singlet excited chlorophyll *a* (^{1}Chl*) and, if there is not enough de-excitation of ^{1}Chl* through non-photochemical quenching (NPQ), the generation of the excited triplet chlorophyll (^{3}Chl*) is unavoidable (Fig. 1). In photosystem II (PSII), ^{3}Chl* can pass excitation energy to molecular oxygen to form singlet oxygen ($^1O_2$), and in the electron transport chain a higher pool of reduced ferredoxin in thylakoids will increase the chances for O_2 to receive electrons from photosystem I (PSI) and become over-reduced, forming superoxide radicals ($O_2\cdot^-$; Asada, 2006). $^1O_2$ is highly reactive and can oxidize lipids and other molecules inside chloroplasts. $O_2\cdot^-$ is rapidly converted to hydrogen peroxide (H_2O_2) by superoxide dismutase, but if not rapidly detoxified, H_2O_2 can give rise to the highly reactive hydroxyl radical (OH·, Asada, 2006). H_2O_2 can oxidize a wide range of molecules inside the chloroplast, leading to the so-called photo-oxidation processes, or can move outside chloroplasts by diffusion (Borisova *et al.*, 2012). Photo-oxidative stress, that is a transient or sustained production of ROS that is not counterbalanced by antioxidant defences, will therefore lead to photo-oxidation processes. If this is controlled by the endogenous antioxidant system, it will be transient and play a positive role in defence and acclimation. But, if photo-oxidation processes are sustained over time and are not counterbalanced by the antioxidant system, photo-oxidative damage in chloroplasts will occur, and yield will ultimately be reduced.

To control photo-induced ROS production effects inside and outside the chloroplast, plants have developed several antioxidant systems that reduce ROS and allow the maintenance of an adequate reduction/oxidation (redox) balance. When plant cells cannot prevent the increase of the ROS pool, an imbalance favouring oxidation reactions occurs. This oxidation chain can affect several macromolecules (including lipids, proteins, and nucleic acids) and will lead to the activation of oxidative signalling cascade mechanisms

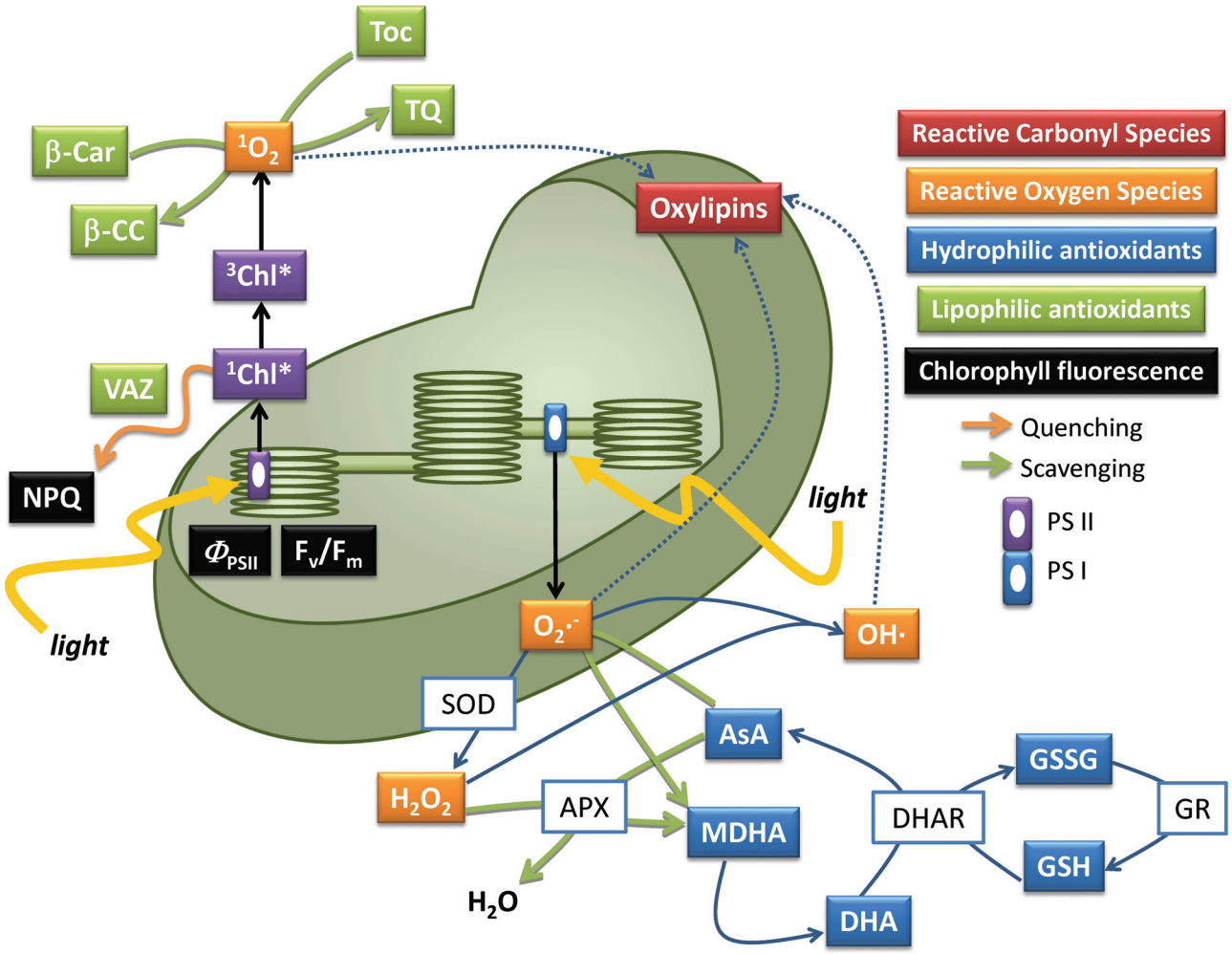

Fig. 1. Localization of photo-oxidative stress markers in the chloroplast. Abbreviations: Chl, chlorophyll; ^{1}Chl*, singlet-state chlorophyll; ^{3}Chl*, triplet-state chlorophyll; $^1O_2$, singlet oxygen; $O_2\cdot^-$, superoxide; H_2O_2, hydrogen peroxide; OH·, hydroxyl radical; PS, photosystem; F_v/F_m, maximum PSII efficiency; NPQ, non-photochemical quenching; Φ_{PSII}, relative PSII efficiency; Toc, tocopherol; TQ, tocopherol quinone; VAZ; xanthophyll cycle; β-Car, β-carotene; β-CC, cyclocitral; AsA, ascorbic acid; DHA, dehydroascorbate; GSH, glutathione; GSSG, oxidized glutathione; SOD, superoxide dismutase; APX, ascorbate peroxidase; DHAR, dehydroascorbate reductase; GR, glutathione reductase. Carotenoids can additionally act to de-excite triplet chlorophyll (the so-called triplet valve) and tocopherols prevent the propagation of lipid peroxidation (not indicated here). Also, the hydroxyl radical is a rather non-specific oxidant that can react with numerous compounds, including glutathione, ascorbate and other low-molecular-weight antioxidants as well as many other cellular metabolites (reactions not included here).

(Foyer and Noctor, 2005; Ahmad *et al.*, 2010). This phenomenon is known as photo-oxidation, and it occurs very frequently when plants grow under stressful conditions and most particularly on leaves at advanced stages of ontogeny leading to an acceleration of senescence (Zimmermann and Zentgraf, 2005; Juvany *et al.*, 2013).

Plants use several strategies to prevent photo-oxidative stress. Leaves and chloroplast movement or solar radiation screening by phenolic compounds (mainly reducing the amount of specific wavelengths, such as UV, that will arrive to the chloroplast) are the first strategies to minimize PSII photoinhibition (Takahashi and Badger, 2011). Once light reaches the reaction centres (Fig. 1), photoprotection mechanisms will allow plant cells to avoid and tolerate the possible excess-light stress consequences by two key steps: preventing ROS formation and scavenging the unavoidably formed ROS. A first step occurs close to PSII, where ^{3}Chl* and $^1O_2$ are produced (Triantaphylidès and Havaux, 2009). The presence of carotenoids at both reaction centres (β-carotene) and light-harvesting antenna complexes (PsbS proteins and xanthophylls) (Siefermann-Harms, 1985; Li *et al.*, 2004) allow a physical quenching of ^{1}Chl*, ^{3}Chl*, and $^1O_2$ by excitation transfer and harmless thermal dissipation (Triantaphylidès and Havaux, 2009). A possible chemical quenching (scavenging) whereby β-carotene forms β-cyclocitral in the reaction centre has also been described (Ramel *et al.*, 2012). A second step occurs when ROS are already formed: scavenging (through chemical reactions) and quenching (a physical process) of $^1O_2$ in PSII and thylakoid membranes is performed by tocopherols and carotenoids, while hydrophilic antioxidants like ascorbate and glutathione, with the help of enzymatic antioxidants such as ascorbate peroxidase or glutathione reductase (and reducing power provided by photosynthetic electron transport) scavenge the H_2O_2 and $O_2^{\cdot -}$ produced in PSI (Asada, 2006).

Photo-oxidative stress is, however, not necessarily negative for the plant. A transient increase in ROS production results in the activation of a plethora of defence-related genes (Karpinski *et al.*, 1999; Pnueli *et al.*, 2003; Apel and Hirt, 2004; Queval and Foyer, 2012; Munné-Bosch *et al.*, 2013). Therefore, redox signalling triggered by a transient ROS formation is considered to be positive to elicit plant stress responses and allow plant acclimation to adverse environmental conditions. However, when produced at high concentrations and sustained over time, ROS can overwhelm the antioxidant defence system and lead to irreversible damage to essential cellular components, from the photosynthethic apparatus to the nuclear gene expression machinery. It is noteworthy that ROS play a dual role in the regulation of leaf senescence. On one hand, a transient accumulation of ROS is needed to trigger leaf senescence during the initiation phase and allow nutrient remobilization during the re-organization phase of senescence, whereas sustained ROS formation seems to occur during the latest stage of leaf senescence (terminal phase, Zimmermann and Zentgraf, 2005; Juvany *et al.*, 2013). As leaf senescence seems to be partly reversible during the re-organization phase, at least in some cases (Zavaleta-Mancera *et al.*, 2007), preventing sustained ROS accumulation will therefore be essential to avoid cell death during the terminal phase of leaf senescence. Consequently, tools to identify sustained ROS production in chloroplasts will undoubtedly help elucidate the degree of photo-oxidative stress in leaves.

This review is aimed at serving as a tool to classify the available techniques to evaluate photo-oxidation processes in crops, with an emphasis on bridging photo-oxidation with abiotic stress and leaf senescence. Not only the advantages and disadvantages of each method will be discussed, but also tools will be given to provide an integrated approach and properly differentiate between abiotic stress (not necessarily leading to leaf senescence) and stress-induced leaf senescence.

Photo-oxidative stress markers

To evaluate the photo-oxidative status of plant tissues, interesting methodologies and technologies have been developed, and are still currently being developed, to estimate and quantify (with the maximum precision) all groups of photo-oxidative molecules, their quenchers/scavengers, and targets. However, information available today is quite segmented and not all markers are used with the same purposes. Here, we report a detailed list of the most frequently used techniques to estimate the degree of photo-oxidative stress both in plant responses to abiotic stress and leaf senescence, and discuss their usefulness in characterizing these two physiological processes. Several markers are discussed depending on the functional trait they are measuring (briefly summarized in Table 1 and Fig. 1).

Photosynthetic pigments: chlorophylls and carotenoids

The quantification of photosynthetic pigments involved in light absorption is a clue to the actual functional capacity of photosystems and therefore of the photosynthetic apparatus in general. Chlorophylls absorb blue and red light used for photosynthesis. Carotenoids, in turn, absorb mainly blue light used for photosynthesis and are additionally involved in (i) energy dissipation by heat through the xanthophyll cycle; (ii) quenching of $^1O_2$ formed during photo-oxidation; (iii) abscisic acid biosynthesis (in the case of neoxanthin and violaxanthin); (iv) strigolactone biosynthesis (Alder *et al.*, 2012); and (v) regulation of thylakoid membrane fluidity (Havaux, 1998). Chloroplastic pigment contents are one of the most frequently used techniques to measure the degree of several abiotic stresses and follow the time-course evolution of leaf senescence.

Spectroscopy analyses are the most common technique used to quantify chloroplastic pigments, as it is a cheap and fast method. Spectrophotometric measurements should be performed in a liquid media extract and each pigment has a corresponding wavelength (or wavelengths) allowing a correspondence between the intensity of the absorption peak and the pigment concentration (Lichtenthaler, 1987). Similarly, near-infrared reflectance spectroscopy (NIRS) has been used to measure chloroplastic pigments (Pintó-Marijuan *et al.*, 2013). The main difference between spectrophotometry and NIRS is the medium, as NIRS does not need any

Table 1. *Summary of the most common techniques used to detect photo-oxidative stress*

Each marker quantification method was evaluated in terms of accuracy, difficulty, time needed, destructiveness, and costs. Colour intensities correspond to higher or lower evaluation result.

Marker	Technique	Accuracy	Difficulty	Time-consuming	Destructiveness	Costs
Photosynthetic pigments						
Chlorophylls and carotenoids	Spectrophotometer					
	NIRS					
	Spectroradiometer					
	Chromatography					
PSII efficiency						
F_v/F_m & Φ_{PSII}	Fluorometer					
NPQ	Fluorometer					
	Chromatography					
Reactive Oxygen Species						
$^1O_2$	Microscopy *in situ*					
β-CC	Chromatography					
PUFAs Ox	Chromatography					
H_2O_2	Spectrophotometer					
	Microscopy *in situ*					
Reactive carbonyl species						
MDA	Spectrophotometer					
	Chromatography					
PUFAs Ox	Autoluminiscence					
	Chromatography					
Antioxidants						
AsA/DHA & GSH/GSSG	Spectrophotometer					
	Chromatography					
Toc & TQ	Chromatography					
Ultrastructure						
	EM					
Transcriptomics						
	Microarray					
	PCR					
Proteomics						
	Westernblot					
	Mass Spectrometry					

Markers: F_v/F_m, maximum PSII efficiency; Φ_{PSII}, PSII efficiency; NPQ, non-photochemical quenching; $^1O_2$, singlet oxygen; β-CC, β-cyclocitral; PUFAs Ox, polyunsaturated fatty acids oxidation; H_2O_2, hydrogen peroxide; AsA, ascorbic acid; DHA, dehydroascorbate; GSH, reduced glutathione; GSSG, oxidized glutathione; Toc, tocopherol; TQ, tocopherol quinone. Techniques: NIRS, near-infrared reflectance spectroscopy; EM, electron microscopy; PCR, polymerase chain reaction.

liquid extraction. However, both techniques are destructive. Spectroradiometers alternatively offer the possibility to perform easy, fast, cheap, and non-destructive measurements of the absorbed (by the quantification of the transmitted and reflected) light by the sample. The market has devices ranging from the easiest equipment with only two wavelengths (used to quantify total chlorophylls) to ones that provide information on the whole range of wavelengths (from where, researchers can easily calculate spectroradiometric indexes). Although spectroradiometric indexes are not frequently used in plant physiology, with only a few examples used to describe photoprotection, such as xanthophyll cycle de-epoxidation in plant stress responses (Peñuelas *et al.*, 2004) and leaf development (Woodall *et al.*, 1998), it is a widely used technique in crop management (Lobos *et al.*, 2013). Finally, chromatographic methods, such as high-performance liquid

chromatography (HPLC), allow the quantification of each chloroplastic pigment molecule separately in a liquid extract. It is a very useful method when high precision is needed in the identification and quantification of the sample components and it is a unique method that additionally estimates xanthophyll cycle de-epoxidation and exact levels of $^1O_2$ quenchers, such as β-carotene. Although the equipment is expensive to buy and maintain, this information is essential to obtain a picture of photo-oxidative processes within the chloroplasts, as will be discussed later. Indeed, measurements of the total levels of chlorophylls and carotenoids only, although widely used in the literature, do not allow differentiation between abiotic stress and leaf senescence. Several abiotic stresses can lead to degradation of photosynthetic pigments, but this does not mean that the leaf has entered senescence. Chlorophyll degradation alone does not even necessarily indicate photo-oxidation processes in all cases, as chlorophylls can also be degraded enzymatically.

PSII efficiency (F_v/F_m and Φ_{PSII}) and non-photochemical quenching

Decreasing PSII efficiency during daylight (relative to the incident light intensity: Φ_{PSII}) or under dark conditions (maximum PSII efficiency: F_v/F_m), as well as the NPQ of chlorophyll *a* fluorescence due to thermal energy dissipation (Fig. 1) are the first indicators of enhanced electron and proton throughput relative to metabolic capacity that may lead to photoinhibition (Demmig-Adams *et al.*, 2012). A widely used technique to detect PSII photoinhibition due to photo-oxidation of the reaction centres and/or the pigments or proteins of the light-harvesting complexes is the modulated chlorophyll fluorescence. Non-destructive, fast, and simple measurements performed with non-expensive equipment allow huge amounts of data to be obtained; in fact, the strongest limitation in the use of chlorophyll fluorescence measurements under field conditions is that too much data is generated (Maxwell and Johnson, 2000; Logan *et al.*, 2007). Experimental designs should include dark-adapted leaves or pre-dawn measurements to obtain F_v/F_m and *NPQ* values (Murchie and Lawson, 2013). An alternative concept to understand *NPQ* measured by chlorophyll fluorescence has been described (Lambrev *et al.*, 2012; Holzwarth *et al.*, 2013), and involves the conversion of time-dependent quenching curves to integrate more precise biochemical information. Among the fluorometers, there are the ones that provide the average information from the whole measured sample, and the ones that provide detailed images of the measured sample, which give extra information to the user and are becoming highly used in the scientific community.

NPQ measurements by fluorometers provide a very useful tool to follow the time-course evolution of photoprotection by the xanthophyll cycle during leaf senescence (Wingler *et al.*, 2004), as NPQ is highly correlated to violaxanthin de-epoxidation (DPS) and excess energy dissipation as heat (Demmig-Adams *et al.*, 2012; Jahns and Holzwarth, 2012). NPQ is an excellent alternative to DPS estimation by HPLC, as it is non-destructive, less time consuming, and much cheaper. However, carotenoid analyses by HPLC provide extra information about photoprotective processes, as they can provide the de-epoxidation state of violaxanthin, and of lutein epoxide, the latter being described only in some species (Garcia-Plazaola *et al.*, 2012). Furthermore, HPLC analyses provide extra information about $^1O_2$ quenchers, such as β-carotene. Therefore, NPQ can be estimated by spectroradiometry, chlorophyll fluorescence, and HPLC (through estimation of xanthophyll cycle estimation). Although Φ_{PSII} and F_v/F_m decrease, and NPQ increases during plant-stress responses and during leaf senescence, their time course evolution during the progression of senescence differs. Φ_{PSII} and NPQ are always altered before a decrease of F_v/F_m occurs during leaf senescence (Wingler *et al.*, 2004). However, changes in all these parameters, even when combined with measurements of photosynthetic levels, will not allow us to fully establish if we are in front of a senescing process. Changes in photosynthetic pigments, Φ_{PSII}, F_v/F_m, and NPQ usually occur during plant responses to abiotic stresses caused by excess light, without necessarily leading to photo-oxidation and leaf senescence.

Reactive oxygen species

ROS formed in the chloroplast during photosynthesis processes are thought to be present at very low concentrations (Apel and Hirt, 2004; Asada, 2006). This is because of three main reasons: (i) a complete set of quenchers are constantly fighting to reduce ROS; (ii) ROS are highly reactive: they react with other molecules within a maximum of a few milliseconds (or less in some cases, such as $^1O_2$ and OH·) after being generated; (iii) ROS can also move outside the chloroplast; in the case of H_2O_2 it then becomes a very important cellular signal. Nevertheless, both abiotic stress and senescence increase ROS concentrations in the chloroplast. When ^{3}Chl* is formed in photosynthetic reaction centres, $^1O_2$ is rapidly formed at high oxygen tensions, particularly when photosynthetic electron acceptors are reduced (Owens, 1996). $^1O_2$ seems to be the major ROS involved in photo-oxidative stress-induced cell death (Triantaphylidès *et al.*, 2008), and is therefore a very interesting reactive species to measure. All techniques to quantify $^1O_2$ are destructive. Even in the cases that measurements are performed *in vivo*, the steps required to detect $^1O_2$ will not allow the natural development of the sample after measurements. In isolated biological preparations three different techniques were described in the early 1990s: (i) spin trapping electron paramagnetic resonance (EPR) spectroscopy (Hideg *et al.*, 1994); (ii) chemical trapping (Telfer *et al.*, 1994); (iii) phosphorescence at 1270 nm (Macpherson *et al.*, 1993). However, samples should be manipulated with caution during their preparation to minimize their oxidation and prevent artefacts. Three methods to directly detect $^1O_2$ use the following fluorescent probes: dansyl-2,2,5,5-tetramethyl-2,5-dihydro-1H-pyrrole (DanePy), 1,8-naphthalimide, and singlet oxygen sensor green (SOSG). Dansyl-based fluorescence double probe (fluorescent and spin) called DanePy, reacts with $^1O_2$ forming the oxidized DanePyO, which can be imaged by confocal laser scanning microscopy (Hideg *et al.*, 2002). Furthermore, the imaging fluorescence UV-A excited

ROS sensor can be combined with a chlorophyll fluorescence detector to obtain parallel data on ROS formation and the efficiency of the photosystems (Hideg and Schreiber, 2007). However, the lack of commercially available DanePy, its variable stability in some biological treatments, and the need for a highly sensitive fluorescence detection system to determine the quenching induced by $^1O_2$, limited the usefulness of the DanePy method. Developed from the DanePy there are double sensor available molecules containing 4-amino substituted 1,8-naphthalimide as a fluorophore (Kálai *et al.*, 2013). However, this method is currently not sensitive enough to detect low levels of $^1O_2$. Alternatively, SOSG is highly selective for $^1O_2$ (with no appreciable response to hydroxyl radicals or superoxide) and emits a green fluorescence with excitation and emission peaks at 504 nm and 525 nm, respectively, which can be readily detected (Flors *et al.*, 2006). These direct methods to measure $^1O_2$ remain, however, technically difficult (Foyer and Noctor, 2011), and are very time consuming, as researchers need time to adapt concentrations of the fluorescent molecules to the different specificities of each plant material and conditions (Triantaphylidès and Havaux, 2009).

Three indirect ways to estimate $^1O_2$ can alternatively or additionally be used by quantifying specific products generated by $^1O_2$ oxidation. $^1O_2$ rapidly reacts with carotenoids, tocopherols, and lipids; therefore, the oxidation products of carotenoids, tocopherols, and polyunsaturated fatty acids (PUFAs) can be a reliable indirect estimation of $^1O_2$ in chloroplasts. Using gas chromatography coupled to mass spectrometry (GC/MS), Ramel *et al.* (2012) detected an early indicator of $^1O_2$ stress resulting from oxidized β-carotene: β-carotene endoperoxides, including mainly β-cyclocitral, but also β-ionone and dihydroactinidiolide. The method is also expensive and time consuming, but the quantification of these oxidized molecules is a very fair marker of photo-oxidative stress mediated by $^1O_2$. Nevertheless, only early-stress, high levels have been identified in leaves of *Arabidopsis thaliana*, not allowing the molecule to be a good marker for more than two-day experiments (Ramel *et al.*, 2012). Measurements of tocopherol oxidation by HPLC are also a possible alternative. Tocopherol quinone (TQ) is formed as a consequence of $^1O_2$ scavenging by tocopherol in thylakoid membranes (Munné-Bosch and Alegre, 2003; Kobayashi and DellaPenna, 2008). Carotenoid and tocopherol oxidation have been used to estimate photo-oxidative stress mediated by abiotic stresses, such as drought or high light stress (Munné-Bosch and Alegre, 2003; Kobayashi and DellaPenna, 2008; Ramel *et al.*, 2012), but no information is yet available as markers of leaf senescence. The third available indirect method to estimate photo-oxidative stress mediated by $^1O_2$ (PUFAs oxidation) has instead been used to determine photo-oxidative stress both in plant responses to abiotic stress and leaf senescence, but will be described later as part of target molecules oxidation.

Another frequently measured ROS is H_2O_2, which is formed in the PSI from $O_2^{\cdot-}$ owing to superoxide dismutase activity, and is detoxified in chloroplasts by ascorbate peroxidase (Fig. 1). The high variability of H_2O_2 results after measurements of this ROS in the literature is probably due to low technical sensitivity and specificity, as well as low stability and efficiency during the H_2O_2 extraction procedure (Queval *et al.*, 2008). Indeed, these authors reported useful advice to obtain the most reliable data on real leaf H_2O_2 content. Quantification of H_2O_2 in whole tissue is based on the oxidation of different substances to obtain altered spectral characteristic products. The ferrous xylenol orange (FOX) assay is based on the chemical specificity of the H_2O_2-dependent reaction complex interactions with iron involving ascorbate and O_2 (Wolff, 1994). Amplex Red (AR, 10-acetyl-3,7-dihydroxyphenoxazine) is converted to the fluorescent resorufin, which is easy to quantify with a fluorescence spectrophotometer (Zhou *et al.*, 1997). In the chemiluminescence reaction, ferricyanide can be used as the catalyst that allows oxidation of luminol (Warm and Laties, 1982). In H_2O_2 quantification, light emission methods (as fluorescence or luminescence) are more sensitive than the absorbance measurements (Queval *et al.*, 2008).

Additionally, *in situ* H_2O_2 quantification techniques have been used to obtain a precise quantification by the infiltration of different probes that should be introduced inside the leaf tissue. Several available infiltration techniques were proved by Snyrychova *et al.* (2009) to determine the more efficient and less harmful way for the probes to move inside the whole plant tissue, with the conclusion that a tiny pinhole is the best technique. Several probes can be used to quantify H_2O_2 *in situ*: (i) DAB (3,3-diaminobenzidine) is a semi-quantitative staining method that polymerizes instantly where peroxidase is active forming a brown precipitate (being an indication of H_2O_2 production), but it is destructive as the precipitate can only be localized in leaves after removing the chlorophyll by using the optical microscope (Thordal-Christensen *et al.*, 1997); (ii) Cerium chloride generating cerium perhydroxide (Bestwick *et al.*, 1997) is another semi-quantitative staining method that does not require pigment degradation before its detection in the transmission electron microscope; and (iii) the fluorescent probe Amplex *Red* can also be applied to detect H_2O_2 *in situ* by using the confocal laser scanning microscope and does not overlap with chlorophylls spectral features (Zhou *et al.*, 1997). These methods need time to optimize the appropriate probe concentration to be detectable and to ensure that it does not react as an antioxidant with H_2O_2 reducing their efficiency. Its use is additionally limited by the fact that H_2O_2 could diffuse to other cell compartments (Borisova *et al.*, 2012) or could be formed outside chloroplasts, so it is not always indicative of photo-oxidative stress, but of oxidative stress within the cell in general.

Although technically difficult and costly in terms of time and money, measurements of ROS are essential to demonstrate photo-oxidation processes in plants. A common mistake found in the literature to estimate the degree of photo-oxidative stress is to consider that leaf extracts are equivalent to chloroplast extracts. Although chloroplasts are one of the most important generators of ROS within the cells (Asada, 2006; Triantaphylidès *et al.*, 2008), other compartments such as peroxisomes, mitochondria, or the apoplast significantly contribute, as well, to the overall cellular ROS formation within the cell (Foyer and Noctor, 2005, 2011; Munné-Bosch *et al.*, 2013). Only direct or indirect estimations of $^1O_2$, but

not of other ROS at the cellular level can be a good estimation of photo-oxidative stress at the chloroplast level, as chloroplasts are the main and virtually unique producers of $^1O_2$ within the cell (Triantaphylidès *et al.*, 2008; Triantaphylidès and Havaux, 2009).

Reactive carbonyl species

Reactive carbonyl species (RCS) belong to the reactive electrophilic species (RES), to carbonyls and oxylipins groups (Fig. 1), and are characterized to contain the α,β-unsaturated carbonyl group. Not only the RCS (such as malondialdehyde, MDA) but also the non-reactive oxylipins (such as the enzymatically-formed jasmonic acid) have been related to photoprotection and stress response signalling (Demmig-Adams *et al.*, 2013). RCS quantification will be important to estimate both leaf senescence and photo-oxidative processes as they can react with proteins and nucleic acids serving as intracellular signals (Montillet *et al.*, 2004; Mueller and Berger, 2009). RCS can be formed by both enzymatic and non-enzymatic oxygenation of PUFAs. Lipoxygenases (LOX) mediate enzymatic oxidative processes leading to important intracellular signals, such as jasmonic acid, which can be involved in leaf senescence but not necessarily result from photo-oxidative processes within chloroplasts. Hence, non-enzymatically formed RCS resulting from ROS accumulation will be excellent indicators of photo-oxidative processes within the chloroplasts. Among the ROS-mediated lipid peroxidation products, $^1O_2$ products will give the most precise and exclusive information on the excess light stress photo-oxidation related processes (Ramel *et al.*, 2012).

MDA is one of the most widely measured non-enzymatically formed RES that researchers tend to correlate with oxidative stress. However, several studies on MDA levels reported in the literature are based on non-specific or without-controls quantifications (Farmer and Davoine, 2007), and in several studies MDA levels do not correlate with the intensity of the applied stress. MDA has been proposed to be a latent RES signal synthesized in the plant cells involved in fast-response gene activation (Farmer and Davoine, 2007; Farmer and Mueller, 2013). However there are several arguments against the appropriate use of MDA as a photo-oxidative marker (Mueller *et al.*, 2006). Although MDA is easily measured spectrophotometrically using the thiobarbituric acid-reactive substances (TBARS) (Hodges *et al.*, 1999) and it is a very easy, cheap and fast method to detect MDA levels, this assay does not give specific information about the origin of the oxidation or the oxidized lipid, as different PUFAs generate different levels of TBARS. Gutteridge and Halliwell (2000) argued that, although it is an easy method, TBARS quantification used alone, is an unreliable index of levels of lipid peroxidation in cells or tissues.

On the other hand, new methods to detect and quantify oxylipins that include gas chromatography (Mueller *et al.*, 2006) or liquid chromatography (Montillet *et al.*, 2004; Triantaphylidès *et al.*, 2008) coupled to mass spectrometry technologies are more precise and trustable, even if more expensive and time-consuming. Moreover, some of these methods (Montillet *et al.*, 2004; Mueller *et al.*, 2006) allow the quantification of specific ROS or enzymatic oxidized PUFAs. Hydroperoxide isomers from 18:2, 18:3 and 16:3 PUFAs allow the discrimination between $^1O_2$-induced lipid peroxidation and other-free-radical-induced peroxidation in plant cells. Hydroxy octadecatrienoic acid (HOTE) isomers, such as 12-HOTE and 16-HOTE, are considered ROS markers, as they have never been observed as LOX products; and 10-HOTE and 15-HOTE are specific markers for $^1O_2$-induced lipid peroxidation, as they are only formed by $^1O_2$ and not by any other ROS (Triantaphylidès *et al.*, 2008).

Oxidized lipids can also be detected by spontaneous ultra-weak emitted autoluminiscence or by thermoluminiscence. Depending on the produced spectral ranges measured in a custom-made apparatus and with a liquid N_2-cooled CCD camera, one can discriminate the molecule that is emitting light (Havaux, 2003; Birtic *et al.*, 2011). However, this technique does not allow the differentiation among all hydroxyl fatty acids to determine the origin of the lipid oxidation (Havaux *et al.*, 2006).

Antioxidant systems

Several enzymatic and non-enzymatic antioxidant systems have been developed during plant evolution to (physically) quench and (chemically) scavenge ROS and RCS within cells (Fig. 1). A plethora of studies describe how antioxidant concentrations and antioxidant enzyme activities are modified by abiotic stresses and leaf senescence. However, two important observations should be taken into account before quantification of antioxidant systems. First of all, the localization of the measured molecule and its role in each cellular compartment will be the key to obtain an accurate discussion. Secondly, the most important antioxidant systems present a reduced and an oxidized form; therefore, not only the total concentration but also the ratio between the oxidized/reduced forms (oxidation state) will allow a correct discussion with appropriate data.

Among the non-enzymatic antioxidants, ascorbic acid (AsA) is the most abundant hydrophilic antioxidant in plant leaves, and 30–40% of the AsA of a plant cell has been described to be localized in the chloroplast (Noctor and Foyer, 1998) being related to photoprotection. AsA scavenges ROS, H_2O_2 in particular, and is a substrate for ascorbate peroxidase, which leads to the formation of the oxidized form of AsA, dehydroascorbate (Foyer and Noctor, 2005; Asada, 2006). Moreover, AsA regenerates α-tocopherol from α-tocopheroxyl radicals (which result from preventing the propagation of lipid peroxidation, Munné-Bosch and Alegre, 2002) and acts as a cofactor for violaxanthin de-epoxidase giving rise to zeaxanthin, which is essential for dissipation of excess energy as heat in chloroplasts (Demmig-Adams *et al.*, 2012). The second most abundant antioxidant, glutathione (reduced: GSH; oxidized: GSSG), helps in AsA recycling *via* the ascorbate-glutathione cycle (Noctor and Foyer, 1998). Apart from all these functions related to photoprotection, glutathione and ascorbate (both oxidized and reduced) have very important roles in several other organelles in the plant

cell as well as in the apoplast (Pignocchi and Foyer, 2003; Noctor *et al.*, 2012; Sierla *et al.*, 2013). Both ascorbate and glutathione can be quantified spectrophotometrically (Queval and Noctor, 2007) or by HPLC (Leipner *et al.*, 2000; Havaux *et al.*, 2009). In both methods, enzymes and molecules that react with either oxidized or reduced forms are used to determine the reduced, oxidized and total ascorbate and glutathione concentrations. To measure the redox state of these two low-molecular-weight antioxidants, spectrophotometric techniques will provide the higher accuracy.

Antioxidant enzymes involved in scavenging ROS in the chloroplast include superoxide dismutase (SOD; EC 1.15.1.1) and ascorbate peroxidase (APX, EC 1.11.1.1) detoxifying $O_2^{\cdot-}$ and H_2O_2 at their site of production by the thylakoid-bound forms and in the stroma by the soluble forms (Niyogi, 1999). On the other hand, there are enzymes found in the stroma only, helping with the recycling of hydrophilic low-molecular-weight antioxidants, including: glutathione reductase (EC 1.6.4.2) and monodehydroascorbate reductase (Fig. 1). Antioxidant enzymes can be quantified spectrophotometrically. Some of the reagents to perform the quantifications are quite expensive and some determinations are very time consuming. It is very important to give antioxidant enzyme activities on a total protein concentration basis (that can also be estimated by spectrophotometric methods).

Carotenoids, discussed previously, and tocopherols are responsible for quenching $^1O_2$ formed in chloroplasts, controlling therefore ROS accumulation in the reaction centres and lipid matrix of thylakoids. Additionally, α-tocopherol, the major tocopherol form present in leaves, can scavenge $^1O_2$ leading to the formation of TQ and other oxidation products (Munné-Bosch and Alegre, 2002, 2003; Kobayashi and DellaPenna, 2008). The major function of tocopherols is however in the inhibition of propagation of lipid peroxidation in thylakoid membranes (Munné-Bosch and Alegre, 2002). Measurement of carotenoids and tocopherols, particularly when they include oxidation products (as discussed before) are reliable indicators of photo-oxidative stress because they accumulate only in chloroplasts. Therefore, chloroplast isolation is not needed in this case to study their involvement in photo-oxidative processes during leaf senescence. Indeed, previous studies indicate that tocopherols are good indicators of the progression of leaf senescence, their levels increasing at early stages of senescence, before decreasing later (Juvany *et al.*, 2013). However, further studies are needed to investigate whether carotenoids and tocopherols are indeed oxidized during the progression of leaf senescence, an aspect that has not been considered thus far.

Ultrastructure

Transmission electron microscopy allows the analysis of plant-cell ultrastructure to detect any changes in the cell organization during the progression of leaf senescence. This technique has not been widely used to detect photo-oxidative stress symptoms associated with leaf senescence, as it is a time-consuming and destructive method, and equipment infrastructures are very expensive. Nevertheless, some studies show the advantages of using this technique for a deep understanding of abiotic stress-induced leaf senescence. Symptoms of leaf senescence include thylakoid distortion, plastoglobuli accumulation in chloroplasts (increasing in number and size), and chromatin condensation in the nuclei (Munné-Bosch *et al.*, 2001; Lim *et al.*, 2007; Mulisch and Krupinska, 2013). Although such ultrastructural changes in chloroplasts can also result from photo-oxidative stress in stressed plants, chromatin condensation is indicative of changes in gene expression and has been identified as a typical hallmark of programmed cell death typically occurring in senescing leaves (Mostowska, 2005). The high costs of time, sample processing and, equipment required to perform such measurements, however, makes this technique unviable for application in large-scale studies in crops.

Transcriptomics and proteomics

To detect either photo-oxidation or senescence, 'omics' techniques are determinant. On one side, the abundance of several transcripts from genes is known to be induced by ROS and specific signals from the chloroplast can modulate nuclear gene expression (Baier and Dietz, 2005). Different molecules are candidates to be signals from the chloroplast to the nucleus: ROS ($^1O_2$ or H_2O_2), oxylipins (Demmig-Adams *et al.*, 2013), oxidized peptides (Moller and Sweetlove, 2010) or low-molecular-weight antioxidants (Queval and Foyer, 2012), among others. Moreover, there are some nuclear genes expressed by the activation of excess light sensing (Li *et al.*, 2009). Therefore, several pathways interconnect photo-oxidation in the chloroplast with transcription regulation in the nucleus, the early light-induced proteins being a clear example of over-expressed proteins during photo-oxidative stress (Hutin *et al.*, 2003).

On the other side, the expression of senescence-associated genes (SAGs) was shown to be upregulated during leaf senescence (Fischer, 2012). A database with 1,145 SAGs has been compiled after performing a literature survey and it has been called "LSD: a leaf senescence database" (Liu *et al.*, 2011). Most importantly, one can assign changes in gene expression or protein levels to specific senescence. For instance, *SAG12* was described as a senescence-associated cell death gene during developmental senescence that was not expressed when senescence was induced by environmental stress (Lim *et al.*, 2007). Regarding proteomics, there are as well several examples of useful markers of senescence. For instance, eIF5A is a post-translationally modified protein activated by covalent modification that is only present in senescing tissues (Thompson *et al.*, 2004).

Measuring the expression of these genes can be performed by targeted analysis of some marker genes (using qRT-PCR, RNA hybridization, etc), by large-scale genes measurement (microarrays), or by identifying and quantifying specific proteins (western blotting, matrix-assisted laser desorption/ionization (MALDI), etc). All techniques are accurate and can give rise to reliable results as markers of leaf senescence or photo-oxidative stress. Although they are quite expensive and sometimes quite time consuming to obtain the final results,

Table 2. *Photo-oxidative markers used in the literature during the last five years characterizing abiotic stress-induced leaf senescence in crops*

		Yield	Photo pig		Chl Flr	ROS		RCS	Antioxidants		US	Transcript		Proteo		
Stress	**Crop**		Chl	Car		H_2O_2	O_2^-	MDA	AsA	Enz	TEM	MA	PCR	WB	PI	**Author**
Water manipulation																
Drought	*Solanum tuberosum* - Potato							■								Wang *et al*. 2013
	Zea mays - Maize				■											Escobar-Gutierrez and
	Panicum miliaceum - Broomcorn millet	■	■				■	■		■						Zhang *et al*. 2012
	Brassica oleracea - Cabbage	■	■		■											Simpson *et al*. 2012
	Oryza sativa - Rice	■	■								■	■	■			Peleg *et al*. 2011
	Agrostis stolonifera - Creeping bentgrass				■			■		■				■	■	Merewitz *et al*. 2011
	Zea mays - Maize		■	■												Vitale *et al*. 2009
	Oryza sativa - Rice											■	■			Degenkolbe *et al*. 2009
Waterlogging																
	Cajanus cajan - Pigeon pea		■			■		■		■						Kumutha *et al*. 2009
	Cajanus cajan - *Pigeon pea*		■			■	■			■			■			Sairam *et al*. 2009
Salt stress																
	Solanum licopersicum - Tomato				■											Albacete *et al*. 2009
	Zea mays - Maize					■		■								Hichem *et al*. 2009
Temperature stress																
High temperature	*Phalaris arundinacea* - Reed canary grass				■											Ge et al. 2012
	Glycine max- Soybean	■	■		■	■	■	■		■						Djanaguiraman *et al*. 2011
	Glycine max- Soybean	■	■		■	■	■	■		■						Djanaguiraman *et al*. 2010
	Agrostis stolonifera - Creeping bentgrass		■					■		■						Xu and Huang 2009
Light manipulation																
	Helianthus annuus - Sunflower		■	■		■				■						De La Mata *et al*. 2013
	Populus cathayana - Poplar		■	■			■	■		■	■					Zhao *et al*. 2009
Ozone stress																
	Quercus mongolica - Mongolian Oak		■		■			■	■	■						Yan *et al*. 2010
Heavy metals stress																
Se	*Vicia faba* - Faba bean		■			■				■						Moussa and Ahmed 2010
Cd	*Lactuca sativa* - Lettuce		■		■			■		■						Monteiro *et al*. 2009
Nutrients modification																
Low micronutrients	*Amomum villosum* - Cardamom	■	■	■	■			■		■						Feng *et al*. 2012
Combined stresses: Drought & Salinity & Zn	*Nicotiana tabacum* - Tobacco		■	■						■						Mytinova *et al*. 2010

Abbreviations: Photo pig, photosynthetic pigments; Chl, chlorophylls; Car, carotenoids; Chl Flr, chlorophyll fluorescence; ROS, reactive oxygen species; RCS, reactive carbonyl species; MDA, malondialdehyde; AsA, ascorbic acid; Enz, enzymatic antioxidants; US, ultrastructure; TEM, transmission electron microscopy; Transcript, transcriptomics; MA, microarrays; Proteo, proteomics; WB, western blot; PI, protein identification.

little material will provide tremendously important information. Four techniques have been described to isolate targeted cells from an organism without causing alterations in the sample (Long, 2011): (i) laser capture microdissection; (ii) fluorescent activated cell sorting; (iii) isolation of nuclei tagged in specific cell types (also known as INTACT); and (iv) immunopurification of mRNA in ribosome complexes. The results obtained compensate the extra time and effort required by far when details at the subcellular level are required (Long, 2011). Analysing changes in nuclear gene expression and better understanding retrograde (chloroplast to nuclei) signalling seems to be very costly, but is essential to characterize abiotic stress-induced leaf senescence. The use of photo-oxidative stress markers cited in the other sections alone is not enough to demonstrate conclusively that leaf senescence is really occurring.

Combined approaches and the importance of space and time

Combined approaches are essential to have a complete picture of processes underlying abiotic stress-induced leaf senescence, including studies from changes in photosynthetic pigments and PSII activity in the chloroplast, to alterations in gene expression in the nucleus. Despite cost limitations, a detailed analysis of physiological processes underlying abiotic stress-induced leaf senescence is only possible when measurements of photo-oxidative stress markers are combined with microscopy and/or gene expression analyses. However, once the senescence process has been described physiologically from the induction to the terminal phase, very cheap photo-oxidative stress markers, such as photosynthetic pigments or chlorophyll fluorescence measurements provide an excellent tool to monitor the physiological stage of crops in large-scale studies. We suggest, therefore, that initial studies include an in-depth analysis of photo-oxidative stress markers combined with transcriptomics to fully characterize the senescing process under a given environmental stress. As a second step, a single photo-oxidative stress marker, such as chlorophyll levels or the F_v/F_m ratio can be used to monitor the physiological status of a crop in the field.

In this regard, it is very important to incorporate, when possible, imaging techniques in the analyses. Imaging techniques, such as chlorophyll fluorescence imaging, allow the detection of heterogeneities in the sample. This is very important to non-destructively characterize the progression of nutrient remobilization during leaf senescence, with a progressive loss of the functionality of PSII from the leaf margins to the centre of the leaf blade (Wingler *et al.*, 2004; Abreu and Munné-Bosch, 2008). At the subcellular level, caution should be taken with whole-tissue preparations, as they are not always valid. For instance, subcellular fractionation to isolate chloroplasts is needed when establishing the potential role of ascorbate and glutathione in photo-oxidative processes, as they are also found outside chloroplasts (Munné-Bosch and Alegre, 2003). Another technique to obtain information on the cellular localization of the physiological process is genetic transformation. For instance, redox-sensitive green fluorescent proteins (roGFPs)-transformed plants allow the quantification of the redox state in the cytosol, mitochondria, or chloroplast (Jiang *et al.*, 2006; Schwarzländer *et al.*, 2009; Rosenwasser *et al.*, 2010; Brossa *et al.*, 2013). When the cysteine residues close to the chromophore oxidize changes in the roGFP, fluorescence properties can be observed with a confocal microscope allowing a non-invasive *in vivo* monitoring of redox status. As recently suggested by Kangasjarvi *et al.* (2012), *in vivo* sensors able to report specific ROS in a reliable, quantitative, and compartment-specific ways are needed to understand which processes are activated, when and where.

A better characterization of the time-course evolution of leaf senescence in response to abiotic stresses is also needed. Sampling the same set of plants at different time points is very costly in terms of time and money; therefore, most studies focus on a few sampling time points or even a single time point to characterize leaf senescence, which is completely insufficient. Usually, the more expensive the technique, the fewer time points are sampled. As leaf senescence can only be fully characterized in an initial stage by using expensive techniques (such as transcriptomic analyses), it is very important that times of measurements are adequately selected. Unfortunately, selecting the adequate time point is only possible for a given marker when the process has been characterized in detail. We propose therefore to initially evaluate the best markers of the senescing process for any given species and abiotic stress, so that when the best marker has been identified, this is used in large-scale studies. Table 1 summarizes the advantages and limitations of each technique used to characterize photo-oxidative stress processes in abiotic stress-leaf senescence. Obviously, the most expensive measurements (e.g. omics, electron microscopy) will be used in initial studies only, with a reduced number of plants and time points, whereas the cheapest markers (e.g. chlorophyll fluorescence, photosynthetic pigment analyses) will be suitable for large-scale studies. It is very important to note, however, that the latter can only be informative of the senescing process when they have been used in parallel with omics to fully characterize the process. Unfortunately, a survey of literature during the last five years (Table 2) shows that studies combining different approaches (including transcriptomic analyses) are scarce. This is particularly alarming when productivity traits are included in the literature search.

Conclusions and perspectives

Here, we have summarized current technologies to evaluate the extent of photo-oxidative stress in plants and have discussed their advantages and limitations in characterizing abiotic stress-induced leaf senescence in crops. We conclude that photo-oxidative stress markers are reliable indicators of leaf senescence, but only when used in combination with transcriptomic analyses. Therefore, initial studies to characterize the process of abiotic stress-induced leaf senescence are needed to establish the potential use of any photo-oxidative

stress marker, which then allows it to be used later on a larger scale. Changes in photo-oxidative stress markers, such as a loss of photosynthetic pigments or chlorophyll fluorescence, are excellent ways to monitor leaf senescence in crops, but only when the response of a given species to a given stress factor has been characterized in detail from the visual symptoms to changes in gene expression. A greater research effort is currently needed to better understand the time-course evolution of senescence markers for their application in large-scale studies in crops.

Acknowledgements

Support for the research of SM-B. laboratory is received through grants BFU2012-32057 and CSD2008-00040 from the Ministry of Economy and Knowledge of the Spanish Government. We would like to thank Judit Vilardosa for her help in preparing the figures.

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