Chapter 9
Photoprotection and Photo-Oxidative Stress Markers As Useful Tools to Unravel Plant Invasion Success

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1 Introduction

Light is essential for life, but also potentially dangerous, particularly for plants. As sessile and photosynthetic organisms, plants benefit from solar irradiation but must also cope with it when too much light is received. The meaning of 'excess light' strictly refers to the amount of energy not used for photosynthesis in chloroplasts of plant leaves. A number of factors determine excess energy in chloroplasts, including not only the amount of solar radiation but also its quality and duration, the plant physiological status (including the development stage), plant stress tolerance, and the availability of other resources for plant growth (Demmig-Adams et al. 2017). As the name of "photosynthesis" itself reveals, light is the main resource for photosynthesis, this is, the conversion of light into chemical energy stored in carbohydrate molecules, synthesized from carbon dioxide and water, releasing oxygen. Under optimal conditions, light is captured by the light harvesting complexes (LHC) at the photosystems (PSI and PSII), which are found at the thylakoid membrane inside the chloroplast of the photosynthetic tissues (Croce and Van Amerongen 2011). Photosynthetic pigments, such as chlorophylls and carotenoids, are responsible of light capture and transference into reaction centres which ultimately allow redox reactions through the electron transport chain (ETC) leading to the ultimate reduction of NADP to NADPH. In addition, this creates a proton gradient across the chloroplast membrane, which is used by ATP synthase in the synthesis of ATP. The NADPH and ATP generated after the ETC are essential for carbon assimilation through their use in the Calvin cycle.

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There are different factors that can lead to suboptimal conditions for photosynthesis. For instance, low concentrations of the substrate for the Calvin cycle, i.e. CO_2, may lead to an accumulation of $NADPH^+$ at the ETC. A common plant response to stress is stomatal closure, which reduces water losses through transpiration but at the same time slows down the photosynthetic machinery. Besides low internal CO_2 concentration, high light itself may collapse the photosynthetic apparatus by an energy excess that cannot be used due to saturation on the ETC components. In PSII, a bound quinone (Q_A) receives the electron transferred from water splitting via the initial acceptor pheophytin. However, Q_A is not able to accept another electron from PSII until it has passed its electron to the next carrier, Q_B (Kalaji et al. 2014). In this state, the reaction centers are considered to be 'closed', leading to an accumulation of molecules of excited chlorophyll (^{3}Chl*). This, in turn, will inevitably cause a decline in quantum efficiency of PSII and damage on it due to the consequent generation of reactive oxygen species (ROS) (Apel and Hirt 2004). After damage on the PSII reaction centre by light excess, it must be dissembled and repaired. The D1 protein is the only compound that, when damaged, needs to be synthesized de novo (Goh et al. 2012). When the oxidation of D1 overcomes its regeneration capacity, photoinhibition occurs, thus leading to a light-induced reduction of the photosynthetic capacity (Takahashi and Badger 2011).

Not only at the PSII, but also at the PSI, the high energy received and the high tensions of oxygen found inside the chloroplast may lead to the formation of ROS, such as singlet oxygen ($^1O_2$), superoxide ion ($^{\cdot}O_2^-$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($^{\cdot}OH$) (Asada 2006). Singlet oxygen is formed at the PSII due to an accumulation of excited chlorophylls (^{3}Chl*) (Havaux and Triantaphylides 2009). Singlet oxygen seems to be the major ROS involved in photo-oxidative stress-induced cell death, and is therefore a very interesting ROS to quantify, despite its high reactivity. The superoxide ion is formed at the PSI rapidly leading to hydrogen peroxide by the action of superoxide dismutase, potentially leading thereafter to the formation of hydroxyl radical, a very reactive ROS (Asada 2006). Here, we will use the term "photo-oxidative stress" as the imbalance between pro-oxidants (such as ROS) and antioxidant defences caused by excess energy in chloroplasts.

If photo-oxidative stress is not properly counterbalanced by antioxidant defences, oxidative damage occurs over different biomolecules, causing peroxidation of lipids, oxidation of proteins, and/or damaging nucleic acids. Photo-oxidative damage is therefore characterized by alterations of the membrane properties (changes in fluidity, ion transport), a loss of enzymatic activity, protein cross-linking, inhibition of protein synthesis, DNA damage and at the end, the death of the cells (Sharma et al. 2012). When this occurs irreversible by lack of sufficient regeneration capacity within cells and organs, this photo-oxidative damage in chloroplasts leads to photo-oxidative damage at the cellular, organ and eventually organism levels.

However, there are multiple photo-protective mechanisms that plants have developed to protect the chloroplast from photoinhibition and photo-oxidative stress (Takahashi and Badger 2011). These include from structural changes that reduce light collection, to an increase in the amounts of antioxidants that quench and/or

scavenge ROS ("quenching" is considered here as the physical process eliminating ROS, while "scavenging" involves a chemical reaction for ROS elimination). All these responses reflect the plant's physiological status and correlate with different stresses intensity, being therefore highly informative to understand stress responses, compare genotypes and give insight into new alternatives to improve environmental management. Likewise, stress markers based on photo-oxidative stress may be helpful on some global ecological problems such as invasive plant species that constitute the second main threat to biodiversity. The utility of photo-oxidative stress markers in invasive plants studies lies in the fact that invasive vigour is determined by their physiological capacity overcoming the native coexistent species.

In this chapter, we aim at compiling existing information on the photoprotective and photo-oxidative stress markers used in plant invasion biology studies: from the study of the light harvesting complexes composition or the photosynthetic efficiency, to ROS formation and the accumulation of antioxidants and its oxidation. Much emphasis will be put on providing the essential information that each marker offers, but also their limitations and the actual and potential use in plant invasion studies.

2 Photoprotection and Photo-Oxidative Stress Markers: How to Measure Them

A **photo-oxidative stress marker** could be considered any molecule, ratio, index or general descriptor that responds to excess light and is related to oxidative stress. The different approaches to quantify photoprotection and photo-oxidative stress comprise the different defense levels that the plants trigger to respond to it and its measurement may include both in situ and ex situ measurements (Fig. 9.1). At the first level, the composition of light harvesting complexes regulates the light capture process at the thylakoid membrane (Walters 2005). Hence, plant pigments play a crucial role on the capacity to transfer light energy into the ETC that will ultimately lead into the production of ATP and storing the reducing power as NADPH. The efficiency by which the electrons are transferred can constitute also a stress marker, providing information on the actual degree of photoinhibition of the photosynthetic apparatus (Kalaji et al. 2014). If the energy exceeds the photosynthetic capacity, ROS are generated and, thus, an estimation of ROS production and/or the accumulation of antioxidants (preferably including their redox state) is another way to get a proxy of the extent of photo-oxidative stress. Finally, the accumulation of oxidation products is also a measure of the degree of oxidative stress. We are going to get through the different approaches to measure photoprotection and photo-oxidative stress and present the most used techniques (resumed in Table 9.1), taking into account what information we are really getting from them, including their limitations and requirements.

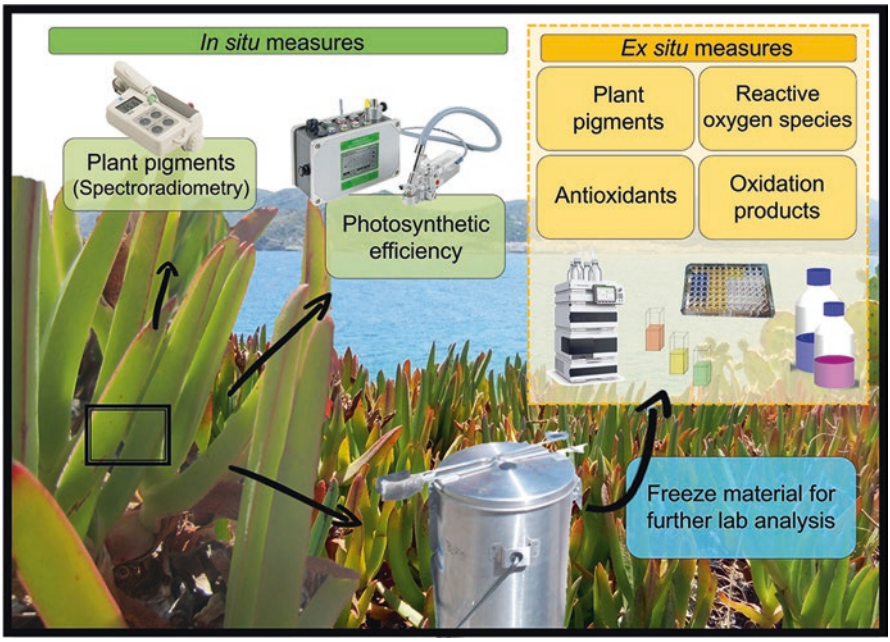

Fig. 9.1 Form field to lab in an invasion biology study, here exemplified with the aggressive invasive plant species *Carpobrotus edulis*, using photo-oxidative stress markers, measured in situ and ex situ. As examples, SPAD (MCL502, Minolta SPAD 502, GIS Ibérica, Spain), MiniPam II (Heinz WalzGmbH, Germany) and an Agilent HPLC are included here

2.1 Sampling Design

Before going through the different approaches and techniques used to measure photoprotection and photo-oxidative stress markers, it is important to point out some common requirements related to the sampling design.

At first, **representativeness** must be seriously considered when designing our experimental set, taking into account the high biological diversity at multiple levels. It is well known that biological diversity is wide, not only at the species level but also among different individuals from the same species, and even at the intraindividual level. For instance, there is an incredible variability considering the different organs within an individual taking into account the cellular structure and its biochemistry.

Choosing an appropriate number of replicates is essential to capture the biological variation, but it may depend on the study scale (growth chamber, common garden, field, ecosystem, international, etc.). In general, pseudo-replications are not recommended if we are after a real representation on the plant response to its environment. The number of replicates must increase after the variability on the environmental conditions and the differences among individuals (age, size, number and

Table 9.1 Summary of techniques and stress markers used to assess photoprotection and photo-oxidative stress in invasion biology and other ecophysiological studies, including a qualitative evaluation of their difficulty, accuracy, costs and dependency on other markers

	Photo-oxidative stress markers	Technique	Difficulty	Accuracy	Costs	Dependency on other markers
Plant pigments	Chl T	Spectrophotometry or HPLC	L H	M H	L H	L
	Chl a/b	Spectrophotometry or HPLC	L H	M H	L H	L
	Car/Chl	Spectrophotometry or HPLC	L H	M H	L H	L
	VAZ	HPLC	H	H	H	L
	DPS	HPLC	H	H	H	L
	Lut	HPLC	H	H	H	H
	β-Car	HPLC	H	H	H	L
	Anthocyanins	Spectrophotometry	L	M	L	M
	SPAD	Spectroradiometry	L	L	L	H
	NDVI	Spectroradiometry	L	L	L	H
	PRI	Spectroradiometry	L	L	L	M
Photosynthetic efficiency	F_v/F_m	Fluorescence	L	H	L	L
	Φ_{PSII} or ETR	Fluorescence	L	H	L	L
	NPQ	Fluorescence	L	H	L	L
ROS	H_2O_2	Spectrophotometry	M	M	H	M
Antioxidants	AsA	Spectrophotometry	M	M	H	L
	AsA/(AsA+DHA)	Spectrophotometry	M	M	H	L
	α-, β-, γ-, δ-Toc	HPLC	H	H	H	L
Oxidation products	LOOH	Spectrophotometry	M	M	H	M
	MDA	Spectrophotometry or HPLC	M H	M H	M H	M
	Protein carbonylation	Spectrophotometry or HPLC	M H	M H	M H	H
	β-CC	GC/MS	H	H	H	H

This last parameter refers to the possibility of understanding photo-oxidative stress with the stress marker alone. The color code refers to the goodness of the qualification, from green (adequate) to orange (not adequate)
L low, *M* medium, *H* high

position of leaves, phenology, reproductive effort, etc.). For measures of photoprotection and photo-oxidative stress markers on ecophysiological studies performed under natural conditions, a number of replicates between 10 and 20 is in general recommended per sampling point, treatment and genotype, with a minimum of at least 8 individuals. The standard deviation can indeed be used as a measure of variation in each particular case.

To guarantee representativeness, some considerations of what and when we should sample are recommended, such as limit our sampling material to **leaves at the same developmental stage** and sample always under **similar environmental conditions**. Attention should be paid to avoid other factors influencing potential differences such as light incidence or biotic stress. We recommend sampling fully-expanded young leaves that receive direct solar radiation to minimize heterogeneity.

As photoprotection and photo-oxidative stress markers are strongly light-dependent, it is crucial to choose similar sampling environmental conditions and time of the day for measurements. We recommend performing **samplings during midday** (when the sun is at its zenith) on clear, sunny days.

Most of the techniques here presented require laboratory analysis (Fig. 9.1) and they need special **considerations to prevent sample degradation** and **additional measures** to estimate the final concentration. During sampling, samples must be immediately frozen in liquid nitrogen and stored at −80 °C until analysis to prevent sample degradation and changes on the cellular redox state. Moreover, as some of the molecules are highly reactive, thermo- or light sensitive, it is recommended to perform all analysis under cold conditions (4 °C) and protecting the samples against direct light. It is necessary to calculate the fresh weight/dry weight ratio of each sample and if possible the leaf mass area (LMA), to present the quantified molecules by fresh weight, dry weight and leaf area.

2.2 Plant Pigments

Among the Viridiplantae subkingdom (vascular plants, mosses and green algae), pigment composition has been shown to be remarkably constant, with chlorophylls *a* (Chl *a*) and *b* (Chl *b*) and six carotenoids: lutein (Lut), β-carotene (β-Car), neoxanthin (Neo), violaxanthin (Vio), antheraxanthin (Ant) and zeaxanthin (Zea), being found in all species (Young et al. 1997). Each of these pigments plays a specific role and is distinctively located within the photosynthetic apparatus (Croce and Van Amerongen 2011; Takahashi and Badger 2011). All these pigments play a dual role by collecting light through the light harvesting complexes (LHC) and offering photoprotection at the photosystem II, where light is initially collected.

Chlorophylls are the main photosynthetic pigments responsible for light capture, constituting therefore good photo-oxidative stress markers. Chlorophylls are found in cyanobacteria, algae and plants and are composed by a large heterocyclic aromatic ring with a magnesium ion at the centre of it. Chl *a* is present in the reaction centres and the antennae of PSI and PSII, whereas the presence of Chl *b* is restricted to light-harvesting systems (Croce and Van Amerongen 2011). Therefore, the ratio Chl *a/b* could be an indicator of the degree of sun/shade acclimation or the intensity of a stress (Esteban et al. 2015). Also, the content of total Chl itself respond to the widest variety of stressors (Esteban et al. 2015). Chlorophyll loss is a process associated with both intense stress and senescence processes (Zimmermann and Zentgraf 2005).

On the other hand, **carotenoids** belong to the category of tetraterpenoids, which take the form of a polyene hydrocarbon chain, which is sometimes terminated by rings. This group of isoprenoids play a dual role in the photosynthetic machinery, as light-harvesting pigments (Bontempo e Silva et al. 2012), but also they protect against photooxidative damage (Lambrev et al. 2012). This group is subdivided into carotenes, of which β-carotene (β-Car) is the most abundant, and xanthophylls, which contain oxygen in is chemical structure and include lutein, violaxanthin, zeaxanthin, antheraxanthin and neoxanthin. β-Car is especially efficient at eliminating the singlet oxygen ($^1O_2$) generated in photosystem II (PSII) from excited triplet chlorophyll (^{3}Chl*) (Ramel et al. 2012). Lutein is the most abundant xanthophyll

species in plants and is essential for protein folding and ^{3}Chl* quenching (Dall'Osto et al. 2006). Moreover, xanthophylls are crucial as physical quenchers that promote thermal dissipation or non-photochemical quenching (NPQ), an efficient energy-dissipation mechanism in plants (Demmig-Adams and Adams 1996). The de-epoxidation of Vx to Ax and Zx (components of the VAZ cycle) responds to different environmental stresses (Demmig-Adams et al. 2012).

There are other plant pigments with an important role on photoprotection and widely distributed among the plant kingdom: **anthocyanins**, a class of flavonoids. These water-soluble pigments consist of an aromatic ring bound to a heterocyclic ring that contains oxygen, which is linked through a carbon-carbon bond to a third aromatic ring (forming the anthocyanidins), in some cases all bound to a sugar moiety (forming the corresponding anthocyanins) (Castañeda-Ovando et al. 2009). Anthocyanins are responsible of screening ultraviolet (UV) light and therefore constitute an important photoprotective defense for plants, as UV comprises 7–9% of the total solar radiation energy (Jansena et al. 1998), protecting plants from PSII damage (Takahashi et al. 2010). Anthocyanins are responsible for some of the colors on leaves, flowers, fruits and seeds, and are not localized on the chloroplast but accumulated in vacuoles, especially in the leaf epidermis cells, together with other phenolic compounds that accomplish the same screening photoprotective function (Takahashi and Badger 2011). The synthesis of phenolic compounds (including anthocyanins) is enhanced under strong light, particularly UV and blue light conditions (Winkel-Shirley 2002).

There are different techniques to measure plant pigments, all based on its specific light absorption spectrum (Table 9.1). With a liquid solvent (methanol, ethanol or acetone with different purity) we can easily extract all plant pigments. Calibration curves have been defined for determination of Chl *a*, Chl *b* and total carotenoids (Car) through **spectrophotometry** using different solvents (Lichtenthaler 1987). It was not until the late 1980s when good protocols for an easy and precise separation of the different carotenoids through **high-performance liquid chromatography** (HPLC), usually employing acetonitrile as the mobile phase, were developed (Thayer and Björkman 1990; Munné-Bosch and Alegre 2000). This is a relatively expensive approach, but it allows quantifying all the carotenes from one extract, offering the possibility to have a deeper understanding on the plant physiological status. Through this methodology, one can quantify how much energy the plant is dissipating through the xanthophyll cycle, by calculating the proportion of de-epoxidated xanthophylls, i.e. the de-epoxidation state ((DPS = (Zx + Ax)/Vx). Not only the DPS but the total amount of Vx, Ax and Zx (so called VAZ) increases in response to stress (Demmig-Adams and Adams 1996).

Anthocyanins can also be measured both by spectrophotometry and HPLC, taking advantage of the absorption range of the spectrum among 500–530 nm of these reddish pigments. There are several methods that show different specificity. At first, the most used method, due to its simplicity, is to estimate total anthocyanins by acidifying the methanol extract with 1% HCl and reading absorbance at 535 nm (Siegelman and Hendricks 1958; Fuleki and Francis 1968), always subtracting unspecific absorbance at 700 nm. Despite the simplicity of this method, it shows

low specificity as all reddish pigments are quantified as anthocyanins, as phlobaphenes (Winkel-Shirley 2002). Another method is the pH differential method or the total monomeric anthocyanin method, designed to measure only single anthocyanin units (Giusti and Wrolstad 2001). Monomeric anthocyanins can change their colour under different acidic conditions, and the lectures at pH 1 and 4.5 comparison allows the removal of the interference of other reddish pigments, being an interesting method for several species (Lee et al. 2005; Dandena et al. 2011). Spectrophotometric methods usually use cyanidin-3-glucoside chloride as a standard, taking its extinction coefficient, as the main anthocyanin found in plants. HPLC methods can be used not only for a more precise quantification but also for identification of the precise anthocyanin composition. Different procedures have been proposed, including an acid hydrolysis that breaks the glyosidic bond of monomeric anthocyanins, releasing anthocyanidins (Lao and Giusti 2016).

Different indices and techniques based on **spectroradiometry** have been described also for chloroplastic pigments determination. Based on leaf transmittance, SPAD (MCL502, Minolta SPAD 502, GIS Ibérica, Spain) is a simple and portable apparatus that determines a relative quantity of chlorophylls by a simple non-destructive leaf measurement (Richardson et al. 2002) that can be measured in situ (Fig. 9.1). The relative measures show a high correlation with the total chlorophyll content and therefore it is a simple and fast alternative to laboratory analysis. However, this measure shows a high variability and needs calibration depending on the species and the environmental conditions. Based on leaf reflectance there are a whole plethora of different defined indexes with different applications. The broadest index used is the normalized difference vegetation index (NDVI), which is known for its good correlation with this chlorophyll content (Richardson et al. 2002). Another commonly used spectral reflectance index is the photochemical reflectance index (PRI) that often strongly correlates with total carotenoids or chlorophyll *a/b*, but also with radiation use efficiency, chlorophyll fluorescence parameters, DPS, net CO_2 uptake, Jmax or water content (Garbulsky et al. 2011). NDVI and PRI can be calculated at different scales, using different platforms where we use the spectroradiometer such as a drone, a balloon, planes or satellites. Spectroradiometric indexes are a promising tool for high-throughput phenotyping, but this technique requires calibration depending on the species, the season, the environmental conditions, etc., and normally there is a huge variability associated.

2.3 Photosynthetic Efficiency

Once a chlorophyll molecule gets excited, it helps the transference of an electron through the different proteinic complexes that form the electron transport chain (ETC). At the end, through the generation of an H^+ gradient, this process will generate ATP and accumulate reducing power as NADPH, necessary for Calvin cycle. An easy, in situ, and non-destructive way to measure the efficiency by which electrons pass through the ETC and detect photoinhibition is the measurement of **chlorophyll**

***a* fluorescence** (Fig. 9.1). The illumination of the photosynthetic tissue with photosynthetic active radiation leads to the emission of fluorescence (680–760 nm), mainly associated with chlorophyll *a* on the PSII. This fluorescence is one of the three ways where chlorophylls excitation energy is distributed, apart from the photochemical reactions on the ETC and the thermal dissipation (explained above). As the three processes are competitive, it is possible to estimate them from the chlorophyll fluorescence measurements. A range of instruments has been developed focusing on different aspects of photosynthesis and on different properties of Chl *a* fluorescence, but most authors are using only a limited set of experimental protocols based on methods that have been developed over time (Kalaji et al. 2014). One of the favorite techniques involves the use of a modulated measuring system, which allows the quantification of the contribution of the photochemical and non-photochemical quenching. In darkness, all the PSII reaction centers are open (all the quinone pool is reduced) and when a leaf is transferred from the darkness into light, PSII reaction centers close progressively. The comparison between the fluorescence emitted after a short duration saturation flash light (that immediately reduces the whole quinone pool) under natural light and after darkness adaptation allows the differentiation between the energy derived to photochemical and non-photochemical processes. In darkness, with all the reaction centers open, the increase on the fluorescence emission ($Fv = Fm - F_0$, variance, maximum and basal fluorescence) due to the saturation flash light indicates the maximum capacity of the PSII to transport electrons. One of the most widely used photo-oxidative stress markers is the maximum efficiency of the PSII (F_v/F_m), calculated from the parameters presented above, and measured by all the modulated fluorimeters. For unstressed leaves, the value of F_v/F_m is highly consistent, with values of ~0.83, and correlates to the maximum quantum yield of photosynthesis (Demmig and Björkman 1987). F_v/F_m below 0.75 reflect damage on the PSII, photoinhibition, and therefore it is an extremely informative stress marker.

More information can be obtained from chlorophyll *a* fluorescence analysis. The same calculation from the basal and maximum fluorescence after a saturating flash pulse under natural light gives the relative efficiency of the PSII (φ_{PSII}) and the electron transport rate (ETR). The latter requires the use of the average ratio of light absorbed by the leaf (around 0.84) and the average ratio of PSII reaction centers to PSI reaction centers (0.50) for calculation. Another parameter obtained from chlorophyll fluorescence analysis is NPQ. This parameter estimates the non-photochemical quenching and its calculation involves both light and dark-adapted measures. NPQ is calculated as $(Fm - Fm')/Fm'$, with the prima parameters corresponding to those taken under light conditions. NPQ strongly correlates with DPS (Demmig-Adams et al. 2012; Jahns and Holzwarth 2012), being a cheaper and non-destructive alternative to the measurement of the xanthophyll cycle by HPLC. The most attractive feature of Chl *a* fluorescence is its non-invasive character, but it is common to commit some pitfalls with the measures. Several reviews have elegantly compiled common pitfalls, questions and conflictive points of view of Chl *a* fluorescence techniques (Maxwell and Johnson 2000; Logan et al. 2007; Murchie and Lawson 2013; Kalaji et al. 2014).

2.4 Reactive Oxygen Species

We define oxidative stress as the imbalance between prooxidants and antioxidants. Therefore, the amount of reactive oxygen species (ROS), among other prooxidants, gives us information about the status of the imbalance during a stress response. Chloroplasts are quantitatively and qualitatively one of the most important sources of ROS in illuminated plant cells (Foyer and Noctor 2003). Thus, the measurement of singlet oxygen ($^1O_2$), superoxide ion ($^{\cdot}O_2^-$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($^{\cdot}OH$) are good markers to evaluate the status of the photosynthetic apparatus.

There are three approaches for measuring ROS in plant tissues: (1) monitoring ROS released into a medium where the cell culture grows, (2) in vivo ROS visualization and (3) quantification of ROS production (Noctor et al. 2016). The third group is indeed the best suited for ecophysiology experiments. Here we will present the measures of hydrogen peroxide as it is the most stable of the group of the four primary ROS (H_2O_2, superoxide ion, hydroxyl radical and singlet oxygen), and therefore it is quantifiable after direct extraction (third approach).

Hydrogen peroxide (H_2O_2) can be quantified through spectral changes of different substances when they are oxidized by this molecule. For instance, the ferrous xylenol orange (FOX) assay is based on the oxidation of ferrous to ferric ions by H_2O_2 producing a chromophore complex which absorbs strongly at 540–600 nm (Cheeseman 2006); however, there are some matrix effects that may be taken into consideration (Queval et al. 2008). Another method is the use of Amplex Red (10-acetyl-3,7-dihydroxyphenoxazine) which is converted to the fluorescent resorufin, easily quantified with a fluorescence spectrophotometer (Zhou et al. 1997). For estimation of the extent of photo-oxidative stress, chloroplasts can be isolated from leaves under reducing conditions and the amount of ROS measured thereafter. This is essential for ROS that can be produced in various cellular compartments, as it occurs with hydrogen peroxide (Munné-Bosch et al. 2013).

2.5 Antioxidants

An antioxidant is a molecule that prevents the oxidation of other molecules. One of the most common responses to stress is the activation of antioxidant defences. We can classify the antioxidants into enzymatic and non-enzymatic, and the latter, depending on their affinity to water can be classified into hydrophilic and lipophilic antioxidants. The enzymatic antioxidants, (such as superoxide dismutase (SOD), ascorbate peroxidase (APX) and glutathione reductase (GR)), among others) are found in chloroplasts, but also in other cellular compartments reducing oxidative stress. Therefore, they are not necessarily only related to photo-oxidative stress and specific chloroplastic isoforms should therefore be investigated to relate them to excess light energy. In contrast, a clear and strong relationship has been established between photo-oxidative stress and the accumulation and tocopherols (or vitamin E) and carotenoids (Car), since both are exclusively located in chloroplasts.

The group of **tocopherols (Toc)** include the α-, β-, γ-, and δ-tocopherols, which are differentiated by the number and position of methyl groups on the chromanol ring. It is specifically this chromanol head that provides the molecule its antioxidant scavenging properties as it can donate electrons to various acceptors such as $^{\cdot}OH$ or $^1O_2$. Tocopherols also deactivate singlet oxygen by (physical) quenching, being this latter function the most important quantitatively, protecting PSII from photo-oxidative damage. Tocopherols, which are located on the thylakoid membrane, but accumulate as well in the plastoglobuli (where they are stored), have also an essential role in preventing the propagation of lipid peroxidation (scavenging lipid peroxyl radicals, Munné-Bosch and Alegre 2002a). The contents of tocopherols, in agreement with their antioxidant function, increase in plants adapted to drought and other abiotic stresses (Munné-Bosch 2005). The four tocopherol homologues can be measured after an extraction with methanol by HPLC with a mixture of n-hexane and p-dioxane as a mobile phase, using a fluorescence detector, emitting at 330 nm and with detection at 295 nm (Amaral et al. 2005). The major homologue found in leaves is the α-tocopherol, followed by its immediate precursor, γ-tocopherol. β-, and δ-tocopherols are usually present at very low concentrations in leaves.

Ascorbic acid (AsA) is the most abundant hydrophilic antioxidant in plant leaves, and it is mainly accumulated in the chloroplast (Queval and Noctor 2007). Ascorbate can be oxidized to monodehydroascorbate radical (MDHA) or dehydroascorbate (DHA). Not only the total amount of AsA, but also the redox state of the ascorbic acid pool (AsA/(AsA + DHA)), particularly when measured in isolated chloroplasts, constitute excellent photo-oxidative stress markers and have been described to be very sensitive to several stresses. The most popular techniques for measuring AsA are based on the molecule's absorbance at 256 nm. To determine the amount of reduced and oxidised AsA is common to use reducing agents such as dithiothreitol (DTT) and ascorbate oxidase (AO), that reduce/oxide the whole sample extract in an acid medium and compare the maximum and the minimum absorbance with the initial one (Queval and Noctor 2007). AsA has an intimal relationship with tocopherols as it mediates their regeneration. At the same time, AsA is regenerated from DHA to AsA by **glutathione (GSH)**, another hydrophilic antioxidant found in most organelles.

2.6 Oxidation Products

As a result of photo-oxidative stress, if ROS are not counterbalanced by antioxidant defences, oxidative damage occurs over different biomolecules. The derived products of this process: oxidized compounds, such as primary or secondary lipid peroxidation products and modified proteins constitute excellent photo-oxidative stress markers focusing on the consequences after the damage. The enhanced production of ROS during environmental stresses can pose a threat to cells by causing peroxidation of lipids, oxidation of proteins, and/or damage to nucleic acids, thus causing enzyme inhibition, alterations of the membrane properties (changes in fluidity,

ion transport), protein cross-linking, inhibition of protein synthesis, DNA damage and at the end, the death of the cells (Sharma et al. 2012).

Over lipids, free radicals or ROS can inflict direct damage, leading to lipid peroxidation that at the same time can inflict damage over DNA or the protein complexes of the PSII (Pospísil and Yamamoto 2017). This is the process under which free radicals attack polyunsaturated fatty acids (PUFAs) of the phospholipidic membrane from the cell or its organelles, essential for cell survival (Ayala and Muñoz 2014). Hydroxyl radical ($HO^{\cdot}$) and hydroperoxyl ($HO^{\cdot}_2$) are the most dangerous ROS for lipids, and a single molecule of ROS can result in multiple peroxided PUFAs as they trigger a cyclic chain reaction that propagates itself very fast (Sharma et al. 2012). The overall process of lipid peroxidation consists of three steps: initiation, propagation and termination (Schneider 2009). During initiation, ROS react with methylene groups of PUFA forming lipid peroxy radicals and hydroperoxides (LOOH). These lipidic products formed are highly reactive and attack other lipids propagating the chain reaction at the propagation phase. After that reactions several reactive species including lipid alkoxyl radicals, aldehydes (malonyldialdehyde, among others), alkanes, lipid epoxides and alcohols are formed by the decomposition of lipid hydroperoxides (Davies 2000). In the termination phase, antioxidants such as vitamin E donate a hydrogen atom to the lipid peroxyl radical ($LOO^{\cdot}$) species forming tocopheroxyl radical that reacts with another $LOO^{\cdot}$ forming nonradical products (Ayala and Muñoz 2014).

Proteins can be affected directly or indirectly by ROS. Direct modifications include modification of its activity trough nitrosylation, carbonylation, disulphide bond formation and glutathionylation, while indirect effects include protein conjugation with lipid peroxidation products (Sharma et al. 2012). Protein carbonylation is defined as an irreversible post-transcriptional modification that yields a reactive carbonyl moiety in a protein, such as an aldehyde or ketone (Fedorova et al. 2014). This is the most common protein modification derived from the oxidation by a ROS, and an enhanced modification of proteins has been reported in plants under various stresses, therefore considering it a major hallmark of oxidative stress (Dalle-Donne et al. 2006). The accumulation of carbonylated proteins results in biomolecule malfunctions that can lead to cell death (Curtis et al. 2013).

Reactive oxygen species, specially $^{\cdot}OH$ and $^1O_2$ constitute the main source of DNA damage resulting in deoxyribose oxidation, strand breakage, removal of nucleotides and a variety of modifications in the organic bases of the nucleotides (Sharma et al. 2012). Despite the fact that ROS can inflict damage to nuclear, mitochondrial and chloroplast DNA, the two last are more susceptible to oxidative damage than nuclear DNA, due to the lack of protective protein, histones, and because they are very close to locations where ROS is produced (Manova and Gruszka 2015).

Among the different biomolecules damaged by ROS, some are better suited than others to become good stress markers. For lipid damage, the accumulation of lipid peroxides or the secondary product malondialdehyde constitute good markers of lipid peroxidation. Protein carbonylation is being also used as a good marker of oxidative stress (Levine et al. 1994). However, neither lipid peroxidation nor protein carbonylation are exclusively formed in chloroplasts and their use as markers of

photo-oxidative stress should be interpreted carefully. Protein carbonyls are in turn more stable (in a scale of hours/days) than lipid peroxidation products, which are removed within minutes (Weber et al. 2015).

The accumulation of **lipid peroxides** (LOOH) are key indicators of the degree of lipid peroxidation, and constitute a good stress marker (Niki 2014). There are multiple approaches to measure the accumulation of LOOH. For plant samples, an easy method can be performed after a methanol extraction through **spectrophotometry** using again the FOX method, which measures the oxidation from ferrous to ferric ions by LOOH, in comparison with an extract where all LOOH are reduced by adding triphenylphospine (TPP). The ferric ions form a chromophore complex with the xylenol orange that absorbs at 540–600 nm (Bou et al. 2008).

Malondialdehyde (MDA) is one of the oxidation products derived from lipid peroxidation and usually measured by several studies assessing the degree of oxidative stress (see some examples in Table 9.2). The assay of thiobarbituric acid-reactive substances (TBARS) is the most used method to assess the breakdown products from lipid peroxidation, including MDA. The TBARS assay includes a liquid extraction with 80% ethanol and measure at 440, 532 and 600 nm with the **spectrophotometer** (Du and Bramlage 1992; Hodges et al. 1999) after an incubation with thiobarbituric acid. Higher precision can be obtained by **HPLC**, using a similar procedure (Iturbe-Ormaetxe et al. 1998; Munné-Bosch and Alegre 2002a).

3 Photoprotection and Photo-Oxidative Stress Markers in Invasion Biology Studies

The economical, demographic and technological development has allowed us to access to almost every biome causing some impacts, altering ecosystems functions. Moreover, globalization has led to the possibility that some species move along with humans, jumping off the geographical barriers that define the realized niche of each species, impacting on native ecosystems by changes on function and composition. Indeed, invasive species are considered the second major threat for the global biodiversity, after habitat loss (Simberloff et al. 2013). Invaders are supposed to have an increased vigor and/or an increased phenotypic plasticity underlying its ability to displace native species (Higgins and Richardson 2014). Therefore, invasive species may have increased physiological performance responding better to the environmental local conditions. In that way, the use of photoprotection and photo-oxidative stress markers may be helpful on invasive studies allowing a better comprehension of the boundaries of the physiological niche by understanding their stress tolerance and adaptation.

Photoprotection and photo-oxidative stress markers may be useful in invasion studies to understand the differences that may lead invasive species to outcompete natives through the description of their capacities under different environmental conditions and the affectation over native species. The comparison of invasive

Table 9.2 Compilation of the plant invasion studies using photoprotection and photo-oxidative stress markers during the last decade (since 2007)

Measurement	Methodology or type	References	Number of studies
Plant pigments	Spectro-photometry	Kim et al. (2008), Li et al. (2008), Liu et al. (2008), Mateos-Naranjo et al. (2008, 2010), Qaderi and Reid (2008), Qaderi et al. (2008), Zhang and Wen (2008), Feng (2008), Feng and Fu (2008), Funk (2008), Andrews et al. (2009), Hussner and Meyer (2009), Küpper et al. (2009), Yang et al. (2009), Feng et al. (2009), Zheng et al. (2012), Kaur et al. (2013), Funk et al. (2013), Castillo et al. (2014), Oliveira et al. (2014), Díaz-Barradas et al. (2015), Al Hassan et al. (2016), Huangfu et al. (2016), Lechuga-Lago et al. (2016), Lyu et al. (2016), Zhang et al. (2016), González-Teuber et al. (2017), Rotini et al. (2017), Souza-Alonso and González (2017), Varone et al. (2017), and Choi et al. (2017)	32
	HPLC	Cela et al. (2009), Song et al. (2010), Cela and Munné-Bosch (2012), Molina-Montenegro et al. (2012), Fleta-Soriano et al. (2015), Lassouane et al. (2016), Fenollosa et al. (2017), and Pintó-Marijuan et al. (2017)	8
	Spectro-radiometry	Ge et al. (2008), Spencer et al. (2008), Hestir et al. (2008), Funk and Zachary (2010), Naumann et al. (2010), Godoy et al. (2011), Roiloa et al. (2013, 2014, 2016), Wang et al. (2016), Yu et al. (2016), Heberling and Fridley (2016), and Roiloa and Retuerto (2016)	13
Photosynthetic efficiency	F_v/F_m only	Wang et al. (2008), Li et al. (2008), Liu et al. (2008), Bihmidine et al. (2009); Naumann et al. (2010); Funk and Zachary (2010), Redondo-Gómez et al. (2011), Immel et al. (2011), Waring and Maricle (2012), Roiloa et al. (2014, 2016), Madawala et al. (2014), Díaz-Barradas et al. (2015), Fleta-Soriano et al. (2015), Lechuga-Lago et al. (2016), Lyu et al. (2016), and Souza-Alonso and González (2017)	17
	F_v/F_m, NPQ, ΦPSII	Qaderi and Reid (2008), Richards et al. (2008), Zhang and Wen (2008), Funk (2008), Mateos-Naranjo et al. (2008. 2010), Cela et al. (2009), Wu et al. (2009), Yang et al. (2009), Song et al. (2010), Cela and Munné-Bosch (2012), Molina-Montenegro et al. (2012, 2016), Roiloa et al. (2013), Funk et al. (2013), Quinet et al. (2015), Li et al. (2015), Roiloa and Retuerto (2016), Lassouane et al. (2016), Pintó-Marijuan et al. (2017), Varone et al. (2017), Fenollosa et al. (2017), and Lukatkin et al. (2017)	23
ROS	H_2O_2	Kaur et al. (2013), Oliveira et al. (2014), and Mamik and Sharma (2017)	3

(continued)

Table 9.2 (continued)

Measurement	Methodology or type	References	Number of studies
Antioxidants	Enzymatic	Lu et al. (2007); Zhang and Wen (2008); Li et al. (2008); Immel et al. (2011), Redondo-Gómez et al. (2011), Morais et al. (2012), Huang et al. (2013), Kaur et al. (2013), Oliveira et al. (2014), Al Hassan et al. (2016), Zhang et al. (2016), and Mamik and Sharma (2017)	12
	Non-enzymatic	Cela et al. (2009), Cela and Munné-Bosch (2012), Huang et al. (2013), Fleta-Soriano et al. (2015), Al Hassan et al. (2016), and Pintó-Marijuan et al. (2017)	6
Oxidation products	MDA	Lu et al. (2007) ,(2008), Zhang and Wen 2008, Li et al. (2008), Immel et al. (2011), Falleh et al. (2012), Huang et al. (2013), Kaur et al. (2013), Oliveira et al. (2014), Quinet et al. (2015), Fleta-Soriano et al. (2015), Al Hassan et al. (2016), Molina-Montenegro et al. (2016), Zhang et al. (2016), Lassouane et al. (2016), and Mamik and Sharma (2017)	16

Measures of carotenoids are included on "Plant pigments" despite some of them have a role as antioxidants. Also, due to the high number of studies measuring the total amount of phenolic compounds, they have not been considered here inside the antioxidants group, although they are non-enzymatic antioxidants

species with coexistent natives had led to the conclusions that invaders have higher capacities to respond to stress or that they have broader physiological niches. Moreover, photo-oxidative stress markers can be helpful to predict plant responses to new environmental conditions, such as climate change. The direction of the community changes due to a new climatic framework can only be predicted with a complete ecophysiological approach. Finally, it is important to describe the extend of the differences between the genotypes from the invasive and the native ranges of one species. An in-depth understanding of these differences with the use of physiological descriptors (such as photo-oxidative stress markers) may undoubtedly help predict new invasions.

Although the interest to study the invasion process **using a complete ecophysiological approach** has increased recently, studies considering in-depth physiological processes are still limited (Pintó-Marijuan and Munné-Bosch 2013). However, photo-oxidative stress markers are being used more and more, and constitute indeed a promising tool for a better understanding of the invasion process. As we can see in Table 9.2, the most common photo-oxidative stress markers measured in plant invasion studies are photosynthetic pigments and chlorophyll *a* fluorescence parameters.

Considering the methodologies used, the most common measurements are photosynthetic pigments through spectrophotometry and the measure only of F_v/F_m. Indeed, only a few studies on the last decade include different measurements of photo-oxidative stress markers, which guarantee a complete understanding of the

plant response. The most common combination is the measurement of photosynthetic pigments through spectrophotometry, the F_v/F_m, and the extend of lipid peroxidation through MDA analysis.

4 Some Limitations and Perspectives

The techniques to measure photo-oxidative stress markers present some common limitations. First, as Pintó-Marijuan and Munné-Bosch (2014) pointed out, it is very difficult to differentiate photo-oxidative damage caused by stress from that caused by **leaf senescence**. Senescence is the physiological deterioration with aging, and some of the hallmarks of senescence are chlorophyll loss and an increase of oxidative stress (Munné-Bosch and Alegre 2002b). Sampling fully-expanded young leaves throughout the experiment is the only way to separate stress- vs. senescence-related effects.

Another point to consider is the **localization** of the measured compound. For example, the measurement of ascorbic acid in leaves. A significant percentage of the ascorbic acid is normally found outside the chloroplast and an increase of this antioxidant can be a consequence of other processes rather than photo-oxidative stress. An easy (but time-consuming) way to ensure that we are measuring a photo-oxidative stress marker is to isolate chloroplasts.

The **matrix effect** must be cheeked every time we work with a new species or a known species under different conditions. Some of the protocols and the authors describing them indeed propose some alternatives to reduce matrix effects. One must keep in mind that some protocols are pH-dependent and the sample pH will depend on the species and its conditions.

It is essential to understand what **information** do we get from each photo-oxidative stress marker, and be aware of the fact that depending on the stress intensity we will see changes on different markers. Sometimes a **combined approach** with different photo-oxidative stress markers would be the most appropriate solution and the selection must follow the question we are trying to answer with our experiment.

As said before, there is a need to use combined stress markers. Photo-oxidative stress is a final consequence of the imbalance of different processes. It is not until antioxidant systems have been taken down that we can measure an accumulation of reactive oxygen species or oxidation products. Therefore, combined markers provide complementary information about the stress response. Here we propose some tips to perform a multiple approach to understand the global plant response.

If we focus on the obtained information, it would be ideal to take one photo-oxidative stress marker from the following groups: plant pigments, photosynthetic efficiency, reactive oxygen species, antioxidants and oxidation products. However, that represents multiple assays and a large amount of samples. If a faster and efficient protocol is needed, it is possible to connect different protocols. For instance, the extraction of plant pigments (chlorophylls, carotenoids and anthocyanins),

tocopherols and lipid hydroperoxides have a common start, and all molecules can be extracted with methanol. Thus, it is possible to save time by performing a common extraction. If the limitation is the economy we can use the cheapest techniques, such as chlorophyll *a* fluorescence and spectroradiometric indexes, such as NDVI or PRI, which with the appropriate models can estimate some photo-oxidative stress markers (Table 9.1). The same techniques are useful if we have a high-scale experimental design (e.g. for large-scale phenotyping).

5 General Conclusions

Photoprotection and photo-oxidative stress are central elements of plant responses to a variety of stresses. Markers based on photoprotection and photo-oxidative stress may be extremely useful for understanding plant acclimation, and constitute a promising tool for the study of invasion success. Working with photoprotection and photo-oxidative stress markers requires the understanding of the meaning of every specific marker within the whole framework of the photoprotective mechanisms. As discussed in this chapter, a combined approach is required to better understand the ecophysiology of invasive vs. native species, using several markers providing complementary information. Here, we have provided some essential tools for a correct choosing of the fittest photoprotection and photo-oxidative stress markers, encouraging its use on invasion studies to help unravelling invaders success.

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