Plant Stress ©2007 Global Science Books

Recommendations for Determining Antioxidant Activity to Study Redox Status and Plant Stress

M. B. Arnao[*] • J. Hernández-Ruiz

Department of Plant Biology (Plant Physiology), Faculty of Biology, University of Murcia, 30100 - Murcia, Spain

Corresponding author: * marino@um.es

ABSTRACT

Antioxidant activity is a very useful parameter for characterizing the potential of biological samples to scavenge reactive oxygen and nitrogen species and other dangerous free radicals in specific reaction media. This parameter has been extensively used in fields such as phytochemistry, food chemistry, food science and technology, human and animal physiology, dietetics and nutrition. It has also been used in plant physiology, but to a lesser extent. Several methodological approaches for measuring antioxidative capacity in plant extracts are presented and the advantages and disadvantages of the most relevant methods from a physiological point of view are discussed. The discrimination between hydrophilic and lipophilic antioxidant activities is very important for identifying the specific antioxidants present in plant samples. The plant redox status of different tissues and its relationship with plant stress situations can be studied by reference to the antioxidant activity, which is an interesting parameter that permits us to investigate the overall antioxidative potential, its distribution, its evolution and its adaptation to different environmental situations. Possible future applications are also presented.

Keywords: antioxidant potential; antioxidant capacity; growth; redox state; ROS; ripening; light-stress; TEAC

Abbreviations: AAPH, 2,2'-azobis(2-amidinopropane) hydrochloride; **ABAP**, 2,2'-azobis(2-amidopropane) dihydrochloride; **ABTS**, 2,2'-azinobis(3-ethylbenzthiazoline-6-sulfonic acid); **DPPH**, 2,2-diphenyl-1-picrylhydrazyl; **HAA**, hydrophilic antioxidant activity; **ROS**, reactive oxygen species; **RNS**, reactive nitrogen species; **LAA**, lipophilic antioxidant activity; **TEAC**, trolox equivalent antioxidant capacity

CONTENTS

INTRODUCTION 32
DEVELOPMENT OF METHODOLOGY 32
METHODS APPLICABLE TO PLANT PHYSIOLOGY STUDIES 33
ABTS assay 33
ORAC assay 33
DPPH assay 33
ANTIOXIDANT ACTIVITY IN PLANT REDOX STATUS AND STRESS 34
CONCLUSION AND FUTURE PERSPECTIVES 35
ACKNOWLEDGEMENTS 35
REFERENCES 35

INTRODUCTION

The determination of antioxidant activity arose as a convenient way of ascertaining the overall content of antioxidative metabolites in a biological sample. Antioxidant activity, also called antioxidant capacity or antioxidant potential, has been related with the presence of compounds capable of protecting a biological system against the potentially harmful effect of processes or reactions normally, involving peroxyl radicals and reactive oxygen species (ROS), which cause excessive oxidation. More recently, other compounds such as reactive nitrogen species (RNS) and a variety of reactive xenobiotic chemicals have been included in this list. The antioxidant activity methodology was first developed in the field of clinical analysis for application to human fluids (plasma, serum, urine, seminal fluid, etc.). While interesting data on the antioxidant activity in these samples was obtained, new methods were also proposed. Antioxidant activity was rapidly identified as a parameter of importance in such diverse fields as food chemistry, phytochemistry, medical chemistry, food technology, nutrition, human and animal physiology and plant physiology. The limited extension of this work does not permit exhaustive mention of the enormous related bibliography, although some interesting reviews are suggested (Rice-Evans *et al*. 1994; Arnao *et al*. 1999; Halliwell *et al*. 2000; Prior *et al*. 2005).

DEVELOPMENT OF METHODOLOGY

The determination of antioxidant activity is generally based on the inhibition of certain reactions in the presence of antioxidants. The most widely used methods involve the generation of a radical compound which subsequently disappears in the presence of antioxidants. The measurement of these radicals, directly or indirectly, by fluorimetric, spectrophotometric, luminiscence or other techniques, and the mode of registering the course of the reaction (by signal inhibition, lag phase, etc.) determine the type of strategy to be used in the antioxidant activity determination. Some interesting review on the progress made in antioxidant activity methodology can be consulted (Arnao *et al*. 1999; Niki 2002; Prior *et al*. 2005). Possibly the first method used to measure anti-

Received: 11 February, 2007. Accepted: 27 February, 2007.

Invited Mini-Review

oxidant activity was that developed by Wayner *et al*. (1985) and called TRAP (Total Reactive Antioxidant Potential) assay, which was applied to human blood plasma. Nevertheless, other approaches had previously been made, although without the same repercussions (Blois 1958). There is no doubt that it was the impulse of medical research that led to the search for a methodology with the desired requirements of sensitivity, accuracy and reproducibility. In the next section, we present the most interesting methods that can be applied to plant physiology studies.

METHODS APPLICABLE TO PLANT PHYSIOLOGY STUDIES

In assays to determine antioxidant activity in biological samples the two main players, oxidants (generated by the assay) and antioxidants (present in the sample), show an extraordinary diversity of physical and chemical characteristics. Generally, antioxidant activity assays attempt to simulate the chemical behaviour of different oxidants with antioxidants in the reaction media. Natural free radicals and oxidants derived from oxygen (ROS) or nitrogen (RNS), such as $O_2^{\bullet-}$, $HO^{\bullet}$, $^1O_2$, H_2O_2, $NO^{\bullet}$, $ONOO^-$, and others derived, such as peroxyl radicals ($ROO^{\bullet}$, $LOO^{\bullet}$), are regarded as suitable for provoking the appearance of oxidative harm in diverse cell components (DNA, lipids, proteins). However, in recent years, ROS and RNS have also been proposed to play an important role in plants as signals in physiological processes (Apel *et al*. 2004; Mittler *et al*. 2004; Mori *et al*. 2004; Gapper *et al*. 2006; Kwak *et al*. 2006). Moreover, antioxidant metabolites can be classified according to their molecular weight: large molecules, mainly proteins, and small molecules, such as ascorbic acid, glutathione, retinoids, tocopherols, carotenoids, polyphenols, polyamines, thiols, ubiquinols, alkaloids, etc. The great variety of small antioxidant molecules, especially in plant samples, means that they may each respond in a different manner to different radical or oxidant sources in the assays used. Furthermore, cooperative effects (synergism) between different antioxidants can occur. Antioxidants can react with any radical by two mechanisms: hydrogen atom transfer (HAT), where the radical is quenched by hydrogen donation, and single electron transfer (SET) where the antioxidant transfers one electron, thereby reducing the free radical (Wright *et al*. 2001). Generally, the methods used for antioxidant activity estimation involve HAT or SET mechanisms, which show different kinetics, limitations and secondary reactions (Halliwell *et al*. 1990; Arnao *et al*. 1996).

The numerous methods developed for antioxidant activity determination (to date, some 25 to 30) can be classified according to different parameters such as: type of radical generated (oxygen radical, free radical, lipid radical, etc.), type of mechanism reaction with antioxidants (HAT or SET), technique used (photometric, fluorimetric, luminiscence, oxygen uptake, gas chromatography, etc.), reaction medium (aqueous, organic), time of analysis, manipulation (simple or complex), versatility (pH, solvent, T^a, etc.) and properties such as sensitivity, accuracy, reproducibility, etc. However, is not the objective of this review to analyze and classify all the methods that actually exist. An exhaustive list of the antioxidant activity methods used in food characterization can be consulted in Prior *et al*. (2005). Only the most significant characteristics of the most important assays used for antioxidant activity determination, according to our experience in food chemistry and plant physiology, are presented. The selected methods are those that have been extensively used to study plant samples in the main, and, among other aspects, we compare their accuracy, reproducibility, standardization and possibility of automation for analyzing a high number of samples.

ABTS assay

This assay, also called TEAC (Trolox Equivalent Antioxidant Capacity), was first reported by Miller and Rice-Evans (Miller *et al*. 1993), although a similar assay was used by Arnao *et al*. (1990) beforehand. It is based on the generation of a metastable chromogen radical, the ABTS radical cation ($ABTS^{\bullet+}$), which shows excellent reactivity with the most common antioxidants (ascorbic acid, trolox, glutathione, tocopherols) and with all the antioxidants described to date, through a mixed SET/HAT mechanism (Wright *et al* 2001). Thus, the presence of antioxidants in a sample leads to the disappearance of $ABTS^{\bullet+}$, which has a characteristic absorption spectrum with maxima at 411, 414, 730 and 873 nm. One of the advantages of this assay is that it makes it possible to estimate the reaction at a high wavelength (730 nm), avoiding the absorption interference of natural compounds such as carotenoids, anthocyanins, etc., and its excellent extinction coefficients (12,947 M^{-1} cm^{-1} in aqueous media at 730 nm) (Arnao 2000). Many variations of the original assay, involving improvements to the sensitivity, the analysis time, the quantification mode and the generation of $ABTS^{\bullet+}$ have been proposed (Arnao *et al*. 1999). This last can be carried out by a long-time chemical reaction using manganese dioxide (Miller *et al*. 1996), potassium persulfate (~16 h) (Re *et al*. 1999), ABAP (60ºC) (Van den Berg *et al*. 1999) or by a faster enzyme reaction using metmyoglogin, hemoglobin (Miller *et al*. 1993) or horseradish peroxidase (Arnao *et al*. 1996; Cano *et al*. 1998). The assays using potassium persulfate and that which uses peroxidase have been the most widely applied to plant samples. The method using peroxidase makes it possible to generate $ABTS^{\bullet+}$ in media at different pH values and in organic media, which is very useful for discriminating between antioxidants of a hydrophilic (ascorbic acid, glutathione, polyphenols, thiols) and lipophilic (carotenoids, tocopherols) nature. This method shortens the analysis time to 2-10 min. The method has been adapted to microplate-reader and to HPLC (Cano *et al*. 1998, 2000; Arnao *et al*. 2002; Cano *et al*. 2002; Chen *et al*. 2004).

ORAC assay

The ORAC (Oxygen Radical Absorbance Capacity) assay was developed by Cao *et al*. (1993) from an original idea of Delange and Glazer (1989). The assay measures the inhibition of fluorescence due to β-phycoerythrin (a protein isolated from *Porphyridium cruentum*) by the presence of antioxidants coupled to the peroxyl radical generation from AAPH and O_2, in a classic HAT mechanism. The original β-phycoerythrin was later substituted by fluorescein or dichlorofluorescein, two more stable probes (Ou *et al*. 2001; Ishige *et al*. 2001). However, the assay requires exhaustive plots to integrate the areas of decay curves to avoid underestimating the antioxidant activity, while careful temperature control is also essential. Recently, the assay has been adapted to measuring lipophilic antioxidants using a complicated water/acetone medium incorporating methylated β-cyclodextrin to solubilize the lipophilic antioxidants. ORAC is possibly the most used assay for the characterization of foodstuffs in the USA, where a database for total antioxidant activity in food will soon be incorporated into the USDA Nutrient Database (Huang *et al*. 2002; Wu *et al*. 2004). Nevertheless, this assay has never been used in plant physiology studies.

DPPH assay

This assay uses the free metastable radical DPPH, which has a maximum absorbance peak at 515 nm with an excellent extinction molar coefficient of 12,500 M^{-1} cm^{-1} in methanolic media (Blois 1958; Brand-Williams *et al*. 1995). DPPH reacts with many antioxidants showing bi-phasic kinetic behaviour involving mixed SET/HAT mechanisms. The assay is very simple and possibly the most widely used because it is not necessary to generate the DPPH radical which can be purchased, and only a photometer is required. Nevertheless, the assay has many drawbacks, the most relevant being the underestimation of antioxidant activity due to sample interferences at 515 nm. Plant compounds

such as carotenes, xantophylls and anthocyanins among others, show substantial overlap at this wavelength, the level of interference clearly being dependent on the plant material in question (Prior *et al*. 2005). This problem can be avoided by using the ABTS assay (Arnao 2000). Also, the insolubility of DPPH in water limits the study of antioxidants in biological buffered media.

Others assays, such as β-carotene bleaching (oxidizing this carotenoid by heat, light, AAPH, etc.) or LDL oxidation, have the advantages of using biological targets, although data interpretation is often complicated. In β-carotene bleaching, temperature control is critical, and for LDL oxidation, it is necessary to obtain homogenous LDL fractions, which is a serious handicap (Halliwell *et al*. 2000).

ANTIOXIDANT ACTIVITY IN PLANT REDOX STATUS AND STRESS

The major source of ROS in plant cells are organelles which show highly oxidizing metabolic activity, such as chloroplasts, mitochondria, peroxisomes and glyosyxomes. The production of ROS is enhanced in plants by limiting conditions such as drought, salinity, light and temperature stress and, in general, by biotic and abiotic induced stress (Mullineaux *et al*. 2006; Rhoads *et al*. 2006; Suzuki *et al*. 2006). For each situation, the steady-state level of ROS in cells needs to be precisely regulated, possibly being this balance, the responsible signal in the regulation of important cellular processes. In *Arabidopsis thaliana*, a network of at least 152 genes is involved in managing the levels of ROS. This network is highly dynamic and encodes ROS-producing and ROS-scavenging agents. The major ROS-scavenging enzymes in plants include superoxide dismutase, ascorbate peroxidase, monodehydro-ascorbate reductase, dehydroascorbate reductase, catalase, glutathione peroxidase, glutathione reductase, NADPH oxidase and peroxiredoxins (Apel *et al*. 2004; Mittler *et al*. 2004). The ROS-scavenging enzymes need a cellular pool of the antioxidants ascorbic acid and glutathione to operate (Potters *et al*. 2002). The reduced state of these antioxidative metabolites is maintained by enzymes capable of using NADP(H) to regenerate oxidized forms of glutathione and ascorbic acid. In addition, the protection of membrane integrity against oxidative stress is guaranteed by the α-tocopherol (vitamin E) pool, which is kept in its reduced state by the pool of ascorbic acid. Also, ascorbic acid and glutathione are metabolites involved in the violaxanthin/antheraxanthin/zeaxanthin system (VAZ cycle), a protective mechanism against light stress (Eskling *et al*. 1997). Thus, in the antioxidative pool of cellular metabolites, compounds other than ascorbic acid and glutathione, such as phenolic compounds or other secondary metabolites, could also act on antioxidative enzymes in specific organelles (Foyer *et al*. 2003; Conklin *et al*. 2004).

One way of monitoring the antioxidative status or antioxidative pool of cells, tissues, organs or of the entire plant could be to determine antioxidant activity. Physiological studies can establish some specific requirements in the antioxidant activity assays applied: i) the most relevant plant antioxidants (ascorbic acid, glutathione, tocopherols, etc.) should be measured; ii) there should be no interference; iii) a clear quantitative response (signal-antioxidant) should be obtained; iv) there should be some versatility with respect to buffered media and pH, and also for organic media; v) it should be possible to measure and discriminate between antioxidant activity due to hydrophilic and lipophilic antioxidants, and *vi)* the assays need to be simple, rapid and low cost, and offer the possibilities of high sample throughput and automatization.

The physiological aspects where antioxidant activity may be an interesting parameter to determine would be cases where antioxidative fluctuations might be expected, such as germination, growth and development, plant ontogeny, fruit ripening and senescence, stress situations caused by biotic or abiotic agents, etc. It could also be useful in the characterization of standard plant materials and varieties.

We have developed and adapted the ABTS assay to study and characterize several physiological situations. The applicability of the antioxidant activity parameter in plant physiology has been demonstrated in many studies. For example, the time-course of hydrophilic antioxidant activity (HAA) and of lipophilic antioxidant activity (LAA) during four ripening-stages of on-vine tomatoes (*Lycopersicum esculentum* Mill.) (green, breaker, pink and red ripe) has been shown. The strong correlation between the levels of ascorbic acid and HAA ($r = 0.90$, $P < 0.1$) and between the lycopene content and LAA ($r = 0.91$, $P < 0.05$), using the same assay, has been demonstrated (Cano *et al*. 2003). Other authors have studied the ripening process of tomatoes using our methods (Lana *et al*. 2006) or a similar ABTS assay (Javanmardi *et al*. 2006). As regards plant material characterization, many authors have used the ABTS assay, until now the most extensively used method in studies with some physiological objective. For example, this assay has been applied in studies of pepper fruit ripening (Jimenez *et al*. 2003), sweet cherry ripening (Serrano *et al*. 2005), kiwifruit ripening (Park *et al*. 2006), apple fruit quality in different farming systems (Peck *et al*. 2006), broccoli head conservation (Serrano *et al*. 2006), fruit characterization of orange and grape varieties (Alcolea *et al*. 2002; Pretel *et al*. 2004), woody plant germplasm of different species (Johnston *et al*. 2006), etc. Also, we studied the growth and development of different varieties of lettuce, determining the changes in HAA and LAA in leaves of different ages and different positions. Thus, LAA increased sharply from stem to outermost leaves, demonstrating the protective role of lipophilic antioxidants in mature and light-exposed leaves (Cano *et al*. 2005). Also, a direct relationship between citrus leaf age and LAA has been described (Arnao *et al*. 2001).

As regards plant stress, not fewer studies have applied the antioxidant activity assays. However, this is a field where expectations are high in this respect. When our methods were applied to the study of the post-fire re-emergence of leaf sprouts of the Mediterranean tree *Quercus ilex* (El Omari *et al*. 2003), high correlations between LAA and certain chloroplast pigments such as β-carotene and the carotenoids of the xanthophyll cycle (VAZ) were obtained. Also, in leaves and stems, a seasonal pattern of antioxidant activity appeared, being twice as great in summer as in spring and winter. This pattern also correlated with the accumulation of small heat-shock proteins, which were clearly related with the general adaptation syndrome described in plants exposed to seasonal alterations in environmental conditions (Verdaguer *et al*. 2003). Thus, antioxidant activity determination, together with other physiological parameters, can provide valuable information for ecophysiological studies. More recently, we studied the de-etiolation transition of seedlings and the behaviour of leaves after light stress induction in oat and wheat. The changes observed in antioxidant activity and ascorbic acid levels clearly reflected variations in environmental conditions. Thus, in oat and wheat seedlings, the HAA increased during the de-etiolation process, reaching an antioxidative status similar to that of evergreen plants of the same age. Ascorbic acid levels and HAA values, on the other hand, were maximum in young leaves and decreased with age, indicating that young leaves were more efficiently prepared to confront oxidative stress. The variations observed in HAA and ascorbic levels in oat and wheat leaves after a high-light treatment confirmed the above assertion. HAA decreased in response to high-light treatment and totally or partially recovered in the dark. However, this capacity depends on age and species, the antioxidant activity acting as indicator in the stress process (Cano *et al*. 2006). Other studies that applied the antioxidant activity assays in stress studies suggested that an optimal redox status or capacity to limit the oxidative damage is very important in plant protection (Ben Amor *et al*. 2006; Johnston *et al*. 2007).

CONCLUSION AND FUTURE PERSPECTIVES

Although in physiological studies, antioxidant activity can contribute to our understanding of the plant redox state, this parameter must be interpreted with caution since it should act as a complement to specific antioxidant determinations. The detailed analysis of overall plant antioxidants involved in defence redox system can be difficult. However, the analysis of the most important individual antioxidants (ascorbic acid, glutathione, antioxidative enzymes) and their variations with respect to antioxidant activity could be a practical approach to studying physiologically normal and stress situations. Many approaches have been developed for there determination, ranging from classical analysis using photometric techniques to sophisticated tools such as electron spin resonance, pulse radiolysis and proteomic markers (Punchard *et al*. 1996; Halliwell *et al*. 2000; Shulaev *et al*. 2006; Navrot *et al*. 2007).

Antioxidant activity determinations could contribute to the study of many physiological aspects, as mentioned above, specially concerning the role that the hydrophilic antioxidant pool plays in plant organelles, such as mitochondria, chloroplasts and peroxisomes (Potters *et al*. 2002; Foyer *et al*. 2003; Asada 2006; Rhoads *et al*. 2006; Shulaev *et al*. 2006; Navrot *et al*. 2007). Such determinations might also throw light on the variations that these antioxidants show in the apoplastic space, related with cellular growth and stress. Another important aspect will be studies of the photosynthetic process. The determination of changes in lipophilic antioxidant activity in chloroplasts of leaves or in chromoplasts in a variety of situations can contribute to this study. Lastly, the use of these assays as a first screening for the antioxidant content of plant materials may be helpful for detecting little understood molecules in plant, such as melatonin, serotonin, catecholamines, lipoic acid, etc. (Navari-Izzo *et al*. 2002 ; Arnao *et al*. 2006; Kulma *et al*. 2007).

ACKNOWLEDGEMENTS

This work was supported by project FUNDACIONSENECA 502/PI/04 and by project MEC-BFU2006-00671 (co-financed by FEDER). JHR has a contract with the University of Murcia.

REFERENCES

Alcolea JF, Cano A, Acosta M, Arnao MB (2002) Hydrophilic and lipophilic antioxidant activities of grapes. *Nahrüng* **46**, 353-356

Apel K, Hirt H (2004) Reactive oxygen species: metabolism, oxidative stress, and signal transduction. *Annual Review of Plant Physiology and Plant Molecular Biology* **55**, 373-399

Arnao MB (2000) Some methodological problems in the determination of antioxidant activity using chromogen radicals: a practical case. *Trends in Food Science and Technology* **11**, 419-421

Arnao MB, Acosta M, Cano A (2002) Antioxidant activity: an adaptation for measurement by HPLC. In: *Encyclopedia of Chromatography*. Marcel Dekker Inc, NY, pp 1-6

Arnao MB, Acosta M, Casas JL, del Río JA, García-Cánovas F (1990) An enzymatic colorimetric method to measuring naringin using ABTS in the presence of peroxidase. *Analytical Biochemistry* **185**, 335-338

Arnao MB, Alcolea JF, Cano A, Acosta M (2001) Estimation of the free radical-quenching activity of leaf-pigment extracts. *Phytochemical Analysis* **12**, 138-143

Arnao MB, Cano A, Acosta M (1999) Methods to measure the antioxidant activity in plant material. A comparative discussion. *Free Radical Research* **31**, S89-96

Arnao MB, Cano A, Hernández-Ruiz J, García-Cánovas F, Acosta M (1996) Inhibition by L-ascorbic acid and other antioxidants of the ABTS oxidation catalyzed by peroxidase. A new approach for determining total antioxidant status of foods. *Analytical Biochemistry* **35**, 255-261

Arnao MB, Hernadez-Ruiz J (2006) The physiological function of melatonin in plants. *Plant Signaling and Behavior* **1**, 89-95

Asada K (2006) Production and scavenging of reactive oxygen species in chloroplasts and their functions. *Plant Physiology* **141**, 391-396

Ben Amor N, Jimenez A, Megdiche W, Lundqvist M, Sevilla F, Abdelly C (2006) Response of antioxidant systems to NaCl stress in the halophyte *Cakile maritima*. *Physiologia Plantarum* **126**, 446-457

Blois MS (1958) Antioxidant determinations by the use of a stable free radical. *Nature* **26**, 1199-1200

Brand-Williams W, Cuvelier ME, Berset C (1995) Use of a free radical method to evaluate antioxidant activity. *Lebensmmittel-Wissenschaft und Technologie* **28**, 25-30

Cano A, Acosta M, Arnao MB (2000) A method to measure antioxidant activety in organic media: application to lipophilic vitamins. *Redox Report* **5**, 365-370

Cano A, Acosta M, Arnao MB (2003) Hydrophilic and lipophilic antioxidant activity changes during on-vine ripening of tomatoes (*Lycopersicon esculentum* Mill.). *Postharvest Biology and Technology* **28**, 59-65

Cano A, Alcaraz O, Acosta M, Arnao MB (2002) On-line antioxidant activity determination: comparison of hydrophilic and lipophilic antioxidant activity using the ABTS assay. *Redox Report* **7**, 103-109

Cano A, Arnao MB (2005) Hydrophilic and lipophilic antioxidant activity in different leaves of three lettuce varieties. *International Journal of Food Properties* **8**, 521-528

Cano A, Hernandez-Ruiz J, Arnao MB (2006) Changes in hydrophilic antioxidant activity in *Avena sativa* and *Triticum aestivum* leaves of different age during de-etiolation and high-light treatment. *Journal of Plant Research* **119**, 321-327

Cano A, Hernández-Ruiz J, García-Cánovas F, Acosta M, Arnao MB (1998) An end-point method for estimation of the total antioxidant activity in plant material. *Phytochemical Analysis* **9**, 196-202

Cao G, Alessio HM, Cutler RG (1993) Oxygen-radical absorbance capacity assay for antioxidants. *Free Radical Biology and Medicine* **14**, 303-311

Chen IC, Chang HC, Yang HW, Chen GL (2004) Evaluation of total antioxidant activity of several popular vegetables and chinese herbs: a fast approach with ABTS/H_2O_2/HRP system in microplates. *Journal of Food and Drug Analysis* **12**, 29-33

Conklin PL, Barth C (2004) Ascorbic acid, a familiar small molecule intertwined in the response of plants to ozone, pathogens, and the onset of senescence. *Plant, Cell and Environment* **27**, 959-970

Delange RJ, Glazer AN (1989) Phycoerythrin flourescence-based assay for peroxyl radicals: a screen for biologically relevant protective agents. *Analytical Biochemistry* **177**, 300-306

El Omari B, Fleck I, Aranda X, Abadia A, Cano A, Arnao MB (2003) Total antioxidant activity in *Quercus ilex* resprouts after fire. *Plant Physiology and Biochemistry* **41**, 41-47

Eskling M, Arvidsson PO, Akerlund HE (1997) The xanthophyll cycle, its regulation and components. *Plant Physiology* **100**, 806-816

Foyer CH, Noctor G (2003) Redox sensing and signalling associated with reactive oxygen in chloroplasts, peroxisomes and mitochondria. *Physiologia Plantarum* **119**, 355-364

Gapper C, Dolan L (2006) Control of plant development by reactive oxygen species. *Plant Physiology* **141**, 341-345

Halliwell B, Gutteridge JMC (2000) *Free Radicals in Biology and Medicine* (3rd Edn), Oxford University Press, NY, 936 pp

Huang D, Ou B, Hampsch-Woodill M, Flanagan J, Deemer E (2002) Development and validation of oxygen radical absorbance capacity assay for lipophilic antioxidants using randomly methylated β-cyclodextrin as the solubility enhancer. *Journal of Agricultural and Food Chemistry* **50**, 1815-1821

Ishige K, Schubert D, Sagara Y (2001) Flavonoids protect neuronal cells from oxidant stress by three distinct mechanisms. *Free Radical Biology and Medicine* **30**, 433-446

Javanmardi J, Kubota C (2006) Variation of lycopene, antioxidant activity, total soluble solids and weight loss of tomato during postharvest storage. *Postharvest Biology and Technology* **41**, 151-155

Jimenez A, Romojaro F, Gomez JM, Llanos MR, Sevilla F (2003) Antioxidant systems and their relationship with the response of pepper fruits to storage at 20ºC. *Journal of Agricultural and Food Chemistry* **51**, 6293-6299

Johnston JW, Dussert S, Gale S, Nadarajan J, Harding K, Benson EE (2006) Optimisation of the ABTS radical scavenging assay for physiological studies of total antioxidant activity in woody plant germplasm. *Plant Physiology and Biochemistry* **44**, 193-201

Johnston JW, Harding K, Benson EE (2007) Antioxidant status and genotypic tolerance of *Ribes in vitro* cultures to cryopreservation. *Plant Science* **172**, 524-534

Kwak JM, Nguyen V, Schroeder JI (2006) The role of reactive oxygen species in hormonal responses. *Plant Physiology* **141**, 323-329

Kulma A, Szopa J (2007) Catecholamines are active compounds in plants. *Plant Science* **172**, 433-440

Lana MM, Tijskens LMM (2006) Effects of cutting and maturity on antioxidant activity of fresh-cut tomatoes. *Food Chemistry* **97**, 203-211

Miller NJ, Diplock AT, Rice-Evans C, Davies MJ, Gopinathan V, Milner A (1993) A novel method for measuring antioxidant capacity and its application to monitoring the antioxidant status in premature neonates. *Clinical Science* **84**, 407-412

Miller NJ, Sampson J, Candeias LP, Bramley PM, Rice-Evans C (1996) Antioxidant activities of carotenes and xanthophylls. *FEBS Letter* **384**, 240-242

Mittler R, Vanderauwera S, Gollery M, van Breusegem F (2004) Reactive oxygen gene network of plants. *Trends in Plant Science* **9**, 490-498

Mori IC, Schroeder JI (2004) Reactive oxygen species activation of plant Ca^{2+} channels: a signaling mechanism in polar growth, hormone transduction,

stress signaling, and hypothetically mechanotransduction. *Plant Physiology* **135**, 702-708

Mullineaux PM, Karpinski S, Baker NR (2006) Spatial dependence for hydrogen peroxide-directed signaling in light-stressed plants. *Plant Physiology* **141**, 346-350

Navari-Izzo F, Quartacci MK, Sgherri C (2002) Lipoic acid: an unique antioxidant in the detoxification of activated oxygen species. *Plant Physiology and Biochemistry* **40**, 463-470

Navrot N, Rouhier N, Gelhaye E, Jacquot JP (2007) Reactive oxygen species generation and antioxidant systems in plant mitochondria. *Physiologia Plantarum* **129**, 185-195

Niki E (2002) Antioxidant activity: are we measuring it correctly? *Nutrition* **18**, 524-525

Ou B, Hampsch-Woodill M, Prior RL (2001) Development and validation of an improved oxygen radical absorbance capacity assay using fluorescein as the fluorescent probe. *Journal of Agricultural and Food Chemistry* **49**, 4619-4926

Park YS, Jung ST, Gorinstein S (2006) Ethylene treatment of Hayward kiwifruits during ripening and its influence on ethylene biosynthesis and antioxidant activity. *Scientia Horticulturae* **108**, 22-28

Peck GM, Andrews PK, Reganold JP, Fellman JK (2006) Apple orchard productivity and fruit quality under organic, conventional, and integrated management. *Hortscience* **41**, 99-107

Pretel MT, Botella MA, Zapata PJ, Amoros A, Serrano M (2004) Antioxidative activity and general fruit characteristics in different traditional orange varieties. *European Food Research and Technology* **219**, 474-478

Prior RL, Wu X, Schaich K (2005) Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. *Journal of Agricultural and Food Chemistry* **53**, 4290-4302

Potters G, de Gara L, Asard H, Horemans N (2002) Ascorbate and glutathione: guardians of the cell cycle, partners in crime? *Plant Physiology and Biochemistry* **40**, 537-548

Punchard NA, Kelly FJ (1996) *Free Radicals: A Practical Approach*, Oxford University Press, NY, 310 pp

Re R, Pellegrini N, Proteggente A, Pannala A, Yang M, Rice-Evans C (1999) Antioxidant activity applying and improved ABTS radical cation decolorization assay. *Free Radical Biology and Medicine* **26**, 1231-1237

Rhoads DM, Umbach AL, Subbaiah CC, Siedow JN (2006) Mitochondrial reactive oxygen species. Contribution to oxidative stress and interorganellar signaling. *Plant Physiology* **141**, 357-366

Rice-Evans CA, Miller NJ (1994) Total antioxidant status in plasma and body fluids. *Methods in Enzymology* **234**, 279-293

Serrano M, Guillen F, Martinez-Romero D, Castillo S, Valero D (2005) Chemical constituents and antioxidant activity of sweet cherry at different ripening stages. *Journal of Agricultural and Food Chemistry* **53**, 2741-2745

Serrano M, Martinez-Romero D, Guillen F, Castillo S, Valero D (2006) Maintenance of broccoli quality and functional properties during cold storage as affected by modified atmosphere packaging. *Postharvest Biology and Technology* **39**, 61-68

Shulaev V, Oliver DJ (2006) Metabolic and proteomic markers for oxidative stress. New tools for reactive oxygen species research. *Plant Physiology* **141**, 367-372

Suzuki N, Mittler R (2007) Reactive oxygen species and temperature stresses: a delicate balance between signaling and destruction. *Physiologia Plantarum* **126**, 45-51

van den Berg R, Haenen GRMM, van den Berg H, Bast A (1999) Applicability of an improved Trolox equivalent antioxidant capacity (TEAC) assay for evaluation of antioxidant capacity measurements of mixtures. *Food Chemistry* **66**, 511-517

Verdaguer D, Aranda X, Jofre A, El Omari B, Molinas M, Fleck I (2003) Expression of low molecular weight heat-shock proteins and total antioxidant activity in the Mediterranean tree *Quercus ilex* in relation to seasonal and diurnal changes in physiological parameters. *Plant, Cell and Environment* **26**, 1407-1417

Wayner DDM, Burton GW, Ingold KU, Locke S (1985) Quantitative measurements of the total peroxyl radical-trapping antioxidant capability of human blood plasma by controlled lipid peroxidation. *FEBS Letters* **187**, 33-37

Wright JS, Johson ER, DiLabio GA (2001) Predicting the activity of phenolic antioxidants: theoretical method, analysis of substituent effects, and application to major families of antioxidants. *Journal of the American Chemical Society* **123**, 1173-1183

Wu X, Gu L, Holden J, Haytowitz DB, Gebhardt SE, Beecher G, Prior RL (2004) Development of a database for total antioxidant capacity in foods: a preliminary study. *Journal of Food Composition and Analysis* **17**, 407-422