Naser A. Anjum · Shahid Umar · Ming-Tsair Chan *Editors*
Ascorbate-Glutathione Pathway and Stress Tolerance in Plants

Plants are sessile organisms that live under a constant barrage of biotic and abiotic insults. Both biotic and abiotic stress factors have been shown to affect various aspects of plant system including the acceleration in the formation of reactive oxygen species (ROS). The ascorbate (AsA)-glutathione (GSH) pathway is a key part of the network of reactions involving enzymes and metabolites with redox properties for the detoxification of ROS, and thus to avert the ROS-accrued oxidative damage in plants.
The present book mainly deals with the information gained through the cross-talks and inter-relationship studies on the physiological, biochemical and molecular aspects of the cumulative response of various components of AsA-GSH pathway to stress factors and their significance in plant stress tolerance.
Advanced students, junior researchers and faculty in Plant Stress Physiology/Plant Biochemistry and concerned fields can be benefited with the present edited volume.

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Chapter 5
Regulation of the Ascorbate–Glutathione Cycle in Plants Under Drought Stress

Adriano Sofo, Nunzia Cicco, Margherita Paraggio, and Antonio Scopa

Abstract Acclimation of plants to drought is often associated with increased levels of reactive oxygen species (ROS), such as superoxide anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), hydroxyl radical (HO·) and singlet oxygen ($^1O_2$), which are toxic for the cells. ROS are by-products of aerobic metabolism, and their production is enhanced during drought conditions through the disruption of electron transport system and oxidizing metabolic activities occurring in chloroplasts, mitochondria and microbodies. Under non-stressful conditions, ROS are efficiently eliminated by non-enzymatic and enzymatic antioxidants, whereas during drought conditions the production of ROS exceeds the capacity of the antioxidative systems to remove them, causing oxidative stress. The non-enzymatic antioxidant system includes ascorbate and glutathione, located both within the cell and in the apoplast. They are two constituents of the antioxidative ascorbate–glutathione cycle which detoxify H_2O_2 in the chloroplasts. Ascorbate (AsA) is a major primary antioxidant compound synthesized on the inner membrane of the mitochondria which reacts chemically with $^1O_2$, $O_2^{\cdot -}$, HO· and thiyl radical, and acts as the natural substrate of many plant peroxidases. Moreover, AsA is involved in other functions such as plant growth, gene regulation, modulation of some enzymes, and redox regulation of membrane-bound antioxidant compounds. Glutathione (GSH) is a tripeptide synthesized in the cytosol and chloroplasts which scavenges $^1O_2$ and H_2O_2, and it is oxidized to glutathione disulfide (GSSG) when acts as an antioxidant and redox regulator. GSH is the substrate of glutathione S-transferases, which have a protective role in the detoxification of xenobiotics, and dehydroascorbate reductase (DHAR). Finally, GSH is a precursor of phytochelatins, which regulate cellular heavy metals levels, and is involved in gene expression. This review, based on the most significant studies published in

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the last decade, focuses on the changes of antioxidant enzyme activities (ascorbate peroxidase, APX; monodehydroascorbate reductase, MDHAR; dehydroascorbate reductase, DHAR; glutathione reductase, GR), and of the levels of some compounds involved in the ascorbate–glutathione cycle (ascorbate and glutathione pools , H_2O_2 and α-tocopherol) in plants grown under water shortage.

Keywords Antioxidant enzymes • Ascorbate-glutathione cycle • Ascorbate peroxidase • Dehydroascorbate reductase • Drought stress • Glutathione reductase • Oxidative stress • Water deficit

1 Introduction

Drought stress is one of the main environmental factors limiting plant growth and yield worldwide, and it is the most prevalent cause of crop yield loss but also the most difficult to tackle because of the strong link between transpiration and photosynthesis (Smirnoff 1998; Posch and Bennett 2009). Acclimation of plants to drought is often associated with increased levels of reactive oxygen species (ROS), such as superoxide anion ($O_2\cdot^-$), hydrogen peroxide (H_2O_2), hydroxyl radical (HO·) and singlet oxygen ($^1O_2$), which are toxic for the cells (Smirnoff 1993; Chaves et al. 2003). ROS are by-products of aerobic metabolism and their production is enhanced during drought conditions through the disruption of electron transport system, and oxidizing metabolic activities occurring in chloroplasts, mitochondria and microbodies (Asada 1999; Van Breusegem et al. 2001).

Under non-stressful conditions, ROS are efficiently eliminated by non-enzymatic and enzymatic antioxidants (Fig. 1), whereas during drought conditions the production of ROS exceeds the capacity of the antioxidative systems to remove them, causing oxidative stress (Smirnoff 1998; Morales et al. 2006).

The non-enzymatic antioxidant system includes ascorbate and glutathione, located both within the cell and in the apoplast (Horemans et al. 2000; Foyer et al. 2001). They are two constituents of the antioxidative ascorbate–glutathione cycle which detoxify hydrogen peroxide (H_2O_2) in the chloroplasts (Asada 1999) (Figs. 1 and 2). Ascorbate (AsA) is a major primary antioxidant compound synthesized on the inner membrane of the mitochondria which reacts chemically with $^1O_2$, $O_2\cdot^-$, HO· and thiyl radical (Noctor and Foyer 1998; Asada 1999), and acts as the natural substrate of many plant peroxidases (Mehlhorn et al. 1996). One of the important functions of AsA is the protection against oxidative damage of plant cells through the scavenging of H_2O_2 mediated by ascorbate peroxidase (APX) which has a higher affinity for H_2O_2 than catalase (CAT) or peroxidase isoforms (Srivalli et al. 2003; Mittler and Poulos 2005). In bright light, or when low temperatures and drought limit CO_2 fixation, the excess excitation energy is dissipated in the light harvesting antennae as heat by zeaxanthin, that is formed by successive de-epoxidation of the xanthophyll cycle pigments violaxanthin and antheroxanthin. The deepoxidase, which is bound to the lumen side of the thylakoid membrane, is

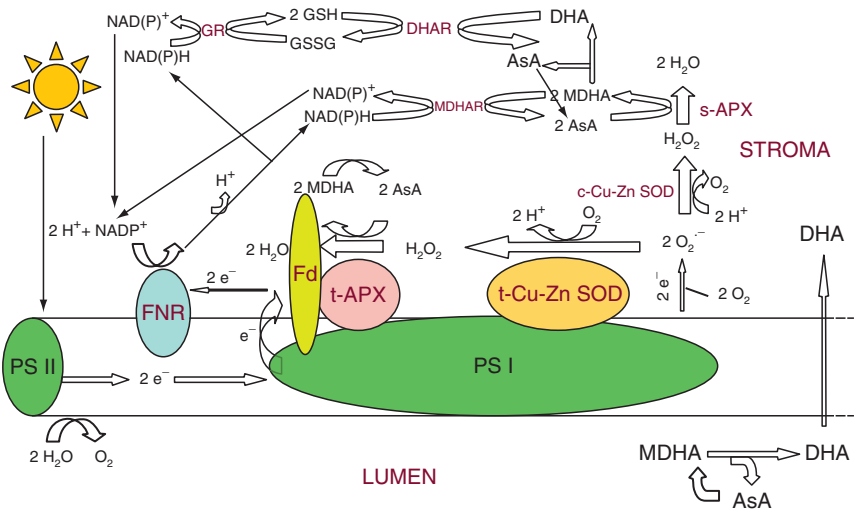

Fig. 1 Antioxidant system of plant chloroplasts.The thylakoidal antioxidant system includes Cu–Zn–superoxide dismutase (t–Cu/Zn–SOD), present on the thylakoidal surfaces (in many plant species, t–Cu–Zn–SOD is substituted by t–Fe–SOD), thylakoidal ascorbate peroxidase (t–APX) and ferredoxin (Fd). Fd reduces monodehydroascorbate (MDHA) directly to ascorbate (AsA). The stromatic antioxidant system is composed by stromatic Cu–Zn–superoxide dismutase (t–Cu/Zn–SOD), stromatic APX (s-APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR) and glutathione reductase (GR). NAD(P)H is used for the reduction of monodehydroascorbate (MDHA), whereas dehydroascorbate (DHA) is photo-generated by ferredoxin-NADP+-oxidoreductase (FNR). MDHA is also produced in chloroplast lumen by violaxantin de-epoxidase or when AsA releases electron to the two photosystems (PS I or PS II). MDHA is rapidly transformed in AsA and DHA. This latter enters the lumen by thylakoidal membranes and is reduced to AsA

dependent on AsA as a cofactor (Smirnoff 2005). Moreover, AsA is involved in other functions such as plant growth, gene regulation, modulation of some enzymes, and redox regulation of membrane-bound antioxidant compounds (Horemans et al. 2000; Foyer et al. 2001). Glutathione (GSH), one of the major redox buffers in most aerobic cells, is a tripeptide synthesized in the cytosol and the chloroplast which scavenges $^1O_2$ and H_2O_2, and is oxidized to glutathione disulfide (GSSG) when acts as an antioxidant and as a regulator of redox status and gene expression (Briviba et al 1997; Smirnoff 1998; Foyer et al. 2001). Furthermore, GSH is the substrate of glutathione S-transferases, which have a protective role in the detoxification of xenobiotics, phospholipid hydroperoxide glutathione peroxidase, that use glutathione to reduce H_2O_2 and lipid hydroperoxides, and dehydroascorbate reductase (DHAR), a key enzyme of the ascorbate–glutathione cycle (Foyer et al. 2001; Yang et al. 2006). Finally, GSH is a precursor of phytochelatins, which regulate cellular heavy metals levels, and is involved in gene expression (Noctor and Foyer 1998). In addition to ascorbate and glutathione, α-tocopherol (α-toc, vitamin E) found in leaf chloroplasts takes part to the ascorbate–glutathione cycle as it deactivates photosynthesis-derived ROS and prevents the propagation of lipid peroxidation by scavenging lipid peroxyl radicals in thylakoid membranes (Munné-Bosch et al. 2001).

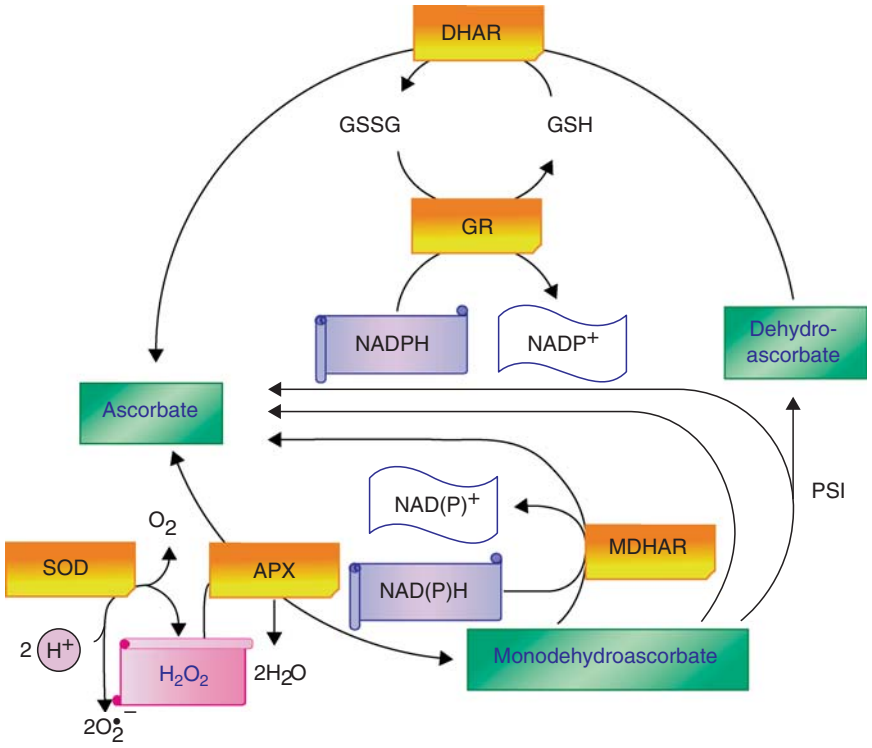

Fig. 2 The ascorbate-glutathione cycle in plants. Ascorbate peroxidase (APX), dehydroascorbate reductase (DHAR), monodehydroascorbate reductase (MDHAR), and glutathione reductase (GR)

Successively, the scavenging of lipid peroxyl radicals results in the formation of tocopheroxyl radicals, which can be recycled back to α-tocopherol by ascorbate.

The enzymatic antioxidant system, that operates both in the chloroplasts and in cytosol, includes the enzymes of the ascorbate–glutathione cycle: ascorbate peroxidase (APX, EC 1.11.1.11), monodehydroascorbate reductase (MDHAR, EC 1.6.5.4), dehydroascorbate reductase (DHAR, EC 1.8.5.1) and glutathione reductase (GR, EC 1.6.4.2) (Figs. 1 and 2). Activities of enzymes in the ascorbate glutathione cycle are increased by drought and low temperature suggesting a requirement for increased activity of the cycle under these conditions (Smirnoff 1996). Furthermore, the mRNAs corresponding to the genes of antioxidant enzymes are induced by drought stress (Reddy et al. 2004b). Ascorbate peroxidise isozymes, able to scavenge the H_2O_2 produced by SOD using ascorbate as the electron donor, are generally located in chloroplasts, but microsomal, peroxisomal and membrane-bound forms, as well as soluble cytosolic and apoplastic isozymes, also exist (Quan et al. 2008). Moreover, APX can scavenge H_2O_2 that is inaccessible for CAT because of their higher affinity for H_2O_2 and presence in different subcellular locations (Van Breusegem et al. 2001). Monodehydroascorbate (MDHA), a free radical intermediate produced by APX catalysis, can disproportionate spontaneously to AsA and

dehydroascorbate (DHA) or be enzymatically reduced to AsA by MDHAR, a FAD enzyme with an high specificity to MDHA which uses NAD(P)H as a reductant (Smirnoff 2005). DHAR is a monomeric thiol enzyme that reduces DHA to AsA using GSH as an electron donor, with the consequent production of GSSG (Foyer et al. 2005). DHAR has been frequently implied as a biochemical indicator of oxidative stress in plant metabolism (Vadassery et al. 2009) but a characterization of DHAR has remained elusive because of rapid loss of enzyme activity. The isoforms of GR are flavoenzymes with a redox cystine residue in their active sites which maintain the intracellular glutathione pool in the reduced status, catalysing the NADPH-dependent reduction of GSSH to GSH (Foyer et al. 2005). Morell et al. (1997) had tried to demonstrate that the regeneration of AsA is not coupled to a glutathione-dependent DHAR, and that GR is not directly involved in the regeneration of AsA but Foyer and Mullineaux (1998) and many successive works definitively proved that both DHAR and GR have a key role against oxidative stress.

Excessive levels of ROS damage cellular structures and macromolecules, causing photoinhibition of photosynthetic apparatus, but also activate multiple defence responses, thus having also a positive role (Van Breusegem et al. 2001; Vranová et al. 2002; Foyer and Noctor 2003; Laloi et al. 2004) (Fig. 3). This dualism

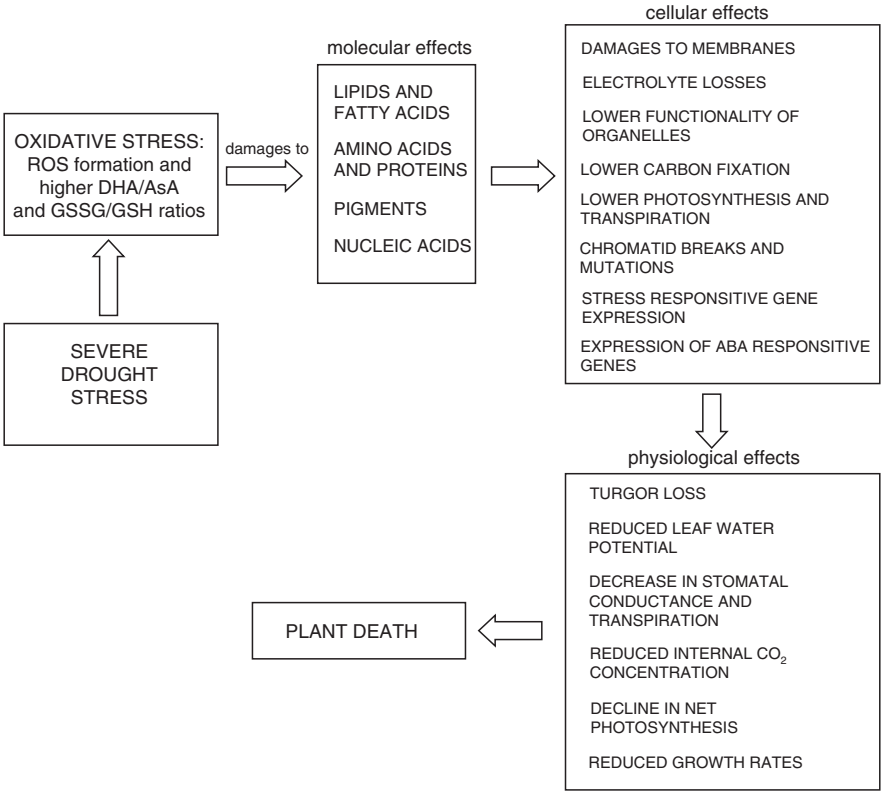

Fig. 3 Molecular and cellular effects of drought-mediated oxidative stress

can be obtained only when cellular levels of ROS are tightly controlled at both the production and consumption levels (Van Breusegem et al. 2001; Quan et al. 2008). Foyer and Noctor (2005) highlighted the crucial role of ROS as second messengers in signal transduction cascades in processes as diverse as mitosis, tropisms and cell death. In particular, the presence of H_2O_2 in the apoplast is toxic for pathogens, is involved in gene transcription and systemic acquired resistance, and slows down the spread of invading organisms by cell death round the infection and a rapid local cross-linking of the cell wall (Horemans et al. 2000; Smirnoff 2000). Other two major low molecular weight antioxidants, such as ascorbate and glutathione determine the specificity of the transduced signal in cells, and are also themselves signal-transducing molecules that can either signal independently or further transmit ROS signals (Foyer and Noctor 2005). For all these reasons, in contrast to this pejorative or negative term, implying a state to be avoided, the presence of ROS in cellular apparatus would be more usefully described as 'oxidative signalling', that is, an important and critical function associated with the mechanisms by which plant cells sense the environment and make appropriate adjustments to gene expression, metabolism and physiology.

The response to water deficit of plant species is a well documented process but relatively few studies highlighted the importance of the enzymes of ascorbate–glutathione cycle associated to drought tolerance and/or resistance, and not much is known about the linkages between drought and the components of the ascorbate–glutathione cycle in some economically important C_3 plant species (e.g., fruit trees) (Scebba et al. 2001; Lima et al. 2002; Chai et al. 2005; Pinheiro et al. 2004; Sofo et al. 2005b; Guerfel et al. 2009). For these reasons, the aim of this work is to give an up-to-date overview of the studies on the changes of antioxidant enzyme activities (APX, MDHAR, DHAR and GR), and of the levels of some compounds involved in the ascorbate–glutathione cycle (ascorbate and glutathione pools, AsA/DHA and GSH/GSSG redox couples, H_2O_2 and α-toc) in plants grown under water shortage. Some of the significant changes in enzymatic and non-enzymatic antioxidants of the ascorbate–glutathione cycle in drought-stressed plants have been summarized in Table 1.

2 Changes in Enzyme Activities and Pools of Non-enzymatic Antioxidants in Drought-Stressed Plants

2.1 Tree Species

Plants are sessile organisms and their only alternative to a rapidly changing environment is a fast adaptation to the abiotic and biotic stresses. This concept is particularly valid for the physiological and biochemical responses (adaptation, avoidance, resistance or tolerance) against water deficit, among which there are the

Table 1 Changes in enzymatic and non-enzymatic antioxidants of the ascorbate–glutathione cycle in drought-stressed plants

No.	Species	Level of drought stress	Antioxidant	Tissue	Change in response of drought stress	Reference
1	*Acer saccharinum*	RWC from 55% to 28%	AsA	Embryo	From 25 to 33 μmol g^{-1} DW	Pukacka and Ratajczak 2006
			DHA		From 45 to 52 μmol g^{-1} DW	
			AsA/DHA		From 4.5 to 2.5	
			GSH		From 0 to 1,100 mmol g^{-1} DW	
			GSSG		From 600 to 1,450 mmol g^{-1} DW	
			GSH/GSSG		From 7.8 to 3.6	
			H_2O_2	Seeds	From 1.04 to 2.58 μg g^{-1} DW	
			$O_2 \cdot^-$		From 0.71 to 1.27 ΔA_{530} g^{-1} DW	
2	*Allium schoenoprasum*	RWC from 70.7% to 53.2%	APX	Leaves	From 1.60 to 2.06 units mg^{-1} protein	Egert and Tevini 2002
3	*Anoda cristata*	6 days of water withholding	APX	Leaves	From 1.1 to 1.3 units mg^{-1} protein	Ratnayaka et al. 2003
4	*Arabidopsis thaliana*	Leaves Ψ_w from −0.65 up to −2.54 MPa	GR	Young leaves	From 0.21 to 0.24 μmol min^{-1} mg^{-1}	Jung 2004
				Mature leaves	From 0.21 to 0.34 μmol min^{-1} mg^{-1}	
5	*Arbutus unedo*	RWC from 83% to 53%	t-Asc	Leaves	From 18 to 30 μmol g DW^{-1}	Munné-Bosch and Peñuelas 2004
			DHA/t-Asc		From 0.09 to 0.14	
6	*Bupleurum chinense*	RWC from 93.02% to 45.78%	APX	Roots	From 19257 to 33262 μmol Vc g^{-1} FW h^{-1}	Zhu et al. 2009
			AsA		From 19.43 to 43.32 μg g^{-1} FW	
			H_2O_2		From 0.35 to 0.95 μmol g^{-1} FW	
7	*Capparis ovata*	RWC from 83.13% to 76.29%	APX	Leaves	From 0.2 to 3.7 units mg^{-1} protein	Ozkur et al. 2009
			GR		From 0.18 to 0.55 units mg^{-1} protein	

(continued)

Table 1 (continued)

No.	Species	Level of drought stress	Antioxidant	Tissue	Change in response of drought stress	Reference
8	*Catharanthus roseus* 'Rosea'	20 days interval drought	APX	Leaves	From 39 to 44 units mg^{-1}	Jaleel et al. 2008a, b
				Roots	From 28 to 32 units mg^{-1}	
			AsA	Roots	From 7 to 9 mg g^{-1} FW	
			GSH		From 8 to 9 mg g^{-1} FW	
			α-toc		From 13 to 15 mg g^{-1} FW	
	Catharanthus roseus 'Alba'	20 days interval drought	APX	Leaves	From 35 to 38 units mg^{-1}	
				Roots	From 20 to 24 units mg^{-1}	
			AsA	Roots	From 11 to 13 mg g^{-1} FW	
			GSH		From 9 to 11 mg g^{-1} FW	
			α-toc		From 12 to 14 mg g^{-1} FW	
9	*Cistus clusii*	RWC from 82% to 64%	t-Asc	Leaves	From 38 to 94 μmol dm^{-1} leaf surface	Hernández et al. 2004
			α-toc		From 120 to 400 nmol dm^{-1}	
10	*Coffea canephora*	Predawn Ψ_w = −3.0 MPa	APX	Leaves	From 0.3–0.5 to 0.5–0.7 μmol AsA min^{-1} mg^{-1} protein	Pinheiro et al. 2004
			DHAR		From 0.020–0.025 to 0.025–0.030 μmol NADPH min^{-1} mg^{-1} protein	
			t-Asc		From 28 to 30 μmol g^{-1} DW	
11	*Coffea canephora*	Predawn Ψ_w = −3.0 MPa	APX	Leaves	From 0.31–0.42 to 0.83–1.34 units mg^{-1} protein	Lima et al. 2002

12	*Cucumis sativus*	PEG solution 10% (w/v) for 3 days	APX	Leaves	From 125 to 170 units g^{-1} DW	Liu et al. 2009
			MDHAR		From 2,150 to 2,650 units g^{-1} DW	
			DHAR		From 1,000 to 1,100 units g^{-1} DW	
			GR		From 200 to 300 units g^{-1} DW	
			AsA		From 1,500 to 1,700 mg g^{-1} DW	
			AsA/DHA		From 3 to 4	
			GSH		From 200 to 650 μg g^{-1} DW	
			GSH/GSSG		From 20 to 40	
			H_2O_2		From 30 to 85 μmol g^{-1} DW	
			$O_2 \cdot^-$		From 40 to 90 nmol min^{-1} g^{-1} DW	
13	*Eucalyptus globules* – clone 'ST5'	Predawn Ψ_w = –2.43 MPa	APX	Roots	From 0.22 to 0.95 mmol H_2O_2 g^{-1} DW min^{-1}	Shvaleva et al. 2005
			GR	Leaves	From 8.2 to 3.9 mmol H_2O_2 g^{-1} DW min^{-1}	
				Roots	From 0 to 8.2 H_2O_2 g^{-1} DW min^{-1}	
14	*Eucalyptus globules* – clone 'CN5'	Predawn Ψ_w = –1.71 MPa	APX	Leaves	From 27 to 7 mmol H_2O_2 g^{-1} DW min^{-1}	
				Roots	From 0.19 to 1.30 mmol H_2O_2 g^{-1} DW min^{-1}	
			GR	Leaves	From 15 to 2 mmol H_2O_2 g^{-1} DW min^{-1}	
				Roots	From 0 to 3.4 H_2O_2 g^{-1} DW min^{-1}	
15	*Fagus sylvatica*	SWC from 30% to 15%	t-Asc	Leaves	From about 6 to 12 μmol g^{-1} DW	Haberer et al. 2008
			α-toc		From 100 to 150 nmol g^{-1}	
			AsA		From 0.54–0.94 to 2.56–3.18 mg g^{-1} FW	
			MDHA		From 0.25–0.39 to 1.12–1.48 mg g^{-1} FW	

(continued)

Table 1 (continued)

No.	Species	Level of drought stress	Antioxidant	Tissue	Change in response of drought stress	Reference
16	*Glycine max*	Predawn Ψ_w = –1.0 MPa + chilling	APX	Leaves	From 10.7 to 27.8 μmol min^{-1} mg^{-1} protein	Riekert van Heerden and Krüger 2002
			GR		From 1.9 to 4.0 μmol min^{-1} mg^{-1} protein	
17	*Gossypium hirsutum*	6 days of water withholding	APX	Leaves	From 0.8 to 1.7 units mg^{-1} protein	Ratnayaka et al. 2003
18	*Helianthus anuus*	RWC from 90% to 40%	APX	Leaves	From 110 to 130 nmol min^{-1} mg^{-1} protein	Zhang and Kirkham 1996a, b
			DHAR		From 5 to 27 nmol min^{-1} mg^{-1} protein	
			GR		From 23 to 35 nmol min^{-1} mg^{-1} protein	
			AsA		From 5 to 1 mg g^{-1} DW	
			DHA		From 0.0 to 0.2 mg g^{-1} DW	
			GSH		From 2.2 to 0.0 mg g^{-1} DW	
			GSSG		From 0.00 to 0.38 mg g^{-1} DW	
19	*Laurus azorica*	RWC from 95% to 50–55%	APX	Leaves	From 1.26 to 7.08 μmol m^{-2} s^{-1}	Sánchez-Díaz et al. 2007
20	*Licopersicum esculentum*	RWC from 95% to 65%	APX	Leaves	From 2.2 to 5.9 units mg^{-1} protein	Nasibi and Kalantari 2009
			GR		From 0.100 to 0.075 units mg^{-1} protein	
			AsA		From 4.84 to 1.79 mg g^{-1} DW	
			DHA		From 0.95 to 2.37 mg g^{-1} DW	
			t-Glu		From 0.19 to 0.05 mmol g^{-1} DW	
			H_2O_2		From 21.05 to 127.88 μmol g^{-1} DW	
21	*Ligustrum vulgare*	Predawn Ψ_w = –0.8 MPa	APX	Leaves	From about 8 to 14 mol min^{-1} g FW^{-1}	Guidi et al. 2008
22	*Malus domestica*	Predawn Ψ_w = –2.0 MPa	AsA	Leaves	From 5.49 to 7.44 mg g^{-1} DW	Šircely et al. 2005, 2007
			GSSG/t-Glu		From 8.9 to 39.2	

23	*Medicago sativa*	PEG solution 35% (w/v) during germination	APX	Shoots	From about 2 to 8 units mg^{-1} protein	Wang et al. 2009
				Roots	From about 3 to 6 units mg^{-1} protein	
			H_2O_2	Shoots	From about 0.3 to 0.5 mol g^{-1} FW	
24	*Morus alba*	Ψ_w = −2.50 MPa	APX	Leaves	From 450–700 to 900–1,500 µmol mg^{-1} chl min^{-1}	Ramachandra Reddy et al. 2004b
			MDHAR		From 180–200 to 420–870 µmol mg^{-1} chl min^{-1}	
			GR		From 160–300 to 280–450 µmol mg^{-1} chl min^{-1}	
25	*Musa* AAA 'Berangan'	LWC from 93% to 72–75%	APX	Leaves	From 46.28 to 67.64 nmol AsA s^{-1} mg^{-1} protein	Chai et al. 2005
			GR		From 0.65 to 1.01 nmol NADPH s^{-1} mg^{-1} protein	
26	*Musa* AA 'Mas'	LWC from 93% to 72–75 %	APX	Leaves	From 43.73 to 44.91 nmol AsA s^{-1} mg^{-1} protein	
			GR		From 1.26 to 1.93 nmol NADPH s^{-1} mg^{-1} protein	
27	*Nicotiana tabacum*	4 weeks of a water-deficit cycle	GR	Leaves	From 500–600 to 100–300 units g^{-1} protein	Synková and Valcke 2001
28	*Olea europaea* 'Coratina'	Predawn Ψ_w = −5.73 MPa	APX	Leaves	From 3.88 to 13.77 units mg^{-1} DW	Sofo et al. 2005a
				Roots	From 0.23–0.36 to 0.34–0.51 units mg^{-1} DW	
29	*Olea europaea* 'Chemlali'	RWC from 95% to 40%	APX	Leaves	From about 0.3 to 1.4 units mg^{-1} protein	Ennajeh et al. 2009
30	*Olea europaea* 'Meski'	RWC from 95% to 40%	APX	Leaves	From about 0.3 to 1.8 units mg^{-1} protein	

(continued)

Table 1 (continued)

No.	Species	Level of drought stress	Antioxidant	Tissue	Change in response of drought stress	Reference
31	*Olea europaea* 'Chemlali'	Leaves Ψ_w at 9:30 h = –4.10 MPa	APX	Leaves	From 23 to 34 nmol AsA mg^{-1} $proteins^{-1}$	Guerfel et al. 2009
			GR		From 0.3 to 0.7 nmol NADPH mg^{-1} $proteins^{-1}$	
32	*Olea europaea* 'Chétoui'	Leaves Ψ_w at 9:30 h = –5.51 MPa	GR	Leaves	From 0.6 to 1.0 nmol NADPH mg^{-1} $proteins^{-1}$	
33	*Oryza sativa*	Leaves Ψ_w at –3.24 MPa at 10.30 h	APX	Leaves	From 0.18 to 0.60 μmol AsA min^{-1} mg^{-1} protein	Srivalli et al. 2003
			GR		From 0.023 to 0.078 umol NADPH min^{-1} mg^{-1} protein	
			AaA/DHA		From 6.57 to 1.00	
			AsA		From 10.18 to 5.24 μmol g DW^{-1}	
34	*Oryza sativa* 'Xiangnuo no. 1' and 'Zimanuo'	PEG-6000 solution 23% (w/v) for 7 days	APX	Leaves	From about 11 to about 6 units g^{-1} DW	Guo et al. 2006
			AsA		From about 22 to 11–13 μmol g^{-1} DW	
			GSH		GSH from about 11 to 4–5 μmol g^{-1} DW	
			H_2O_2		From about 2.0 to about 4.0–6.0 μmol g^{-1} DW	
35	*Oryza sativa* 'Xiangzhongxian no. 2' and 'IR50'	PEG-6000 solution 23% (w/v) for 7 days	APX	Leaves	From about 11 to about 13 units g^{-1} DW	
			AsA		From about 22 to 24 μmol g^{-1} DW	
			GSH		GSH from about 11 to 12 μmol g^{-1} DW	
			H_2O_2		From about 1.5 to about 3.0 μmol g^{-1} DW	
36	*Picea asperata*	RWC from 76.3–87.7% to 65.9–66.1%	APX	Leaves	From 6.61–6.89 to 9.30–10.60 units g^{-1} FW	Duan et al. 2005

37	*Picea asperata*	Field capacity from 100% to 30%	APX	Leaves	From 0.30 to 0.71 μmol min^{-1} mg^{-1} protein	Yang et al. 2008
			GR		From 0.39 to 1.14 μmol min^{-1} mg^{-1} protein	
			t-Asc		From 1.44 to 1.61 mg g^{-1} FW	
			H_2O_2		From 8.50 to 15.70 μmol g^{-1} FW	
			GR		From 53.8 to 156.4 μmol m^{-2} s^{-1}	
			t-Asc/(t-Asc + DHA)		From 0.6 to 0.7	
38	*Pinus canariensis*	Ψ_w at noon = –0.44 MPa	GSSG/t-Glu	Leaves	From 10% to 20%	Tausz et al. 2001
39	*Pisum sativum*	Predawn Ψ_w = −1.0 MPa	APX	Leaves	From 0.13 to 0.48 μmol ascorbate min^{-1} mg^{-1} protein	Zabalza et al. 2008
			GR		From 0.063 to 0.068 μmol NADH min^{-1} mg^{-1} protein	
40	*Poa pratensis*	RWC from 95% to 68%	APX	Leaves	From 900 to 1,500 nmol min^{-1} mg^{-1} protein	Bian and Jiang 2009
			MDHAR		From 65 to 150 nmol min^{-1} mg^{-1} protein	
				Roots	From 260 to 360 nmol min^{-1} mg^{-1} protein	
			DHAR	Leaves	From 60 to 180 nmol min^{-1} mg^{-1} protein	
				Roots	From 275 to 150 nmol min^{-1} mg^{-1} protein	
			GR		From 75 to 110 nmol min^{-1} mg^{-1} protein	
			H_2O_2	Leaves	From 2.2 to 3.5 μmol g^{-1} FW	
				Roots	From 1.1 to 2.0 μmol g^{-1} FW	

(continued)

Table 1 (continued)

No.	Species	Level of drought stress	Antioxidant	Tissue	Change in response of drought stress	Reference
41	*Poncirus trifoliata*	RWC from 75% to 55%	APX	Roots	From 3.98 to 5.63 units mg^{-1} FW	Wu et al. 2006
			GR	Leaves	From 8.81 to 9.52 units mg^{-1} FW	
			t-Asc		From 9.32 to 6.04 μmol g^{-1} FW	
			GSH		From 2.30 to 1.43 μmol g^{-1} FW	
			H_2O_2		From 105.94 to 139.02 μmol g^{-1} FW	
42	*Populus kangdingensis*	Field capacity from 100% to 50%	APX	Leaves	From 1.27 to 1.73 μmol H_2O_2 min^{-1} g^{-1} FW	Ren et al. 2007
	Populus cathayana	Field capacity from 100% to 50%	APX	Leaves	From 0.30 to 0.41 μmol H_2O_2 min^{-1} g^{-1} FW	
43	*Populus euramericana*	$\Delta\Psi_w = -0.224$ MPa	c-APX	Leaves	From 140 to 195 nmol ascorbate oxidized min^{-1} mg^{-1} protein	Edjolo et al. 2001
44	*Populus przewalskii*	Field capacity from 100% to 25%	APX	Leaves	From 8 to 27 μmol AsA min^{-1} mg^{-1} protein	Lei et al. 2006
			GR		From 0.6 to 2.1 μmol NADH min^{-1} mg^{-1} protein	
			AsA		From about 350 to about 900 μg g^{-1} DW	

45	*Prunus spp.*	Predawn Ψ_w = −3.30 MPa	APX	Leaves	From 0.1–0.7 to 1.6–2.3 units mg^{-1} protein	Sofo et al. 2005b
			MDHAR		From 50–130 to 220–550 units mg^{-1} protein	
			DHAR		From 45–60 to 120–170 units mg^{-1} protein	
			GR		From 45–55 to 60–200 units mg^{-1} protein	
			AsA		From about 0.06 to 0.12–0.17 μmol g^{-1} FW	
			DHA		From about 0.50 to 1.25–1.80 μmol g^{-1} FW	
			GSH		From 0.18–0.28 to 0.23–0.46 μmol g^{-1} FW	
			H_2O_2		From about 0.025 to 0.125–0.150 μmol g^{-1} FW	
46	*Salvia officinalis*	RWC from 67% in June to 32% in August	α-toc	Leaves	From 24 to 14 μg g DW^{-1}	Munné-Bosch et al. 2001
47	*Solanum tuberosum*	RWC from 90% to 70%	GSSG/t-Glu	Leaves	From 19 to 30	Broin et al. 2000
48	*Sorghum bicolor*	RWC from 90% to 50%	APX	Leaves	From 100 to 280 nmol min^{-1} mg^{-1} protein	Zhang and Kirkkham 1996a, b
			MDHAR		From 10 to 50 nmol min^{-1} mg^{-1} protein	
			DHAR		From 1 to 5 nmol min^{-1} mg^{-1} protein	
			GR		From 14 to 45 nmol min^{-1} mg^{-1} protein	
			AsA		From 2 to 4 mg g^{-1} DW	
			DHA		From 0.3 to 0.5 mg g^{-1} DW	
			GSH		From 1.8 to 2.5 mg g^{-1} DW	
			GSSG		From 0.35 to 0.18 mg g^{-1} DW	

(continued)

Table 1 (continued)

No.	Species	Level of drought stress	Antioxidant	Tissue	Change in response of drought stress	Reference
49	*Triticum aestivum*	8 days of water withholding	APX	Leaves	From 160–230 to 210–370 μmol AsA min^{-1} g^{-1} FW	Sairam and Saxena 2000
			GR		From 0.7–2.2 to 1.8–3.8 μmol A_{412} min^{-1} g^{-1} FW	
50	*Triticum aestivum*	RWC from 82–91% to 61–68%	GR	Leaves	From 2.0–5.5 to 8.5–10.5 ΔA_{412} min^{-1} mg^{-1} protein	Sairam and Srivastava 2001
			t-Asc		From 27–54 to 16–27 μmol g^{-1} DW	
			H_2O_2		From 2.3–3.4 to 2.6–3.7 μmol g^{-1} DW	
51	*Triticum aestivum*	PEG-6000 solution 10% (w/v) for 10 days	APX	Leaves	From 0.5 to 1.2 units mg^{-1} protein	Qiu et al. 2008
			t-Asc		From 8.3 to 6.7 mg g^{-1} FW	
			GSSG		From 75 to 70 μg g^{-1} FW	
			H_2O_2		From 37 to 32 μmol g^{-1} FW	
52	*Triticum aestivum*	Predawn Ψ_w = −1.49 MPa	GR	Leaves	From 75 to 63 nmol mg^{-1} protein min^{-1}	Gong et al. 2005
			H_2O_2		From 5.3 to 7.0 μmol g^{-1} DW	
53	*Triticum aestivum*	Ψ_w = −0.5 MPa	APX	Leaves	From 15 to 30 μmol g^{-1} DW	Tian and Lei 2007
			H_2O_2		From 0.25 to 0.35 μmol H_2O_2 mg^{-1} protein min^{-1}	
54	*Triticum aestivum*	Predawn Ψ_w = −1.5 MPa	APX	Leaves	From 0.10 to 0.15 μmol AsA s^{-1} g^{-1} FW	Nayyar and Gupta 2006
			DHAR		From 0.4 to 0.7 μmol min^{-1} g^{-1} FW	
			GR		From 0.5 to 0.7 μmol min^{-1} g^{-1} FW	
			t-Asc		From 8 to 12 μmol g^{-1} DW	
			GSH		From 300 to 420 μmol g^{-1} DW	
			H_2O_2		From 10 to 35 μmol g^{-1} DW	
			APX	Roots	From 0.13 to 0.22 μmol AsA s^{-1} g^{-1} FW	
			DHAR		From 0.5 to 0.7 μmol min^{-1} g^{-1} FW	
			GR		From 0.7 to 0.9 μmol min^{-1} g^{-1} FW	

55	*Triticum aestivum*	PEG-6000 solution 10% (w/v) for 10 days + laser pre-treatment	APX	Leaves	From 0.5 to 1.2 Units mg^{-1} protein	Qiu et al. (2008)
56	*Triticum aestivum* 'Moti'	Leaves Ψ_w from −1.40 to −1.65 up to −2.40 MPa (acclimated)	H_2O_2	Leaves	From about 2.0–2.5 to 4.0 μmol g^{-1} DW	Khanna-Chopra and Selote 2007
		Leaves Ψ_w from −1.40 and then directly −2.40 MPa (non-acclimated)	H_2O_2	Leaves	From about 2.0–2.5 to 4.5 μmol g^{-1} DW	
	Triticum aestivum 'C306'	Leaves Ψ_w from −1.40 to −1.65 up to −2.40 MPa (acclimated)	MDHAR DHAR H_2O_2	Leaves	From 25 to 40 μmol g^{-1} DW From 20 to 25 μmol g^{-1} DW From about 2.0–2.5 to 3.0 μmol g^{-1} DW	
		Leaves Ψ_w from −1.40 and then directly −2.40 MPa (non-acclimated)	APX GR H_2O_2	Leaves	From 190 to 280 μmol g^{-1} DW From 35 to 70 μmol g^{-1} DW From about 2.0–2.5 to 4.5 μmol g^{-1} DW	
57	*Triticum aestivum* and *Triticum durum*	6 days of water withholding	GR α-toc t-Asc GSH H_2O_2	Leaves	From 2–5 to 4–8 μmol $NADPH_2$ min^{-1} From 600–800 to 900–1,200 μg g^{-1} FW From 9 to 15 μmol g^{-1} DW From 380 to 450 μmol g^{-1} DW From 12 to 43 μmol g^{-1} DW	Keleş and Öncel 2002
58	*Trifolium repens*	RWC from 80% to 60%	APX GR	Leaves	From about 20 to 60 μg g^{-1} DM min^{-1} From about 20 to 50 μg g^{-1} DM min^{-1}	Bermejo et al. 2006
59	*Vaccinium myrtillus*	Environmental drought stress	GR	Leaves	from 0.8–1.3 pkat g^{-1} DW in June to 0.2–0.4 pkat g^{-1} DW in December	Tahkokorpi et al. 2007

(continued)

Table 1 (continued)

No.	Species	Level of drought stress	Antioxidant	Tissue	Change in response of drought stress	Reference
60	*Zea mays*	Predawn $\Psi_w = -1.5$ MPa	APX	Leaves	From 0.14 to 0.23 μmol AsA s^{-1} g^{-1} FW	Nayyar and Gupta 2006
			DHAR		From 0.4 to 0.6 μmol min^{-1} g^{-1} FW	
			GR		From 0.6 to 0.8 μmol min^{-1} g^{-1} FW	
			t-Asc		From 9 to 15 μmol g^{-1} DW	
			GSH		From 280 to 450 μmol g^{-1} DW	
			H_2O_2		From 8 to 26 μmol g^{-1} DW	
			APX	Roots	From 0.13 to 0.32 μmol AsA s^{-1} g^{-1} FW	
			DHAR		From 0.5 to 1.6 μmol min^{-1} g^{-1} FW	
			GR		From 0.7 to 1.5 μmol min^{-1} g^{-1} FW	
			t-Asc		From 9 to 15 μmol g^{-1} DW	
			GSH		From 380 to 530 μmol g^{-1} DW	
			H_2O_2		From 12 to 31 μmol g^{-1} DW	
61	*Zea mays*	RWC from 95% to 70%	AsA	Leaves	From 260 to 0 μmol m^{-2}	Aroca et al. 2003
			t-Glu		From 2 to 5–6 μmol m^{-2}	
			H_2O_2		From about 50 to 90–13 μmol m^{-2}	
62	*Zea mays*	PEG-6000 solution 20% (w/v) for 2 days	APX	Leaves	From 47 to 55 nmol min^{-1} mg^{-1} protein	Rapala-Kozik et al. 2008
			GR		From 36 to 78 nmol min^{-1} mg^{-1} protein	
			α-toc		From 1.3 to 2.0 μmol g DW^{-1}	

Abbreviations α-toc, α-tocopherol; APX, ascorbate peroxidase; AsA, reduced ascorbate; c-APX, cytosolic ascorbate peroxidase; DHA, dehydroascorbate; DHAR, dehydroascorbate reductase; DW, dry weight; FW, fresh weight; GR, glutathione reductase; GSH, reduced glutathione; GSSG, oxidized glutathione; MDHAR, monodehydroascorbate reductase; t-Asc, total ascorbate; t-Glu, total glutathione

antioxidant defenses (Foyer et al. 2005; Smirnoff 2005; Morales et al 2006). Trees carry on the same processes as other seed plants, but their larger size, slower maturation, and much longer life accentuate their susceptibility to drought-mediated oxidative stress in comparison to smaller plants having a shorter life span (Pallardy 2008). For this reason, the antioxidant response of woody plants is of key importance and will be herein discussed in detail.

Among tree species, poplar (*Populus* spp.) is a model plant for its economic importance and relative short life cycle. Therefore, poplar genome was entirely sequenced and the antioxidative responses to abiotic stresses were studied in detail. Cuttings of *Populus kangdingensis* and *P. cathayana* originating from high and low altitudes in south-west China, respectively, were used to determine the effect of drought and enhanced UV-B radiation [daily UV-B supplementation = 4.4 kJ m^{-2} day^{-1} ($UV\text{-}B_{BE}$)] and their combination on plant growth and physiological traits in a greenhouse during one growing season (Ren et al. 2007). In both species, cuttings grown under drought conditions exhibited reduced growth. Drought and enhanced UV-B radiation, separately or together, significantly reduced plant growth, and increased APX activity. As higher APX activity was observed in *P. kangdingensis* when compared to *P. cathayana*, an interesting adaptative effect was observed by the authors: *P. kangdingensis*, originating from high altitude exhibited greater tolerance to drought and enhanced UV-B radiation than did *P. cathayana* originating from lower altitude. Lei et al. (2006) found that in a dry climate-adapted population of *Populus przewalskii* Maximowicz exposed to three different watering regimes, drought significantly induced the entire set of antioxidative systems including the increase of AsA content and the activities of APX, and GR. Poplar trees under drought stress were choosen to determine the presence and the activities of cytosolic and plastidial forms of some enzymes of the ascorbate–glutathione cycle, in order to test the ROS-scavenging system in this species. For this purpose, Edjolo et al. (2001) determined APX and GR activities in a drought-tolerant *Populus euramericana* clone (Dorskamp). Because ROS were mainly generated in illuminated chloroplasts, cytosolic and chloroplastic APX and GR were followed in seedlings exposed for 12 h to control or 100 mmol L^{-1} mannitol. Whatever the treatment, the activities of plastidial APX and GR were lower than those of cytosolic fractions (140–200 and 10–60 nmol ascorbate oxidized min^{-1} mg^{-1} protein for APX in cytosol and chloroplasts, respectively; 10–20 and 5–7 nmol NADPH oxidized min^{-1} mg^{-1} protein for GR for APX in cytosol and chloroplasts, respectively). Mannitol treatment significantly increased cytosolic APX activity. The direct linear plot of 1/V against 1/S (where V is the velocity and S is the substrate concentration in AsA, GSSG, and NADPH) was used to estimate the apparent K_m values of APX and GR. In stressed plants, the apparent K_m value for AsA decreased for both APX isoforms (this indicates an increased affinity for AsA in both cell compartments), while K_m for GSSG and NADPH increased for GR isoforms, so demonstrating the different behaviors of the two enzymes observed in cytosolic and chloroplastic subcellular compartments.

The distinction of oxidative stress levels in different of tree species, and in particular in roots, is quite rare to find in the past and recent literature. This is likely

due to the difficulty of sampling metabolically active roots of the trees (usually fine roots, with a diameter <1 mm) and maintaining uniform conditions in the soils, and to the presence of interfering substances for enzyme isolation and enzyme activities determination. In a work on *Eucalyptus globulus* Labill., Shvaleva et al. (2005) compared the metabolic responses of leaves and roots of two clones differing in drought sensitivity to a slowly 7-week imposed water deficit. In addition to the general decrease in growth caused by water deficit, a decrease in osmotic potential was observed at severe drought stress. In both clones, these decreases were greater in roots than in leaves, consistent with the observed increases in concentrations of soluble sugars and proline in these organs. Leaf GR activity in both clones was significantly ($P < 0.001$) decreased by drought stress. The water-stress treatment also decreased APX activity, but only in CN5. In roots, the effects of drought stress on antioxidant enzymatic activities were more marked than in leaves. The activity of APX and GR in roots increased significantly ($P < 0.001$) in both the clones in response to drought stress. Some of the molecular and cellular effects of drought-mediated oxidative stress have been outlined in the Fig. 3.

The enzymatic antioxidative response of tree species having a high degree of tolerance against drought, such as scleropyllous Mediterranean species or xerophytic boreal conifers, is particularly interesting. Duan et al. (2005) examined the responses of photosynthetic gas exchange, chlorophyll fluorescence, activities of antioxidant enzymes, and lipid membrane peroxidation of two contrasting *Picea asperata* Mast. populations to 30% of full sunlight (shade conditions obtained by neutral shade clothes; photosynthetically active radiation, PAR = 300 μmol m^{-2} s^{-1}), and full sunlight (PAR = 900 μmol m^{-2} s^{-1}) were investigated under well-watered and drought conditions. The two contrasting populations came from the wet and dry climate regions in China, respectively. For both populations tested, drought resulted in lower needle relative water content (RWC), gas exchange and photosynthetic efficiency, SOD and APX activities as well as malondialdehyde (MDA) levels and electrolyte leakage in sun plants, whereas these changes were not significant in shade plants. In particular, in the population from the wet climate, APX activity in sun plants increased less than in the population from the dry climate. No significant differences were observed between control and drought-stressed shaded plants in both the populations. This physiological and biochemical response is in accordance to the data of Sofo et al. (2004, 2005b, 2009), that found in olive and *Prunus* spp exposed to the synergic effects of drought and shade. Also in this case, it seems that the lower expression of the enzymatic antioxidant system in shade plants may be due to a reduced need of ROS removal. On the contrary, in sun plants, higher enzyme activities are required for a better protection against a more pronounced oxidative stress. Recently, Yang et al. (2008) also pointed out that *Picea asperata* plants under high light condition and drought significantly increased biomass partitioning to roots, and increased the foliar levels of H_2O_2, total ascorbate content (t-Asc), and APX and GR activities. Sánchez-Díaz et al. (2007) studied photoprotection and antioxidative protection in the three major species of the Canarian laurel forest (*Laurus azorica* (Seub.) Franco, *Persea indica* (L.) K. Spreng and *Myrica faya* Aiton). Trees were exposed to drought under controlled conditions by withholding

water until leaf RWC reached 50–55%. Drought reduced photosynthetic rate, and the effect was associated with decreased quantum yield of photosystem II (PSII), electron transport, and increased non-photochemical quenching in *L. azorica* and *M. faya*. Drought-treated trees of *L. azorica* had the highest de-epoxidation state of the xanthophyll cycle and the highest zeaxanthin concentration, suggesting that this species had more effective photoprotective mechanisms than *M. faya* and *P. indica*. Moreover, β-carotene remained unaltered in *L. azorica* trees during drought, suggesting that the chloroplasts of this species are better protected against oxidative stress than those of *M. faya* and *P. indica*. Increased antioxidation by leaf APX, SOD and GR in *L. azorica* removed ROS generated during drought treatment. Although *M. faya* was able to increase its energy dissipation rate by forming zeaxanthin, and thus increasing the de-epoxidation state of the xanthophyll cycle, it did not respond to drought-induced oxidative stress with the result that β-carotene degradation occurred. *Persea indica* did not activate an energy dissipation mechanism in response to drought treatment, hence formation of ROS was likely high in the drought-treated trees. In general, *L. azorica* appeared to be most resistant, and *P. indica* most sensitive to photoinhibition and oxidative stress during drought.

In the last years, most of the research on antioxidants was focused on the genetic amelioration of fruit tree cultures particularly subjected to environmental changes. One of these is coffee tree, a species with a low genetic diversity, and for this reason very susceptible to drought or pathogens. Pinheiro et al. (2004) submitted four clones of *Coffea canephora* (Robusta coffee), representing drought-tolerant (14 and 120) and drought-sensitive (46 and 109A) genotypes, to a slowly imposed water deficit, until predawn leaf water potential (ψ_w) approximately –3.0 MPa was reached. Drought-tolerant clones were better able to maintain their leaf water status than drought-sensitive clones after withholding irrigation. Regardless of the clones investigated, the net carbon assimilation rate decreased under drought stress. Drought triggered increases in SOD, APX, CAT and POD, and also in DHAR, t-Asc and ascorbate redox state [t-Asc/(t-Asc + DHA)]. Activity of MDHAR was not induced in drought-stressed plants. In another paper (Lima et al. 2002), the effects of water deficit on photochemical parameters and activities of SOD, CAT and APX, as well as cellular damages, were investigated in two clones of *Coffea canephora* differing in drought tolerance. After 6 days without irrigation, predawn ψ_w fell to −3.0 MPa, and this was accompanied by the suppression of net photosynthesis in both clones. Under drought conditions, activities of SOD, CAT and APX increased to a greater extent in the drought-tolerant clone (0.42–1.34 units mg^{-1} protein) than in the drought-sensitive one (0.31–0.83 units mg^{-1} protein). This seemed to be matched with higher protection against oxidative stress, as judged from the lower levels of lipid peroxidation and electrolyte leakage in the drought-tolerant clone. Whereas Pinheiro et al. (2004) did not observe a general link between protection against oxidative stress with differences in clonal tolerance to drought, Lima et al. (2002), concluded that the ability to increase the antioxidant system activity in order to limit cellular damages might be an important attribute linked to the drought tolerance in *C. canephora*. Another crop with a low level of heterozigosis is banana tree. Chai et al. (2005) investigated oxidative

injury and antioxidant responses in two banana genotypes (*Musa* AAA 'Berangan' and *Musa* AA 'Mas') subjected to a drought stress period for 14 days induced by polyethylene glycol (PEG). PEG treatment resulted in oxidative injury, as expressed in increased lipid peroxidation and reduced membrane stability index, in both cultivars; however, greater oxidative injury was detected in 'Mas'. Under PEG treatment, leaf CAT activity and GR activity were enhanced in both cultivars. Leaf APX activity was enhanced in 'Berangan' under drought stress, but was unaffected in 'Mas'.

Olive tree (*Olea europaea*) is one of the most typical and economically important tree culture species belonging to the Mediterranean basin, where water shortage occurs with regularity, often lasting throughout the spring–summer period. This evergreen sclerophyllous tree shows a high degree of drought tolerance, a parsimonious consumption of soil water and a higher ratio of transpiration rate to leaf surface area in comparison with other fruit tree species in both ideal, and in water shortage conditions (Sofo et al. 2009). Olive tree is able to resist drought stress by lowering the water content and water potentials of its tissues, establishing a high water potential gradient between leaves and roots, stopping shoot growth, and reducing transpirative and photosynthesis-related processes (Sofo et al. 2004, 2005a; Ennajeh et al. 2009; Guerfel et al. 2009). For all these reasons, the antioxidant response of olive tree to drought stress has been well documented. The first studies on olive tree's antioxidant enzymes were carried out by Sofo et al. (2004, 2005a) on olive Italian cv. 'Coratina'. They pointed out that olive tree is able to up-regulate the enzymatic antioxidant system when subjected to drought stress (very low predawn ψ_w up to −5.37 MPa), and in particular APX showed marked and significant increases in leaves, medium roots, with a diameter between 1 and 5 mm (from 0.23 to 0.34 units mg^{-1} dry weight [DW]), and in fine roots, with a diameter <1 mm (from 0.36 to 0.51 units mg^{-1} DW). After reaching the maximum level of drought stress, the same plants were subjected to a rewatering treatment for 30 days, under both environmental irradiance and semi-shade conditions of about 60% of PAR (Sofo et al. 2004). In fact, the water recovery after a period of drought is a normal condition after the dry season in the Mediterranean regions. The activity of APX decreased during the rewatering period in both leaves and roots and these decrements were faster in plants rewatered in semi-shade conditions than in plants under environmental light. A similar behavior has been also found in olive (cv. Coratina) during a drought stress period (predawn ψ_w up to −5.7 MPa), in which APX activity and H_2O_2 content were higher in fully irradiated plants than in plants under 67% of PAR (Fig. 4).

The authors concluded that the lower APX expression in shaded plants with respect to non-shaded ones may be due to a reduced need of activated oxygen species removal, whereas in non-shaded plants, higher APX activity is required for a better protection against a more pronounced oxidative stress. Similar results have been found by Ennajeh et al. (2009) in two Tunisian olive cultivars, 'Chemlali' and 'Meski'. In these cultivars, an increase of leaf APX activity with decreasing leaf RWC. In the Tunisian olive cvs. 'Chemlali' and 'Chétoui' experiencing 30 days without irrigation (Guerfel et al. 2009), GR activity increased in both the cultivars,

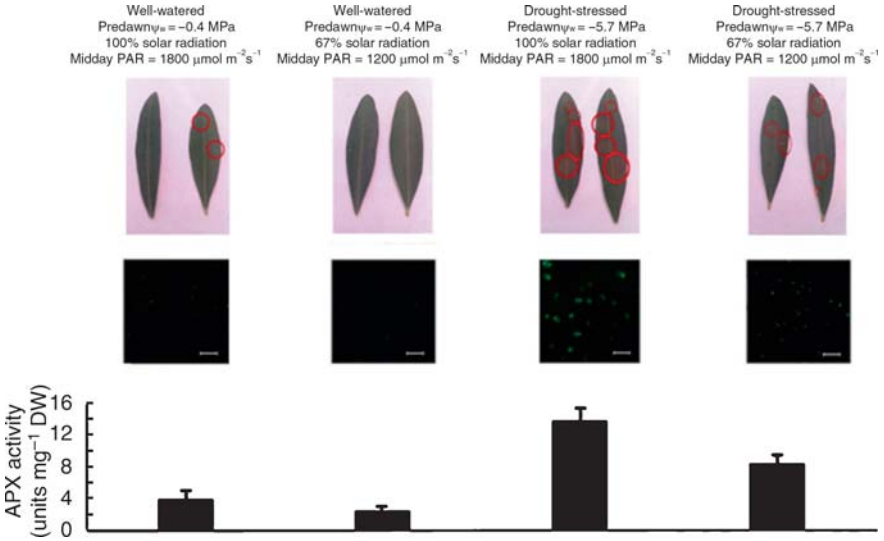

Fig. 4 Oxidative damages (in the *red circles*), H_2O_2 generation (white bar = 10 μm) and APX activity (± standard error) in olive (cv. 'Coratina') epidermal fragments of well-watered and drought-stressed plants subjected to 100% and 67% solar radiation. The levels of H_2O_2 were monitored using the H_2O_2-sensitive fluorescent probe H_2DCFDA (Sigma, MO, USA) as described by Desikan et al. (2004). The images show that the drought-stressed and fully-irradiated olive plants were found to be highly sensitive to the oxidative stress caused by H_2O_2, whereas the drought-stressed and semi-shaded plants were more tolerant to oxidative stress damage. Little visible oxidative damage was observed in well-watered fully-irradiated plants and no damage was observed in well-watered semi-shaded plants (A. Sofo)

whereas APX activity increased only in 'Chemlali'. This result is important as it indicates that enzyme activity regulation can be cultivar-specific. The different values of APX activity found in olive leaves, fine roots and medium roots by Sofo et al. (2004, 2005a) confirm their different functions: leaf tissues showed more pronounced changes (about a fourfold increase), due to the synergic effect of high irradiance levels and loss of cellular water; fine roots were more sensitive to drought stress and its consequent effects, while medium roots maintained a prolonged functionality and presented less reactivity, even at severe drought stress.

It is know that mycorrhiza infection of roots gives benefits to plants. Arbuscular mycorrhizal (AM) symbiosis can positively affect the water relations of many plants and this effect is often more pronounced in plants grown under drought stress than under well-watered conditions. The mechanisms by which AM act in plants are not completely known but there is evidence that AM symbiosis might increase the drought tolerance of plants by promoting antioxidant enzymes (Wu et al. 2006). The effect of the AM fungus, *Glomus versiforme*, on growth and reactive oxygen metabolism of trifoliate orange (*Poncirus trifoliata*) seedlings was studied in potted plants under both well-watered and drought stressed conditions (Wu et al. 2006). Drought stress significantly decreased root colonization. Shoot dry weight, plant height and stem diameter were higher in AM than in non-AM seedlings regardless

of the water status. Inoculation with *G. versiforme* increased root dry weight and leaf number per plant of well-watered seedlings. There was less MDA concentration in leaves and roots of AM seedlings, as well as lower H_2O_2 and $O_2^{\cdot -}$ concentrations in AM roots. AM inoculation did not affect the H_2O_2 and $O_2^{\cdot -}$ concentrations of WW leaves. Whether drought-stressed or not, AM symbiosis notably increased the GR activity of leaves and APX activity of roots. AM infection also markedly increased the APX activity of WS leaves. Soluble proteins and glutathione in leaves and roots, and t-Asc in leaves were higher in well-watered AM than in well-watered non-AM seedlings. AM infection also enhanced the ASC and GSH contents of leaves and roots in drought-stressed seedlings. Cross-tolerance might occur in AM plants and could be enhanced by AM symbiosis. These results suggest that the increased concentrations of antioxidant enzymes and non-enzymatic antioxidants found in AM plants may serve to protect the organism against oxidative damage, enhancing drought tolerance.

2.2 Shrub Species

Plants that occur in semi-arid and arid regions are usually physiologically and biochemically adapted to cope with high levels of solar irradiation and drought, but also possess morphological adaptation (e.g., schlerophyllia, presence of trichomes and waxes on leaf surface, deep root systems, high root/shoot, prevalence of lignified tissues). A good number of species are characterized by a shrub habitus with a high degree of resilience after having experienced environmental stresses. The studies on the regulation of antioxidant enzymes under different levels of light are particularly important in these species, as in mutable semi-arid and arid environments, and light can be an additional abiotic stresses in addition to drought.

Guidi et al. (2008) studied the interactive effects of drought stress and solar irradiance on physiological and biochemical traits in *Ligustrum vulgare*, with special emphasis on antioxidant enzymes and flavonoids. Measured were carried out in plants growing in 12% (shade) or 100% (sun) sunlight, and supplied with 100% or 40% of daily evapotranspiration-demand over a 4-week period. The mild drought stress treatment caused predawn ψ_w and RWC to decline on average by –0.2 MPa and 4.5%, respectively. In response to the drought stress treatment, photosynthetic rates decreased more in sun plants than in shade plants, likely because of declines in PS II photochemistry, coupled with significant reductions in stomatal conductance. Antioxidant enzymatic activities, which were much greater in sun leaves than in shade leaves under well-watered conditions, increased (in particular the enzymatic activities associated with H_2O_2 removal, such as CAT and APX) in response to drought stress only in shade leaves. The authors suggest that assimilated carbon in sun plants was used largely to support an effective antioxidant system capable of countering water-stress-induced oxidative damage, an example of cross tolerance. Another hypothesis is that in shade

plants, carbon was also diverted from growth to counter oxidative damage driven by the mild drought stress treatment. Both findings are consistent with the nearly exclusive distribution of *L. vulgare* in well-watered, partially shaded Mediterranean areas. The effects of separately or simultaneously induced dark chilling and drought stress were evaluated in *Glycine max* 'Merrill' cv. (Riekert van Heerden and Krüger 2002). For the induced drought treatment of 9 days, plants were maintained at normal growth temperatures without irrigation. For the simultaneously induced dark chilling and drought stress treatment, plants were dark chilled (incubated at 8°C during a dark period) without irrigation. All treatments caused similar decreases in predawn ψ_w, but resulted in distinct physiological and biochemical effects on photosynthesis.

Caper (*Capparis ovata* Desf.) is a xerophyte perennial shrub and drought resistant plant which is well adapted to Mediterranean Ecosystem. In a recent study, Ozkur et al. (2009) investigated the plant growth, RWC, chlorophyll fluorescence (F_V/F_M), lipid peroxidation (evaluated by the levels of TBA-reactive substances content) as parameters indicative of oxidative stress in relation to the tolerance to PEG drought stress in *C. ovata* seedlings. Total activity of antioxidative enzymes SOD, APX, POD, CAT, and GR were investigated in *C. ovata* seedlings under PEG mediated drought. For induction of drought stress, the 35 days seedlings were subjected to PEG 6000 of osmotic potential −0.81 MPa for 14 days. Lipid peroxidation increased in PEG stressed seedlings as compared to non-stressed seedlings of *C. ovata* during the experimental period. With regard to vegetative growth, PEG treatment caused decrease in shoot fresh and dry weights, RWC and photosynthetic efficiency but the decline in PEG-treated plants was more prominent on day 14; furthermore, both APX and GR activities increased under the drought period.

Inoculation of autochthonous drought tolerant fungal strains could be an important strategy that assured the greatest tolerance drought stress contributing to a better plant growth under drought. Marulanda et al. (2007) compared the effectiveness of four arbuscular mycorrhizal (AM) fungal isolates (two autochthonous drought-tolerant *Glomus* spp., and two allochthonous drought-sensitive strains) on a drought-adapted plant (*Lavandula spica*) growing under drought conditions. The autochthonous AM fungal strains produced a higher lavender biomass, specially the root biomass, and a more efficient N and K absorption than with the inoculation of similar allochthonous strains under drought conditions. The autochthonous strains of *Glomus intraradices* and *Glomus mosseae* increased root growth by 35% and 100%, respectively, when compared to similar allochthonous strains. These effects were concomitant with an increase in water content and a decline in t-Asc, GSH and H_2O_2. The low cell accumulation of ascorbate and glutathione in plants colonized by autochthonous AM fungal strains is an indication of high drought tolerance. Non-significant differences between antioxidant activities such as GR (GR activity was about 0.15–0.20 μmol NADPH oxidized g^{-1} fresh weight [FW] min^{-1}), CAT and SOD in colonized plants were found. Thus, these results do not allow the generalization that GR, CAT and SOD were correlated with the symbiotic efficiency of these AM fungi on lavender drought tolerance.

2.3 Cereals

Among herbacesous plants, cereals were the most studied for their use as food and economic importance. It seems that drought tolerance in cereals is mainly due to higher membrane stability, chlorophyll and carotenoid contents, lower lipid peroxidation, and higher antioxidant enzyme activity (Nayyar and Gupta 2006; Qiu et al. 2008). The degree of oxidative stress and antioxidant activity seems to be closely associated with the tolerance/susceptibility of a genotype to drought stress.

The role of plant antioxidant systems in drought stress tolerance was studied in three contrasting wheat genotypes (*Triticum aestivum*) (Sairam and Saxena 2000). Drought stress, imposed for 8 days at different stages after anthesis, resulted in an increase in lipid peroxidation, and a decrease in membrane stability, chlorophyll and carotenoid contents. The antioxidant enzymes APX, GR and non-specific peroxidase also increased significantly under drought stress. Genotype PBW 175, which had highest APX, GR and POD activity, showed low lipid peroxidation, and high chlorophyll and carotenoids content under drought stress, while the susceptible genotype WH 542 exhibited the lowest antioxidant enzyme activity, membrane stability and contents of chlorophyll and carotenoids and the highest lipid peroxidation.

Another experiment was conducted on five wheat (*Triticum aestivum*) cultivars, 'C 306', 'PBW 175' (tolerant to drought stress), 'DL 153–2' (moderately tolerant to drought stress), 'HD 2428' and 'HD 2329' (susceptible to drought stress, and so recommended for well-watered conditions), under pot culture conditions. The effect of 7 days of drought stress, starting at 17 days after anthesis, on oxidative injury and antioxidant activity was evaluated (Sairam and Srivastava 2001). In this study, drought stress significantly decreased RWC, t-Asc content and membrane stability, and increased H_2O_2 and MDA content, a measure of lipid peroxidation, and activities of antioxidant enzymes in all genotypes. Drought stress tolerant genotypes 'C 306' and 'PBW 175', closely followed by 'DL 153–2', were superior to 'HD 2428' and 'HD 2329' in maintaining high RWC, t-Asc content and membrane stability, and lower H_2O_2 content and lipid peroxidation (in terms of MDA) under drought stress. The highest activities of GR and CAT under drought stress were observed in 'C 306', 'PBW 175' and 'DL 153–2', and the lowest activities in 'HD 2428' and 'HD 2329' at all the stages. It is apparent that drought stress induces an increase in H_2O_2 content, and consequently lipid peroxidation and membrane injury, that in turn indicate a reduced membrane stability.

Keleş and Öncel (2002) investigated the effects of environmental stress combinations on soluble metabolic compounds in *Triticum aestivum* (cvs. 'Bezostaya-1', 'Seri-82' and 'Kıraç-66'), and *Triticum durum* Desf. (cvs. 'Kiziltan-91', 'Kunduru 414–44' and 'Ç. 1252'). The seedlings were grown at normal (24/16°C), low (LT, 5/−5°C) and high (HT, 40/30°C) temperature conditions, and then exposed to drought stress. Seedlings responses to cross interactions between temperature and drought stresses were investigated. Root and shoot elongation significantly decreased under drought and salt stresses. The content of α-toc significantly increased under drought stress but this increase was inhibited under HT stress, while CAT

activity decreased especially in *T. durum* genotypes, and GR activity increased under drought.

The metabolic reasons associated with differential sensitivity of C_3 and C_4 plant species to drought stress are not well understood. In the deep and important study of Nayyar and Gupta (2006), 15-day-old wheat (*Triticum aestivum*) and maize (*Zea mays*) plants, representatives of C_3 and C_4 plants, respectively, were subjected to a drought stress (predawn $\psi_w = -1.5$MPa) induced by PEG-6000 for 7 days under controlled conditions (Nayyar and Gupta 2006). Both the roots and leaves of these species were evaluated for oxidative damage and antioxidants along with stress injury (as electrolyte leakage), water content and abscisic acid (ABA). While at mild stress, both the plant species did not vary significantly from each other for stress injury, moderate and high stress levels caused considerably more damage to wheat as compared to maize. The oxidative damage in terms of MDA and H_2O_2 content was markedly higher in wheat as compared to maize at moderate and high stress levels. Relatively, maize had significantly higher content of non-enzymatic (ascorbate and glutathione) and enzymatic antioxidants (APX, DHAR and GR, especially in its leaves). In contrast, wheat possessed more activity of CAT in roots as well as leaves in comparison to maize. Thus, leaves of both the species experienced more damage than roots, likely because more subjected to the environmental stresses. These findings suggested that differential sensitivity of C_3 and C_4 plants to drought stress appear to be partially governed by their ability to counter oxidative stress, involving ascorbate and glutathione. The levels of t-Asc/GSH appear to have greater involvement in regulating this response. Manipulation of endogenous expression of antioxidants through genetic means might elevate the defense ability of these plant species, especially of C_3 plants to drought stress. The results of Nayyar and Gupta (2006) showed that increased activity of antioxidants in leaves may be more important for stress tolerance than in roots.

In order to determine the role of laser in drought stress resistance of spring wheat (*Triticum aestivum*), Qiu et al. (2008) exposed seed embryos to CO_2 laser radiation for 5 min, and treated 12-day-old seedlings with 10% (w/v) PEG-6000 solution for 10 days. This is the first investigation reporting the use of CO_2 laser pretreatment to enhance drought stress resistance of spring wheat. Changes in the concentration of MDA, H_2O_2, t-Asc, GSH, GSSG, carotenoid, zeaxanthin, the production rate of $O_2{\cdot}^-$, the activities of APX, POD, CAT, SOD and GR, glutathione peroxidase, glutathione-S-transferase, and the growth parameters of seedlings (plant height, leaf area and dry weight) were measured to test the effects of laser pretreatment. The results showed that suitable laser pretreatment of embryos enhanced drought stress resistance in wheat seedlings by decreasing the concentration of MDA and H_2O_2, GSSG, the production rate of $O_2{\cdot}^-$, leaf area and increasing the activities of APX, glutathione peroxidase, glutathione-S-transferase and POD and t-Asc, carotenoid and zeaxanthin concentration. It is suggested that those changes in MDA, O_2^-, H_2O_2, anti-oxidative enzymes, and anti-oxidative compounds were responsible for the increase in drought stress resistance observed in the experiments. The results also showed that the laser had a long-term positive physiological effect on the growth of drought stress seedlings.

During life cycle, wheat crop may experience water deficit cycles that induce oxidative stress. An interesting study of Khanna-Chopra and Selote (2007) was conducted to evaluate the role of oxidative stress management in the leaves of two wheat (*Triticum aestivum*) cultivars, 'C306' (drought-resistant) and 'Moti' (drought-susceptible), when subjected directly to severe drought stress (non-acclimated plants) or to drought stress cycles of increasing intensity (ψ_w from control at −1.40 MPa to −1.63/−1.67 MPa at mild stress to −1.93/−2.13 MPa at severe stress; instead, directly to −2.70/−1.97 MPa in severe-stressed non-acclimated plants) with an intermittent rewatering (drought-acclimated plants). Mild drought stress during vegetative growth enabled 'C306' to acclimatize better than 'Moti' during subsequent drought stress of severe nature during post-anthesis period. The drought-acclimated 'C306' leaves maintained favorable water relations and lower membrane injury due to low H_2O_2 accumulation than non-acclimated 'C306' plants during severe drought stress. This is due to systematic increase in the activity of APX and POD, and maintenance of ascorbate and glutathione redox pool by efficient functioning of GR enzyme in the drought-acclimated 'C306' plants. MDHAR and DHAR activities increased with increasing drought stress, but only in drought-acclimated plants. In contrast, both acclimated as well as non-acclimated 'Moti' plants exhibited loss in turgor potential, high H_2O_2 levels and poor antioxidant enzyme response leading to enhanced membrane damage during severe drought stress conditions. Generally, total AsA content and AsA/DHA ratio decreased in both stress treatments for both the cultivars, and a similar trend was observed for total glutathione (t-Glu), GSH and GSH/GSSG. It is interesting to note that a drastic drought stress appears to cause more changes than a slower one, and that non-acclimated plants are more subjected to drought stress oxidative damages. Failure in the induction of APX and ascorbate–glutathione cycle enzymes makes the chloroplast susceptible to oxidative stress in non-acclimated plants. Non-acclimated plants protected the leaf mitochondria from oxidative stress by up-regulating SOD, APX, and GR activities. Rewatering led to rapid enhancement in all the antioxidant defense components in non-acclimated plants, which suggested that the excess levels of H_2O_2 during severe drought stress conditions might have inhibited or down-regulated the antioxidant enzymes.

Aroca et al. (2003) studied the photosynthetic performance and protective mechanisms against oxidative stress in two maize (*Zea mays*) genotypes differing in chilling sensitivity ('Z7', tolerant and 'Penjalinan', sensitive), subjected to 5°C for 5 days, with or without a drought pretreatment. In 'Penjalinan' plants, the drought pretreatment decreased the symptoms of chilling injury, estimated as necrotic leaf area and maximum quantum yield of PS II. Furthermore, drought pretreatment diminished the level of lipid peroxidation caused by chilling in 'Penjalinan' plants. After 1 day of recovery from chilling the 'Z7' and drought-pretreated 'Penjalinan' plants showed higher net photosynthesis rates than the non-drought-pretreated 'Penjalinan' plants, thereby decreasing the probability of generating ROS. The greater net photosynthesis was correlated with the greater NADP-malate dehydrogenase activity. No differences in either the de-epoxidation state of the xanthophyll cycle or the antioxidant enzyme activities (APX and GR) were found among the

drought and drought-chilled groups of plants. However, a drastic decrease in AsA content was observed in chilled 'Penjalinan' plants without drought pretreatment (from 260 to 0 μmol m^{-2}). As the authors found an increase of H_2O_2 content after drought pretreatment, they suggested its involvement as a signal in the drought-enhanced chilling tolerance of maize. Recently, Rapala-Kozik et al. (2008) used *Zea mays* seedlings as a model system to analyze for any relation between the plant response to abiotic stress, and the properties of thiamine biosynthesis and activation. Conditions of drought were induced by PEG-6000 at 20% for 2 days, and increases in the activities of APX and GR were found. In an experiment on rice (*Oryza sativa*, cv. 'Tulsi'), Srivalli et al. (2003) subjected plants to three cycles of drought stress of increasing stress intensity. Each stress cycle was terminated by rewatering the plants for a 48-h period. The response of the antioxidant metabolites ascorbate and glutathione was analyzed in terms of activity and isozyme pattern for each cycle of stress and recovery. It was observed that drought stress caused increases in APX and GR activities, and there was a better management of toxic H_2O_2 levels. These increases were parallel to a lower AaA/DHA and to a decrease in AsA. Guo et al. (2006) investigated about the responses of antioxidative defense systems to chilling and drought stresses in four cultivars of rice (*Oryza sativa*) differing in sensitivity, two of them ('Xiangnuo no. 1' and 'Zimanuo') are tolerant to chilling but sensitive to drought, and the other two ('Xiangzhongxian no. 2' and 'IR50') are tolerant to drought but sensitive to chilling. The seedlings of rice were transferred into growth chamber for 5 days at 8°C as chilling treatment, or at 28°C as control, or at 28°C but cultured in 23% PEG-6000 solution as drought stress treatment. Under drought stress, the elevated levels of electrolyte leakage, and the contents of H_2O_2 and MDA in 'Xiangzhongxian no. 2' and 'IR50' are lower than those in 'Xiangnuo no. 1' and 'Zimanuo'. Activities of SOD, CAT, APX, and the contents t-Asc and GSH were measured during the stress treatments. APX activity, AsA and GHS showed a slight increase until 3 days after drought stress in the two drought-tolerant cultivars, or after chilling stress in the two chilling-tolerant cultivars. On the other hand, activities of antioxidant enzymes and contents of antioxidants were greatly decreased in the drought-sensitive cultivars after drought stress, and in the chilling-sensitive cultivars after chilling stress. The results indicated that tolerance to drought or chilling in rice is well associated with the enhanced capacity of antioxidative system under drought or chilling condition, and that the sensitivity of rice to drought or chilling is linear correlated to the decreased capacity of antioxidative system.

2.4 Other Herbaceous Species

The investigations on enzymatic and non-enzymatic antioxidants of the ascorbate–glutathione cycle in drought-stressed herbaceous plants in the last 10 years (excluding cereals) have been mainly focused on species having a known antioxidant effect and with a strong degree of drought tolerance (such as *Euphorbia, Vaccinium,*

Allium, Bupleurum, Catharanthus, Anoda spp.). A good number of studies have also regarded grasses used as cover crops in cultivated fields (*Trifolium, Medicago* spp.) or 'model' plants (*Arabidopsis thaliana*). This studies highlight the importance of antioxidant enzymes in the plant growth in different phenological stages under drought conditions. In the perennial weed leafy spurge (*Euphorbia esula*), GR specific activity increased almost 60% during drought, whereas APX activity only showed a transient increase after 3 days of drought (Davis and Swanson 2001). Tahkokorpi et al. (2007) studied the influence of drought-related winter stress on growth, oxidative responses, and possible after-effects in bilberry (*Vaccinium myrtillus*) in field conditions favorable for desiccation in winter. Bilberry plants were subjected to low temperatures, drought and irradiance, grown under snow (control plants), under a grey plate without snow, under a transparent plate without snow, and under either grey or transparent plates in the absence of snow. Tissue water content, GR activity as well as soluble proteins decreased as frost hardening approached in the three treatments (from 0.8–1.3 pkat g^{-1} DW in June to 0.2–0.4 pkat g^{-1} DW in December). Bilberry plants activate their metabolism in early spring due to increasing temperature, in agreement with the rising of tissue water content and increments in anthocyanins, photosynthetic pigments, GR activity and soluble proteins in March. GR activity increase was lower in the plants that wintered without a snow cover, which points to delayed activation of metabolism, resulting from multiple stresses (low temperatures, drought, light) that acted simultaneously. In another study (Egert and Tevini 2002), drought caused by withholding water led to significant reductions in the relative water content (−17.5%) of chives (*Allium schoenoprasum*) leaves, a significant rise in the osmolarity of the leaf sap (+18.9%) and a loss of leaf transpiration (leaf diffusion resistance >20 s cm^{-1}). While it did not affect specific POD activity, drought increased the specific activity of APX significantly by almost 29%, and reduced the specific lipoxygenase activity significantly by 60%. Recently, Zhu et al. (2009) investigated on roots of *Bupleurum* spp., one of the most popular ingredients in many oriental medicinal preparations. Potted *Bupleurum chinense* DC seedlings were subjected to progressive drought stress by withholding irrigation for 9 days, followed by a rewatering phase, and the changes in antioxidant system, H_2O_2 and $O_2{\cdot}^-$ contents were investigated. Additionally, the antioxidant activity of root extracts was evaluated. The results showed that *B. chinense* root appeared highly resistant to water deficit. Increasing levels of drought stress were accompanied by enhanced $O_2{\cdot}^-$ content and SOD, CAT and APX activity until severe drought stress.

Interference and drought are two major causes of limited productivity in plants. Since weeds reduce the amount of water available to the crop, drought stress can alter crop performance under crop-weed interference differently from that under weed-free conditions. Among weeds commonly found in cotton, spurred anoda is also a member of the Malvaceae family with a growth pattern similar to cotton. For this reason, the influence of plant interference and mild drought stress on gas exchange and oxidative stress was investigated (Ratnayaka et al. 2003) on the cotton species (*Gossypium hirsutum* cv. 'Delta Pine 5415'), and on spurred anoda (*Anoda cristata*). An individual cotton plant was grown alone, or three cotton plants

were planted per pot for intraspecific interference studies. For interspecific interference, a single cotton plant was grown with two plants of spurred anoda. Without interference, cotton and spurred anoda had similar net photosynthesis but different pigment profiles. Stomatal conductance and transpiration rate were greater in spurred anoda than cotton. Net photosynthesis and biomass in cotton were reduced more by spurred anoda interference than by intraspecific interference. With interference, the xanthophyll cycle conversion state and α-toc increased in cotton, but remained unchanged in spurred anoda. The activities of CAT, APX and GR were not influenced by plant interference. Mild drought increased APX activity both in cotton and spurred anoda. Upon drought recovery, drought-induced APX activity was still higher in cotton if compared to well-watered control (1.0 and 0.6 units mg^{-1} protein, respectively), and GR activity compared with well-watered plants was higher in previously drought-stressed cotton (430 and 300 units mg^{-1} protein, respectively), and spurred anoda plants (540 and 480 units mg^{-1} protein, respectively). The authors concluded that the greater impact of spurred anoda interference than intraspecific interference on cotton biomass was due mainly to reduced carbon gain in cotton.

The variations in antioxidant potentials and indole alkaloid content were studied by Jaleel et al. (2008a, b), in two varieties (Rosea and Alba) of *Catharanthus roseus*, an important plant used in traditional as well as modern medicine, exposed to water deficit stress (20 days interval drought starting from 30 days after sowing). The antioxidant and alkaloid profiles were estimated from root, stem, leaf, flowers and pods. The antioxidant potentials were examined in terms of level of non-enzymatic antioxidants and activities of antioxidant enzymes. The non-enzymatic antioxidant molecules studied were ascorbate (AsA), α-toc and GSH, whereas the estimated antioxidant enzymes were SOD, APX, CAT, POD and polyphenol oxidase (PPO). The antioxidant concentrations and activities of antioxidant enzymes were high under water deficit stress in all parts of the plants. The very high concentration of AsA in the root system is surprising and indicates the very high antioxidant ability in this species.

Bermejo et al. (2006) evaluated the changes in water status in two *Trifolium repens* cv. 'Regal' biotypes, tolerant and sensitive to ozone, subjected to a to a short-term drought. The evolution of both soil and plant water status along with leaf gas exchange parameters, leaf APX and GR enzymatic activities for an equal number of clones of each biotype under drought and control treatments was registered for 5 days. The results obtained show that when the short-term drought is imposed, symptoms of oxidative stress and a negative impact on most of the measured parameters are displayed at an earlier stage in the sensitive biotype, proving it to be more sensitive to low water availability. Therefore, it is suggested that these differences might be linked with the existing variability in ozone sensitivity between the biotypes and that, at the same time, they could result in a poorer performance of this biomonitoring system in field assays under conditions of high evapotranspiratory demand, such as those registered during summer time in Mediterranean areas. To understand the adaptability of alfalfa (*Medicago sativa*) to environmental stresses, Wang et al. (2009) have recently analyzed the activity of several antioxidant

enzymes, including SOD, POD, APX and CAT, in alfalfa shoots and roots subjected to salt and drought stresses during germination. The germination rate of six alfalfa cultivars was comparatively studied under a 200 mM NaCl or a 35% PEG treatment. 'Alfalfa Xinmu No. 1' and 'Northstar' varieties were selected as stress-tolerant and -sensitive cultivars, respectively, and were used for further characterization. After NaCl or PEG treatment, 'Xinmu No. 1' showed enhanced seedling growth, compared with 'Northstar'. 'Xinmu No. 1' also exhibited low levels of H_2O_2 production and lipid peroxidation, compared with 'Northstar'. In addition, 'Xinmu No. 1' showed higher enzymatic activity of SOD, APX, CAT, and POD in its shoots and roots than 'Northstar'. These results seem to indicate that 'Xinmu No. 1' cultivar's tolerance to salt or drought stresses during germination is associated with enhanced activity of antioxidant enzymes.

In an interesting research on *Arabidopsis* (Jung 2004), young and mature leaves of 4-week-old plants were exposed to drought stress from −0.65 up to −2.54 MPa of ψ_w by withholding water supply for 7 days. The drought-induced increase in non-enzymatic antioxidants in young and mature leaves, enzymatic antioxidants including CAT, POD, SOD and GR substantially increased only in drought-stressed mature leaves. Plants recovered rapidly 24 h after resupplying water, as indicated by the values of ψ_w, photosynthetic efficiency and pigment contents, however, the activities of POD, SOD and GR remained high. Young leaves were rather not affected in drought responses. The fact that drought-stressed mature leaves suffer more stress than drought-stressed young leaves suggests that developmental stages of leaves might contribute to the differential prevention of oxidative damage in plants exposed to drought.

2.5 Non-enzymatic Antioxidant Pools

The studies reported in this paragraph deal with the changes in the level of non-enzymatic antioxidants (ascorbate and glutathione in their reduced or oxidized status, and α-toc) that act within the ascorbate–glutathione cycle. From a general analysis of the most significant studies published in the last decade on this topic, it emerges that there was found no incontestable and definitive indication that a particular degree of metabolic tolerance to drought is related to the absolute level of some non-enzymatic antioxidants. This is in contrast to the fact that the action of drought stress on antioxidant enzyme activities seems to be clear and direct (see the paragraphs above, in which the up-regulation of the enzymes appeared to be ubiquitous among species).

More interestingly, the increase of the oxidized forms of ascorbate (DHA) and glutathione (GSSG) pools are often symptoms of the increased enzymatic activity of APX, DHAR, MDHAR, and GR in response to lower water content. High GSH/GSSG ratios (10:1) are required for the metabolism of the cytosol and chloroplast, and the high content of GSH and GSH-synthesizing enzymes in *Arabidopsis* cells indicate a specific role in drought stress tolerance (Foyer et al 2001). Posch and

Bennett (2009) found that in *Allocasuarina luehmannii* seedlings severe drought stress (predawn ψ_w = –6.0 MPa) decreased stomatal conductance and CO_2 assimilation rate to 5% and 15% of the control values, respectively; while t-Asc and t-Glu concentrations remained unaffected by drought treatments, and ascorbate became more oxidized (DHA). In leaves of potato plants subjected to drought (70% RWC), glutathione oxidation ratio increased by about 58%, showing an increase in GSSG/t-Glu ratio from 19% to 30% (Broin et al. 2000). Šircelj et al. (2005, 2007) investigated the influence of gradual water deprivation on potted apple trees (*Malus domestica* Borkh.) up to predawn ψ_w = −2.0 MPa. Mild drought (predawn ψ_w = −0.4 MPa) did not significantly affect the chosen stress indicators. Moderate drought (predawn ψ_w = −1.3 MPa) increased the concentrations of ascorbate and GSSG/t-Glu ratio, indicating the adaptation to oxidative stress in apple trees. Severe drought (predawn ψ_w = −2.0 MPa) negatively affected vitality of apple trees, and caused decreases in ascorbate together with the increase in GSSG concentration, indicated severe damage due to oxidative stress. A mild drought is a potential oxidative stressor due to the production of ROS in illuminated chloroplasts which lack CO_2 due to stomatal closure (Morales et al. 2006). Photoprotective pigments (e.g. xanthophyll cycle) may avoid this situation through light energy dissipation, and antioxidants such as ascorbate, tocopherols, and glutathione, may detoxify ROS (Smirnoff 2005). Tausz et al. (2001) subjected potted *Pinus canariensis* seedlings to mild drought by withholding irrigation for one week. This treatment induced a reduction in maximum stomatal conductance (50 mmol m^{-2} s^{-1}) compared to irrigated controls (130 mmol m^{-2} s^{-1}). Concentrations of ascorbate, glutathione, chlorophyll, and the xanthophyll cycle carotenoids were minimal in the evening (under low light) compared to light-saturated conditions. These short-term changes were not affected by drought but the glutathione pool was more oxidized in needles of non-irrigated trees, whereas the redox state of ascorbate remained stable.

Although ascorbate is a high-abundance metabolite, relatively little is known about the factors controlling its accumulation in leaves. To address this issue, Bartoli et al. (2005) examined the role of L-galactono-1,4-lactone dehydrogenase (GalLDH), the enzyme which catalyses the last step of this pathway, in the control of ascorbate content under optimal and stress conditions (drought was imposed on 3-week-old plants by withholding watering until the pots reached a soil ψ_w of −1.5 MPa). In a range of species, no clear relationship between ascorbate content and leaf GalLDH protein and activity was found under optimal growth conditions. To explore the effect of drought stress on GalLDH activity and protein content, wheat (*Triticum aestivum*) was selected for detailed analysis, using two cultivars that differ in their constitutive AsA level. Neither leaf DHA content nor activities of AsA regenerating enzymes were modified by drought. Although drought caused a substantial increase in GalLDH protein and activity in the low AsA in cv. ‘CM’, this treatment had no effect on these parameters in cv. ‘BCH’. Notably, leaf AsA content was unaffected by drought. These results suggest that GalLDH protein and activity cannot be used as an indicator for changes in the capacity for ascorbate biosynthesis, and that ascorbate biosynthesis is constrained by other factors under stress.

The role of α-toc in plants under drought stress is of primary importance within the ascorbate–glutathione cycle (see introduction). In fact, α-tocopherol plays an important role in protecting chloroplastic membranes from the deleterious effects of lipid peroxy radicals and singlet oxygen. It is usually recycled back by ascorbate or GSH following oxidation by lipid peroxy radicals. However, it can be irreversibly converted to the corresponding quinine and quinone epoxides after reacting with singlet oxygen. In a interesting investigation, Munné-Bosch et al. (2001) have studied that the endogenous α-toc levels in a drought-recovery cycle in leaves of sage (*Salvia officinalis* subs. *officinalis*), a drought-susceptible Mediterranean species. The relative leaf water content of the sage plants fell markedly when exposed to drought during the summer. As the drought progressed (RWC from 67% in June to 32% in August), α-toc levels decreased progressively with drought. Therefore, the leaves contained smaller pools of antioxidant defences to counteract oxygen toxicity during the drought, and this explains, among other biochemical and structural features, the susceptibility of this species to stress. These results suggest that it is the complete set of antioxidants, and not a single mechanism, that is responsible for avoiding drought-induced damage in plants. In another study, Munné-Bosch and Peñuelas (2004) investigated the endogenous concentrations of xanthophyll cycle pigments, α-toc, and reduced and oxidized ascorbate in 2-year-old strawberry tree (*Arbutus unedo*) plants exposed to a combination of water deficit, high light, and high temperatures. In the same plants under severe stress, α-toc levels markedly increased, zeaxanthin concentrations increased by 75%, ascorbate increased from 18 to 30 μmol g DW^{-1} and its redox state shifted towards its oxidized form, and chlorophylls, lutein and β-carotene decreased by 63%, 61% and 75%, respectively. To gain insight into the role of flavonoids in the antioxidant defense system of *Cistus clusii* Dunal, Hernández et al. (2004) evaluated drought-induced changes in flavonoids in leaves and compared the response of these compounds with that of ascorbate, α-toc and carotenoids. Total ascorbate (t-Asc) and α-toc concentrations increased to a similar extent in response to a 50-days drought period, even if the kinetics of the drought-induced increases differed. Haberer et al. (2008) quantified ascorbate, glutathione, and α-toc in fine roots of mature *Fagus sylvatica* under free-air canopy ozone (O_3) exposure (twice ambient O_3 concentration, $2 \times [O_3]$) during two growing seasons that differed in the extent of summer drought (exceptional drought year 2003, average year 2004). This design allowed authors to test whether O_3 exposure or drought, or both, affected root antioxidants during the growing season. In both years, root ascorbate and α-toc showed a similar relationship with volumetric soil water content (SWC): t-Asc concentrations on a root dry mass basis increased when SWC dropped from 25% to 20%, whereas α-toc increased at SWC values below 20%. Root glutathione showed no relationship with SWC or differences between the dry and the average year. The results were inconclusive as to whether shoot–root translocation of glutathione or glutathione production in the roots was diminished. Phloem glutathione concentrations in the canopy remained constant, but reduced transport velocity in the phloem and, as a consequence, reduced mass flow of glutathione cannot be ruled out.

3 Effect of Drought Stress on the Whole Ascorbate–Glutathione Cycle

The degree of drought stress at which the up-regulation of the ascorbate–glutathione cycle increases is extremely variable among several plant species, and even between two cultivars of the same species (Reddy et al. 2004b). The level of response depends on the species, the development, and the metabolic state of the plant, as well as the duration and intensity of the stress. The distribution and the level of activity of the enzymes of the ascorbate–glutathione cycle are also known to be differently distributed among all photosynthetic cells in higher plants. In maize leaves, GR and DHAR are exclusively localized in mesophyll cells, APX is mainly localized in mesophyll and bundle sheath cells, and MDHAR is approximately equally distributed between mesophyll and bundle sheath cells (Foyer et al. 2001). Most of the studies on C_4 plants indicate that oxidative damage under stressful conditions is not uniformly distributed between mesophyll and bundle sheath cells of C_4 plants, suggesting that it is restricted to bundle sheath tissue (Nayyar and Gupta 2006).

The whole ascorbate–glutathione system was studied during desiccation of recalcitrant seeds of the silver maple (*Acer saccharinum*) (Pukacka and Ratajczak 2006). The desiccated seeds (seeds were dried at 21°C and 35–40% RH for 14 days) gradually lost their germination capacity and this was strongly correlated with an increase in electrolyte leakage from seeds. Simultaneously the increase of $O_2 \cdot^-$ and H_2O_2 production in seeds was observed. The results indicate that remarkable changes in the concentrations and redox status of ascorbate and glutathione occur in embryo axes and cotyledons. At the first stages of desiccation, up to a level of 43% of moisture content, AsA and GSH content in embryo axes increased. The enzymes of the ascorbate–glutathione pathway: APX, MDHAR, DHAR and GR increased their activity during desiccation, but mainly in embryonic axes.

Lu et al. (2007) investigated the synergic effects of high temperature, low temperature, and water deficiency on the activities of seven antioxidant enzymes in crofton weed (*Eupatorium adenophorum* Spreng.), an invasive weed in southwestern China. The changes in activities of SOD, CAT, POD, APX, GR, MDHAR, and DHAR were evaluated to determine physiological aspects of the crofton weed that might render the plant vulnerable to environmental stress. In the drought-treated plants, APX activity increased to 202% of the control whereas GR activity increased to maximum levels and was more than 23% greater than the controls. The activity of MDHAR decreased significantly ($P < 0.01$) in the heat and drought treatments, whereas DHAR activity increased from day 0 to 4, reaching the highest levels (210% greater than the controls) on day 4.

Reddy et al. (2004a) determined foliar ascorbate content, and antioxidant enzyme activities in five mulberry (*Morus alba*) cultivars subjected to drought stress by withholding water until the ψ_w reached −2.50 MPa. The activities of antioxidant enzymes, which include SOD, CAT, APX, POD, GR and MDHAR were significantly high in the water-stressed leaves of all the cultivars. The contents

of AsA and MDHA in the leaf extracts of all the five cultivars of mulberry also showed variations in response to water deficit. In order to determine if the enzymes of the ascorbate–glutathione cycle were differently affected by drought in C_3 and C_4 plants, Zhang and Kirkkham (1996a, b)) grew *Sorghum bicolor* (C_3) and *Helianthus anuus* (C_4) under dry conditions in a growth chamber. Levels of leaf enzymatic antioxidants (APX, CAT, POD, DHAR, MDHAR, GR and SOD), non-enzymatic antioxidants (ascorbate, glutathione and carotenoids) and stress parameters (chlorophyll and MDA) were determined under watered and drought conditions. Under watered conditions, inherent levels of antioxidants were not consistently higher or lower in sorghum than in sunflower. In response to drought, levels of antioxidants decrease or remained unchanged depending on crop, duration of drought and kind of antioxidants. Duration of drought was divided arbitrarily into three stages. At a late stage of drought (watering had stopped for 7–8 days) when leaf RWC had markedly decreased in sorghum and sunflower, the parameters studied resulted affected by drought. Because of the differential effect of drought, levels of antioxidants were not consistently higher or lower in sorghum than in sunflower under drought. These results show that, under both drought and watered conditions, sorghum does not have consistently higher or lower antioxidant levels than sunflower, and that antioxidant responses to drought differ in C_3 and C_4 plants.

Sofo et al. (2005b) studied the activities of APX, MDHAR, DHAR and GR, as well as the levels of ascorbate pool, glutathione pool and H_2O_2 in plants of four interspecific hybrids of *Prunus* spp. rootstocks subjected to water deficit and shade conditions. The genus *Prunus* comprises more than 400 species adapted to temperate areas and cultivated in Europe. In particular, commercial stone fruit crops, such as peach, plum, almond, apricot, and cherry tree are usually grafted plants with a lower part, the rootstock and an upper grafted part, which is the genotype of the commercial variety. Rootstocks have different genetic background compared to the commercial varieties, and can be used to confer various traits, such as drought stress resistance. After 70 days of water shortage, plants were subjected to a rewatering treatment. During water recovery, leaves fully exposed to sunlight and leaves in shade conditions of about 30% of environmental irradiance were sampled. After 70 days without irrigation, mean predawn ψ_w of all the hybrids fell from −0.34 to −3.30 MPa, and marked decreases in net photosynthesis and transpiration occurred. Generally, APX, MDHAR, DHAR and GR activities were up-regulated during the drought phase and successively down-regulated during the rewatering phase. Furthermore, enzyme activities in shaded leaves were lower than those found in non-shaded leaves. The levels of AsA, DHA, GSH and H_2O_2 were directly related to the increase of drought stress and subsequently decreased during rewatering. In the first 13 days of the drought phase, the ratios of AsA to DHA were similar to those of well-watered plants, whereas, at high level of water deficit, ASA/DHA ratio decreased and subsequently showed an increase in the last days of the drought period. Generally, the ratios of GSH/GSSG increased during high levels of water deficit. The results highlighted the capacity of *Prunus* hybrids to withstand drought conditions by regulating the ascorbate–glutathione cycle. The results obtained in

this investigation, underlining the important role of some antioxidant enzymes and compounds in protecting cellular apparatus during water deficit conditions, may be useful for the selection for drought resistance in *Prunus* rootstocks material. This could lead to the characterization of different genotypes with this important characteristic. The same conclusions of Sofo et al. (2005b) were drawn from an unpublished study on various species of almond (*Prunus* spp.) during drought stress and subsequent rewatering (K. Sorkheh et al., 2010, personal communication).

4 Transcriptional Regulation of Genes Encoding Antioxidant Enzymes in Drought-Stressed Plants

In drought-stressed plants, the induction of the proteins by drought stress is strictly regulated (Fig. 5). If compared to the studies on enzyme activities, the investigations on the regulation of the transcription of antioxidant enzymes genes under drought stress are very scarce. Furthermore, the most of them are only qualitative and not quantitative, as these researches have often been conducted by standard PCR-based methods. Recently, quantitative differences in transcription levels in plants under drought conditions or between drought-tolerant and drought-sensitive have been identified by Real Time-PCR-based methods and microarray techniques (Guo et al. 2009; Vadassery et al. 2009). The gene expression of the enzymes of ascorbate–glutathione cycle profile is affected by the intensity and duration of drought stress. For example, the levels of expression of the *APX2* gene is dependent on drought stress degree and light intensity (Tamaoki et al. 2004).

Besides genes encoding for APX, one of the most studied enzymes is glutathione reductase, that play a key role in the restoration of the post-stress redox state of the cytosolic glutathione pool (Foyer et al. 2005). Two isoform of GR cDNA have been cloned and sequenced from pea (*Pisum sativum* cv. 'Birte') (Stevens et al. 1997). The cytosolic GR cDNA (*GOR2*) is significantly different at the DNA level from the chloroplastidial/mitochondrial GR (*GOR1*). *GOR2* maps to linkage group 6 on the pea genome map and it seems likely that this is the only locus for this gene. In contrast to *GOR1*, transcript levels of *GOR2* increase in the rewatering (post-stress) by about ten- and threefold, respectively. Key regulators and signalling components involved in high light-mediated oxidative stress may lead to cross protection against drought, as many high light-regulated genes are also induced under drought conditions. Molecular analyses of *Arabidopsis thaliana*, ecotype Columbia, have revealed a number of genes whose expression changes in response to high light, including the H_2O_2 scavenger *APX2*, and have provided evidence for common steps in drought and high light stress response pathways (Rossel et al. 2006). The authors described the drought tolerant mutant *alx8*, which has constitutively higher *APX2* expression and higher levels of foliar ABA than wild type. In fact, exogenous ABA increased *APX2* expression and the *APX2* promoter contains ABA response elements. The *alx8* mutant exhibits improved water-use efficiency and the up-regulaton of a number of drought-tolerance genes, including *APX2*.

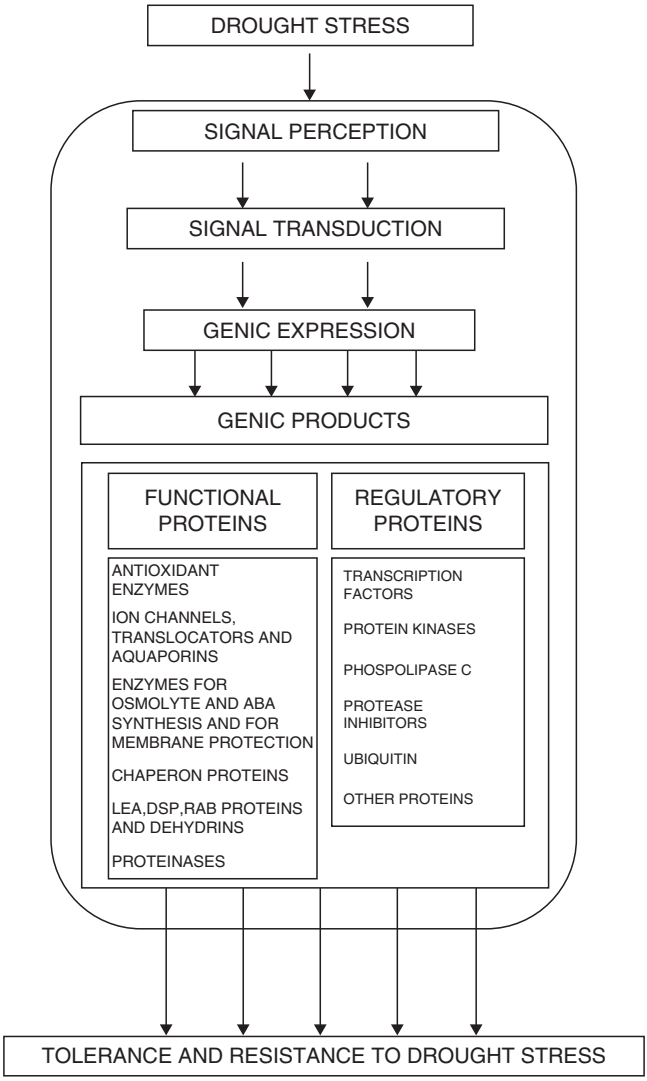

Fig. 5 Functions of the proteins induced by drought stress. The induced proteins are divided in two groups: functional proteins involved in stress tolerance and stress cellular adaption, and regulatory proteins with a role in genic expression and signal transduction in response to drought

The study of Jin et al. (2006) suggests that the regulation of APX at the transcript level may be involved in the response to water deficit stress. Cut rose (*Rosa hybrida*) cv. 'Samantha' flowers were pretreated for 12 h with 6 mM ascorbate (AsA), 5 mM β-aminophenol, or water (control) prior to exposing to water deficit stress for 24 h, and then were placed into water for recovery and vase life. Vase life, flower development, ψ_w, MDA content, and SOD and APX activities were then determined until end of vase life. Water deficit stress reduced vase life and inhibited flower development.

AsA pretreatment alleviated deterioration, while β-aminophenol pretreatment increased the deterioration. AsA pretreatment also decreased MDA content, and increased SOD and APX activities, but the opposite effects were found for the β-aminophenol pretreatment. A cDNA encoding cytosolic APX was isolated from petals, and named *Rh-APX1*. Gene expression in control petals increased in the first 9 h, then decreased until the end of water deficit stress; it recovered when water was resupplied, and peaked again on the third day after placing flowers in water. Compared with the control, the gene expression was enhanced substantially by AsA pretreatment throughout water deficit stress, water recovery, and throughout vase life. In contrast, the expression was inhibited by β-aminophenol. The changing patterns of *Rh-APX1* gene expression paralleled those of APX activity.

The objective of the recent study of Bian and Jiang (2009) was to investigate accumulation of ROS, antioxidant enzyme activities, and gene expression patterns of antioxidant enzymes of Kentucky bluegrass (*Poa pratensis*) under drought stress and recovery. Grass (cv. Midnight II) was subjected to soil drying for 5 days and then rewatered for 1 day in growth chamber. Drought stress increased $O_2 \cdot^-$ production of leaves and H_2O_2 content of the leaves and roots. Recovery enhanced leaf $O_2 \cdot^-$ production and root H_2O_2 content. Drought stress increased the leaf activities of APX, MDHAR and DHAR, and the root activities of GR and MDHAR, while reducing the root activities of DHAR. The increased leaf activities of APX, MDHAR, DHAR and GR, and the root activity of APX and MDHAR were also maintained after rewatering. For the leaves, the expression of DHAR was down-regulated by drought stress but recovered to control level after rewatering, while the expressions of GR and MDHAR were up-regulated and maintained high transcript levels also after water recovery. For the roots, the expressions of cytosolic APX, GR, and DHAR were down-regulated under drought stress but recovered except for GR and DHAR, while MDHAR expression was up-regulated. Antioxidant enzymes and their gene expressions may be differentially or cooperatively involved in the defense mechanisms in the leaves and roots of Kentucky bluegrass exposed to drought stress and recovery. MDHAR and DHAR are the two main enzymes that maintain ascorbate in its reduced state. *MDAR*2 (At3g09940) and *DHAR5* (At1g19570) expression is up-regulated in the roots and shoots of *Arabidopsis* seedlings co-cultivated with the root-colonizing endophytic fungus *Piriformospora indica*, or that were exposed to a cell wall extract or a culture filtrate from the fungus (Vadassery et al. 2009). In fact, growth and seed production were not promoted by *Piriformospora indica* in *mdar2* (SALK_0776335C) and *dhar5* (SALK_029966C) T-DNA insertion lines, while colonized wild-type plants were larger and produced more seeds compared to the uncolonized controls. After 3 weeks of drought stress, growth and seed production were reduced in *Piriformospora indica*-colonized plants compared to the uncolonized controls, and the roots of the drought-stressed insertion lines were colonized more heavily by the fungus than were wild-type plants. Upregulation of the message for the antimicrobial PDF1.2 protein in drought-stressed insertion lines indicated that MDAR2 and DHAR5 are crucial for producing sufficient ascorbate to maintain the interaction between *Piriformospora indica* and *Arabidopsis* in a mutualistic state.

5 Proteomic Studies on Antioxidant Enzymes in Drought-Stressed Plants

Proteomic analysis provides a broad view of plant responses to stress at the level of proteins. In recent years, this approach has increased in sensitivity and power as a result of improvements in two-dimensional polyacrylamide gel electrophoresis (2DE), protein detection and quantification, fingerprinting and partial sequencing of proteins by mass spectrometry (MS), bioinformatics, and methods for gene isolation (Fig. 6). Salekdeh et al. (2002) detected more than 2,000 proteins from drought-stressed and well-watered leaves of rice (line CT9993-5-10-1-M, upland japonica). Among these proteins, the three most marked changes were seen with actin depolymerizing factor, a homologue of the S-like ribonucleases and the chloroplastic glutathione-dependent DHAR. The data showed an increase of about 60% in the abundance of DHAR in drought-stressed plants. Desert plants are exposed to a combination of environmental stress conditions, including low water availability, extreme temperature fluctuations, high irradiance and nutrient deprivation. Studying desert plants within their natural habitat may therefore reveal novel mechanisms and strategies that enable plants to resist stressful conditions. Mittler et al. (2001) studied the acclimation of *Retama raetam*, an evergreen stem-assimilating desert

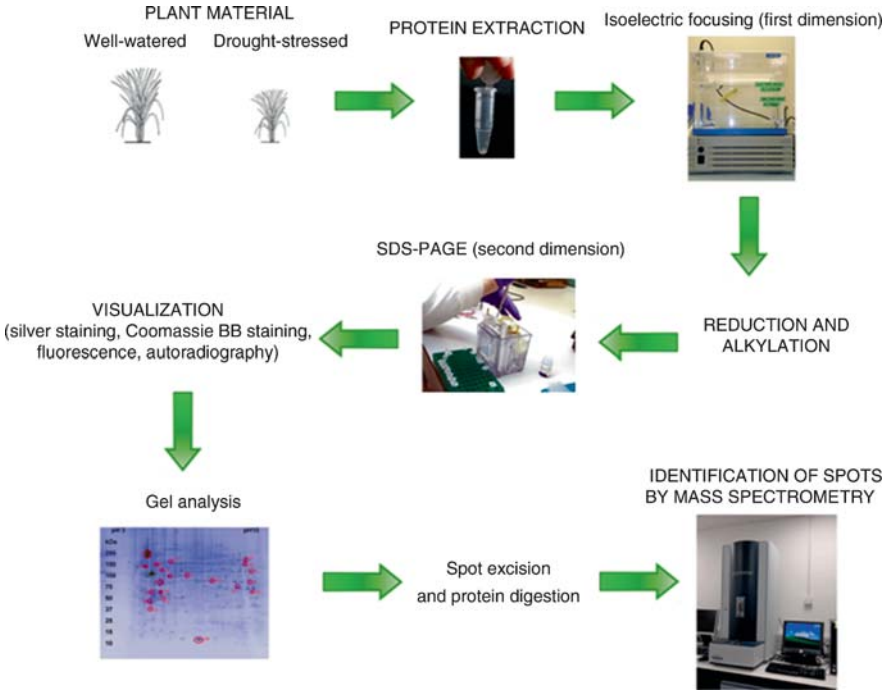

Fig. 6 Scheme for a differential protein display utilizing 2D gel electrophoresis coupled with mass spectrometry

plant, to growth within an arid dune ecosystem. *Retama raetam* contained two different populations of stems: those of the upper canopy, exposed to direct sunlight, and those of the lower canopy, protected from direct sunlight. During the dry season, stems of the upper canopy contained a very low level of a number of essential proteins, including the large and small subunits of rubisco, APX, and the D1 subunit of the reaction centre of photosystem II. Upon rewatering, as well as following the first rainfall of the season, these 'photosynthetically suppressed' stems recovered and accumulated essential proteins within 6 ± 24 h. In contrast, stems of the lower canopy contained the essential proteins throughout the dry season. The authors suggest that *R. raetam* uses an acclimation strategy of 'partial plant dormancy' in order to survive the dry season. 'Dormancy', as evident by the post-transcriptional suppression of gene expression, as well as the suppression of photosynthesis, was induced specifically in stems of the upper canopy which protect the lower canopy by shading.

In order to investigate the unique contribution of individual wine grape (*Vitis vinifera*) berry tissues and water-deficit to wine quality traits, Grimplet et al. (2009) carried out an investigation on tissue-specific differences in protein and selected metabolites, using pericarp (skin and pulp) and seeds of berries from vines grown under well-watered and water-deficit stress conditions. Water-deficit stress altered the abundance of approximately 7% of pericarp proteins, but had little effect on seed protein expression. About half of the 32 metabolites surveyed showed tissue-specific differences in abundance with water-deficit stress affecting the accumulation of seven of these compounds. In particular, the skin displayed a notable increase in the relative abundance of cytosolic APX.

6 Studies on Drought-Tolerant Transgenic Plants

In one of the first experiments on transgenic plants designed to analyze the potential of ROS scavenging system of chloroplasts during drought stress, Shikanai et al. (1998) introduced *Escherichia coli* CAT into tobacco chloroplasts. Photosynthesis of transgenic plants was tolerant to high irradiance (1,600 μmol m^{-2} s^{-1}) under drought conditions, while the wild plants suffered severe damage in photosynthesis under the same conditions. Irrespective of responses to the stress, chloroplastic APX was completely inactivated both in the transgenic and wild-type plants (from about 60 to 0 nmol mg^{-1} protein min^{-1}, and from about 70 to 0 nmol mg^{-1} protein min^{-1} in wild and transgenic plants, respectively). Considering the enzyme stability under oxidative stress conditions, however, the authors concluded that CAT is much superior to APX to confer stress-resistance. These preliminary findings were contrary to the established idea that the APX-mediated antioxidative system protects chloroplasts from oxidative stress but the most of the studies of the last decade pointed out that APX plays an important role in the metabolism of H_2O_2 in higher plants. Recently, Bhatnagar-Mathur et al. (2009) investigated on transgenic plants

of peanut over-expressing the *AtDREB1A* transgene, driven by a stress-inducible promoter (*Atrd29A*) when exposed to progressive drought stress conditions. This authors found APX and GR activities were higher in the transgenic plants than in the untransformed counterparts without the promoter. Moreover, in the transgenic plants, the antioxidative machinery in plants over-expressing the *AtDREB1A* transcription factor under water-limiting conditions indirectly caused improved gas exchange and water use efficiency. Li et al. (2009) studied the effect of over-expressing a *Populus* peroxisomal APX (*PpAPX*) gene under the control of the cauliflower mosaic virus 35S promoter or the rd29 promoter in transgenic tobacco. High levels of *PpAPX* expression were observed in 35S-PpAPX transgenic plants, with a 50% increase in APX activity. The constitutive expression of *PpAPX* in the tobacco exhibited no morphological abnormalities, while significantly increased root growth was observed in transgenic plants, when compared to control plants. APX activity was nearly 80% higher in the leaves of transgenic plants in response to drought or salt stresses. Moreover, the transgenic tobacco also showed significantly improved drought resistance and salt tolerance at the vegetative stage. RNA blot analysis indicated that the *PpAPX* transcript level was very low under normal growing conditions in rd29Ap–PpAPX plants, but significantly increased under drought stress. The authors concluded that that *PpAPX* does not play a significant role under normal growing conditions, but did ameliorate oxidative injury under abiotic stress. Bhatnagar-Mathur et al. (2009) recently investigated on transgenic plants of peanut over-expressing the *AtDREB1A* transgene, driven by a stress-inducible promoter (*Atrd29A*) when exposed to progressive drought stress conditions. These authors found APX and GR activities were higher in the transgenic plants than in the untransformed counterparts without the promoter. Moreover, in the transgenic plants, the antioxidative machinery in plants over-expressing the *AtDREB1A* transcription factor under water-limiting conditions indirectly caused improved gas exchange and water use efficiency.

The effects of re-watering after drought stress and the capacity of plants to resume well after a mild drought have rarely been studied in experiments on transgenic plants. In two interesting works, two cDNAs of the enzyme GR encoding a dual-targeted isoform (dtGR) and a cytosolic isoform (cGR), were cloned from leaves of common bean (*Phaseolus vulgaris*) (accession DQ459505 for dtGR and DQ459504 for cGR) (Contour-Ansel et al. 2006; Torres-Franklin et al. 2008). Moderate drought stress (ψ_w measured at 10 a.m. = −1.5 MPa) followed by re-watering was applied to common bean cultivars, one tolerant to drought ('IPA'), the other susceptible ('Carioca') and to cowpea (*Vigna unguiculata* Walp) cultivars, one tolerant to drought (EPACE-1), and the other susceptible (1183). The results showed that mRNA levels were much higher for *cGR* than for *dtGR* in all cases. Moderate drought stress induced an up-regulation of the expression of *cGR* in the susceptible cultivars. On the contrary, *dtGR* expression decreased. In the tolerant cowpea EPACE-1, GR gene expression remained stable under drought. Total GR activity in bean leaves was 18, 15 and 32 nmol min^{-1} mg^{-1} protein in control, drought-stressed and re-hydrated 'IPA' plants; and 21, 13 and 17 nmol min^{-1} mg^{-1} protein in control, drought-stressed and re-hydrated 'Carioca' plants. The higher

GR activity can be so associated to the higher tolerance of Carioca plants against drought. It is important to note that during recovery from drought, an up-regulation of the two GR isoforms expression occurred, with a peak at 6–10 h after re-hydration. Plant response to re-watering was very rapid: except for *dtGR* in IPA, expression of both GR isoforms was enhanced as soon as 6 h re-hydration for all cultivars. This suggests that moderate drought stress may lead to a hardening process and acclimation tolerance to a subsequent more severe drought. Glutathione has a primary importance for acting also as a substrate of enzymes that take no part in the ascorbate–glutathione cycle. For example, glutathione peroxidase (GPX)-like proteins (GPX-1 and GPX-2) of *Synechocystis* PCC 6803 (S. PCC 6803) reduce unsaturated fatty acid hydroperoxides using NADPH. Gaber et al. (2006) used transgenic *Arabidopsis* plants overexpressing S. PCC 6803 GPX-2 in the cytosol (AcGPX2) or chloroplasts (ApGPX2). Both the transgenic lines showed enhanced tolerance to oxidative damage caused by treatment with H_2O_2 (10 mM), Fe ions (200 mM) or methylviologen (50 mM) and drought for 12 days. The data reported by the authors indicated that the expression of S. PCC 6803 GPX-2 contributes to the reduction in unsaturated fatty acid hydroperoxides using NADPH in situ under stress conditions in the transgenic plants.

Sometimes, a transgenic approach on genes that indirectly affect the expression of enzymatic antioxidant system can be successful, as in the case of the work of Synková and Valcke (2001). The fact that P*ssu-ipt* tobacco, despite a permanent water deficit, can maintain almost unaffected photosynthesis suggested to the authors that some efficient protecting mechanisms exist. The response of antioxidant enzymes to cyclic drought was studied in control non-transformed tobacco (*Nicotiana tabacum* cv. 'Petit Havana SRI') and two types of transgenic P*ssu-ipt* tobacco (grafted on wild rootstock and poorly rooted progeny of Fl generation) grown under different conditions of irradiation (greenhouse, referred as high light, versus growth chamber, referred as low light). Transgenic P*ssu-ipt* tobacco contains the *ipt* gene, encoding for the isopentenyl transferase, a key enzyme of the citokinins biosynthesis. Drought stress cycles (a period of 4 weeks of a water-deficit cycle, i.e., 3 days withholding irrigation followed by re-watering) started with plants at two contrasting developmental stages, i.e., at the stage of vegetative growth (young), and at the onset of flowering (old). Drought reduced the growth of 'SRI' plants compared with transgenic ones, particularly, when treatment started in earlier stage of plant development. Relative leaf water content was significantly lower (below 70%) in all transgenic grafts and plants compared with the wild type, irrespective of age, drought, and growth conditions. The response of antioxidant enzymes was significantly dependent on plant type and plant age; nevertheless, growth conditions and drought stress also affected enzyme activities. Contrary to non-transgenic tobacco, where significant changes of GR activity were found between control and drought-stressed plants grown in a greenhouse, both transgenic types exhibited unchanged activities throughout plant stress treatment. In contrast to non-transgenic and P*ssu-ipt* rooted plants, peroxidase activities (APX, POD and syringaldazine peroxidase) in older P*ssu-ipt* grafts were up to four times higher, irrespective of growth and stress, nevertheless, the effect seemed to be

age-dependent. The differences observed in activities of enzymes of intermediary metabolism (i.e., malic enzyme and glucose-6-phosphate dehydrogenase) revealed that transgenic grafts probably compensated differently for a decrease of ATP and NADPH than control and transgenic rooted plants under stress.

In a recent research, Zhang et al. (2009) demonstrated that the adaptive responses of plants to drought stress is due to a better antioxidant response. For the experiment, they used a transgenic tobacco line Over-expressing the 9-*cis*-epoxycarotenoid dioxygenase gene (*SgNCED1*), with increased ABA content, and tolerance to drought and salt stresses. H_2O_2 and nitric oxide (NO) contents were enhanced in guard cells and mesophyll cells of the transgenic plants, accompanied with increased transcripts and activities of antioxidant enzymes including SOD, CAT, APX and GR. The abundance of H_2O_2 and NO levels, and of the transcripts and activities of antioxidant enzymes in the transgenic plants was blocked by pre-treatments with inhibitor of ABA biosynthesis, scavengers of H_2O_2 and NO, and inhibitors of NADPH oxidase and NO synthase. The elevated production of NO in the transgenic plants was blocked by scavenger of H_2O_2 and inhibitors of NADPH oxidase, whereas H_2O_2 level was not affected by scavenger of NO and inhibitor of NOS-like, indicating that H_2O_2 is essential for the elevated production of NO. The results demonstrate that the increased drought and salt tolerance in the transgenic plants is associated with ABA-induced production of H_2O_2 via NADPH oxidase and NO via NOS-like, which sequentially induce transcripts and activities of SOD, CAT, APX and GR.

7 Some External Applications on Plants for a Better Drought Tolerance

The positive effects of pesticides, exogenous ascorbate, sodium nitroprusside, salicylic acid, silicon or UV-B radiation on drought tolerance of plants were often associated with the increase of antioxidant defense abilities, therefore alleviating oxidative damage of cellular functional molecules induced by over produced ROS under drought and maintaining many physiological processes of stressed plants (Gong et al. 2005).

In order to examine whether paraquat modifies the functioning of antioxidants and oxidative stress levels in drought-stressed plants, Liu et al. (2009) carried out an experiment with *Cucumis sativus* (cv. 'Yuexiu no. 3') grown hydroponically. Drought stress, increased the contents of $O_2 \cdot^-$ and H_2O_2 in cucumber leaves, while pretreatment of paraquat combined with drought increased them in a lower extent. Drought stress and paraquat application both increased the activities of antioxidants such as SOD, CAT, POD, APX, DHAR, MDHAR, GR, GSH and AsA. Furthermore, the combined effect of paraquat application and drought stress resulted in the highest activities of enzymatic and non-enzymatic antioxidants. In conclusion, paraquat is able to moderate the activities of scavenging system enzymes, and to influence oxidative stress intensity under drought stress induced by PEG. Jaleel et al. (2007) conducted a pot culture experiment to estimate the drought stress mitigating effect

of ketoconazole (KCZ), a fungicide cum plant growth regulator, in *Catharanthus roseus* plants. The plants under pot culture were subjected to drought stress (10, 15 and 20 days) and drought stress with KCZ from 30 days after sowing and regular irrigation was kept as control. Antioxidant contents and activities of antioxidant enzymes were estimated from roots and leaves of both control and treated plants. Individual and combined drought stress and KCZ treatments increased ascorbate and α-toc contents, SOD, APX, CAT and PPO activities when compared to control. Unfortunately, the authors did not report the values of the control plants, so it is impossible to do a precise comparison. Another investigation was carried out to find out the extent of changes occurred in groundnut (*Arachis hypogaea* cv. 'ICG 221') in response to paclobutrazol (PBZ) treatment under water deficit (Sankar et al. 2007). Individual treatment with PBZ (10 mg l^{-1}) and drought stress showed an increase in foliar t-Asc and α-toc contents, and GSH, SOD, APX and CAT activities. PBZ with drought stressed plants determined higher levels of antioxidant and scavenging enzymes than drought alone. In the study of Manivannan et al. (2007), a pot culture experiment was conducted to estimate the ameliorating effect of propiconazole (PCZ) on drought stress in cowpea (*Vigna unguiculata*) plants. From 30 days after sowing, the plants were subjected to 9 days of drought stress, and to drought stress with 15 mg l^{-1} PCZ. The plants were separated into root, stem and leaf for estimating the antioxidant contents and activities of antioxidant enzymes. Individual and combined drought stress and PCZ treatments increased t-Asc and α-toc contents, SOD, APX, CAT and PPO activities when compared to control. The PCZ treatment mitigated the adverse effects of drought stress by increasing the antioxidant potentials and thereby paved the way for overcoming drought stress in *V. unguiculata* plants. A pot-culture experiment was conducted to estimate the ameliorating effect of triadimefon (TDM) on drought stress in sunflower plants (*Helianthus annuus*) (Manivannan et al. 2008). Triazole compounds such as TDM, hexaconazole, uniconazole and paclobutrazol, etc., are widely used as fungicides, and they also possess varying degrees of plant-growth regulating properties, mediated by their interference with the isoprenoid pathway and subsequent shift in the balance of important plant hormones, including GA, ABA and cytokinins. The plants were subjected to 3, 6, and 9 days of drought stress and drought stress with TDM at 15 mg l^{-1}. The plant samples were collected and separated into root, stem and leaf for estimating the activities of antioxidant enzymes. Individual and combined drought stress, and TDM treatments increased APX activity when compared to control plants. From the results of this investigation, it can be concluded that the application of TDM caused a partial amelioration of the adverse effects of drought stress by its influence on antioxidant potentials of *H. annuus* plants.

Although ascorbate has been firmly associated with antioxidant response, recent studies have suggested that the functions of ascorbate are related primarily to developmental processes (Smirnoff 2005). Nitrogen fixation in legumes is dramatically inhibited by drought stresses, and this reduction is often associated with oxidative damage. Zabalza et al. (2008) examined the hypothesis that ascorbate is involved in alleviating the oxidative damage to nodules caused by an

increase in ROS under drought stress. The hypothesis was tested by supplying 5 mM ascorbate to pea plants (*Pisum sativum*) experiencing moderate drought stress (predawn $\psi_w = -1.0$ MPa). A supply of exogenous ascorbate increased the nodule t-Asc, whereas the levels of AsA, DHA, GSH, GSSG, GSH + GSSG were not affected. AsA application did not significantly modulate the response to drought stress of APX, whereas modulated that of GR. The effects of water-deficit stress and foliar application of ascorbate were also studied in leaves of *Zea mays* (Dolatabadian et al. 2009). Foliar application was performed by backpack sprayer and four concentrations of AsA (0, 50, 100 and 150 ppm) were applied. The activity of some antioxidant enzymes was clearly increased by water-deficit stress (soil ψ_w of −1.3 MPa). Foliar application of ascorbate reduced stress-induced and antioxidative enzymes activities. It seems that, ascorbate application helps the plants for better resistance under the stress by inactivation and scavenging of free radicals. The authors concluded that ascorbate treatment reduced the damaging action of drought and decreased enzyme activity due to ROS scavenging; thereupon it may be effective for the improvement of stressed plants in arid and semi-arid regions.

In a recent research of Nasibi and Kalantari (2009) on tomato, sodium nitroprusside (SNP) was used as NO donor in control and drought-stressed plants, and the role of NO in reduction of oxidative damages was investigated. The authors observed that SNP pretreatment (plants were sprayed with SNP 100 μM) prevented drought-induced decrease in RWC, membrane stability index, increase in lipid peroxidation and lipoxygenase activity, and increase in H_2O_2 content. However, pretreatment of plants with SNP and phenyl 4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (a NO scavenger) (plants were sprayed with PTIO 200 μM) reversed the protective effects of SNP suggesting that protective effect by SNP is attributable to NO release. In addition, the relationship between these defense mechanisms and activity of antioxidant enzymes were checked. Results showed that in drought-stressed plants APX and CAT activities were elevated over the well-watered controls, while GR decreased under drought condition. The activity of APX and GR increased under SNP pretreatment and it seems that under this condition APX had a key role of detoxification of ROS in tomato plants. This result corresponded well with AsA, DHA and total acid-soluble thiols content. Therefore, reduction of drought-induced oxidative damages by NO in tomato leaves is most likely mediated through either NO ability to scavenge active oxygen species or stimulation of antioxidant enzyme such as APX. Exogenous salicylic acid has been also shown to confer tolerance against biotic and abiotic stresses. In the work of Horváth et al. (2007), the ability of salicylic acid to increase abiotic stress tolerance was demonstrated: it improved the drought tolerance of the winter wheat (*Triticum aestivum*) cv. 'Cheyenne'. The induction of drought tolerance in Cheyenne was correlated with an increase in APX activity (from 0.04 $\Delta A\ mg^{-1}$ protein in non-treated plants to almost 0.06 $\Delta A\ mg^{-1}$ protein in treated plants).

Drought-induced changes in oxidative damage to photosynthetic pigments, proteins and lipids, some enzyme activities and photosynthesis were investigated in wheat (*Triticum aestivum*) plants grown in pots applied with or without silicon

under drought stress (Gong et al. 2005). Three treatments were prepared: well-watered plants, drought-stressed plants and drought-stressed plants + silicon (2.11 mmol of sodium silicate kg^{-1} soil). The results showed that application of silicon improved the water status of drought stressed plants. Compared with the non-silicon treatment, application of silicon increased the foliar activities of SOD, CAT, and GR, the fatty acid unsaturation of lipids, and the contents of photosynthetic pigments and soluble proteins as well as total thiols under drought, whereas the content of H_2O_2 and oxidative stress of proteins were decreased by applying silicon compared with those of non-silicon treatments under drought. The activities of POD and APX showed no significant difference between silicon treated and untreated plants. In addition, application of silicon also increased the net CO_2 assimilation rate of wheat leaves under drought. Physiological and biochemical responses of wheat seedlings to drought, UV-B radiation, and combined stress were investigated by Tian and Lei (2007). Oxidative damage caused by mild drought (ψ_w of −0.5 MPa), UV-B (3.5 kJ m^{-2} UV-B radiation), and combined stresses retarded seedling growth by 26.5%, 29.1%, and 55.9%, respectively. The activities of SOD, POD, and APX increased under drought, UV-B, and the combination of stresses, while CAT activity decreased under the combined stress as compared to the control. The combination of drought and UV-B caused more severe damage to wheat seedlings than stress factors applied separately. Thus, the combined application of drought and UV-B had more strong adverse effects on wheat seedlings. The addition of 0.2 mM sodium nitroprusside (SNP) enhanced wheat seedling growth under drought, UV-B, and combined stress, likely, due to decreasing the accumulation of H_2O_2 and lipid peroxidation as well as activating the antioxidant enzymes.

8 Conclusions and Perspectives

The last decade of research on enzymatic and non-enzymatic antioxidants within the ascorbate–glutathione cycle have revealed that photoprotection against drought-mediated oxidative stress is as complex and intricately regulated. A conspicuous increase in the studies on antioxidant enzymes and their trasnscripts, and on transgenic plants with enhanced antioxidant defenses is expected in the next years. It is not easy to predict if this new knowledge will lead to the creation of varieties with enhanced drought tolerance, as antioxidation does not readily lend itself to improvement via single-gene transgenic up-regulation (Logan 2005).

One of the most studied antioxidant enzyme will probably be ascorbate peroxidase, as it is a unique class I peroxidase found mainly in photosynthetic algae and plants, with a high structural homology to yeast cytochrome c peroxidase (Mittler and Poulos 2005), and uses ascorbic acid as its preferred reducing substrate. Most of the studies herein reported have demonstrated that APX, due to its presence in every compartment of the plant cell and its highly regulated expression (transcriptional and posttranscriptional) is a key defense enzyme involved in the removal of H_2O_2 in plants. In addition, the studies discussed show that ascorbate and glutathi-

one are highly abundant metabolites in plants and that they have many diverse and important functions, such as the H_2O_2-mediated signal transduction cascades. Finally, the intra-cellular distribution of the enzymes of the ascorbate–glutathione cycle is still not completely clear, and more experimentation is required to determine whether the chloroplast and cytosol enzymes fulfill different roles. Further studies are required to elucidate how the function of the different genes/enzymes of the ascorbate–glutathione cycle are coordinated and whether or not they have additional functions in plants.

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