Ramamurthy Mahalingam *Editor*

Combined Stresses in Plants

Physiological, Molecular, and Biochemical Aspects

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Preface

The renowned Greek philosopher Heraclitus' famous quote "Change is the only constant in life" seems very apt in a plant's life. Plants, being sessile in nature, are exposed to a wide variety of environmental perturbations from seed germination to senescence. These environmental changes can be caused due to abiotic and biotic factors. Abiotic factors includes physical aspects of a plant's environment such as soil moisture conditions, soil nutrients, and climatic components such as light, temperature extremes, air pollutants, UV-radiation, and wind. Biotic factors encompass pathogens, pests, parasites, animals, and humans. It is also apparent that the various biotic and abiotic factors are constantly changing during the life cycle of a plant. Furthermore, these external factors co-occur in nature. Plants have to make decisions about fine-tuning their responses to allocate resources efficiently for responding to the more serious threats at any given point in time. Paradoxically, most studies of stress responses in plants focus on a single inciting agent. From the point of view of conducting a well-controlled experiment it is the most ideal strategy. However, the results from such studies may not necessarily mimic the response that a plant would elicit under realistic field conditions where multiple factors are simultaneously operating. In recent years several research groups working on different stress combinations and in different plant species have shown that plants evoke a "unique response" to combined stresses. In other words, combined stress response is not just an additive effect of the responses elicited when the stresses are imposed singly.

The unique responses to combined stresses in plants have been observed at the physiological, biochemical, and molecular levels. The chapters in this book address all the three levels of change in various plants in response to various combinations of stresses.

Chapter 1 provides a general review of the combined stress paradigm. Chapters 2 through 4 focus on the impact of higher CO_2 levels in combination with other stresses (temperature, salinity, and soil contaminants). In Chapters 5 through 8 drought stress is examined in conjunction with other abiotic factors (salinity, heat, and ozone) in different crop plants. Chapters 9 and 10 examine the combination of biotic and abiotic factors. The impact of combined stresses in forest ecosystems are discussed in Chapters 11 and 12.

It is my sincere appeal that the plant stress community embraces the concept of combined stress in their future research. A much-needed second green revolution can become a reality when we incorporate the concept of combined stresses in plant stress research.

This book would not have been possible without the contributions of the experts who were willing to share their knowledge in various stress combinations, and my heartiest thanks to each of them. I would like to convey my thanks to Mr. Eric Stannard of Springer Science+Business Media for broaching the theme of combined stress in plants for a book. I would also like to extend my thanks to my production editor, Mr. Joseph Quatela, along with the entire production team for their efforts in bringing out this book. I would like to convey my sincere thanks to Dr. John Gustafson, professor and head of the department of Biochemistry and Molecular Biology at Oklahoma State University for his encouragement and support for taking up this book project.

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Abbreviations

A	Net photosynthetic rate
ABA	Abscisic acid
ABF	ABRE binding factor
ABRE	ABA response element
AB-QTL	Advanced backcross—quantitative trait locus
AOC	Allene oxide cyclase
AOS	Allene oxide synthase
APX	Ascorbate peroxidase
AREB	ABA response element binding protein
AsA	Ascorbate
ASM	Available soil moisture
ATAF1	Arabidopsis thaliana activating factor 1
ATL4	Arabidopsis Toxicos En Levadura
BABA	Beta-amino-butyric acid
BCAA	Branched chain amino acids
BM	Biomass
CAT	Catalase
CBF	C-repeat binding factor
CCA1	Circadian clock associated 1
CDPK	Calcium-dependent protein kinase
CE-MS	Capillary electrophoresis-mass spectrometry
CH_4	Methane
Chl	Chlorophyll
Chl*	Excited chlorophyll
CN	Cyst nematode
CO_2	Carbon dioxide
COI1	Coronatine insensitive 1
Cyt	Cytochrome
DAF	Days after flowering
DAS	Days after sowing
DH	Doubled haploid
DMNT	(*E*)-4,8-dimethyl-1,3,7-nonatriene

DREB	Dehydration response element binding protein
E_s	Soil evaporation
EREB	Ethylene responsive element binding protein
ET	Ethylene
ET	Evapotranspiration
ETc	Crop evapotranspiration
FAC	Fatty acid chains
FACE	Free-air CO_2 enrichment
FC	Field capacity of soil
FDR	False discovery rate
FLN	Flower number
FT-ICR-MS	Fourier transformation cyclotron resonance mass spectrometry
GABA	Gamma-aminobutyric acid
GC-MS	Gas chromatography-mass spectrometry
GLV	Green leaf volatiles
GR	Glutathione reductase
GSH	Glutathione
H_2O_2	Hydrogen peroxide
HCL	Hierarchical clustering analysis
HI	Harvest index
HSF	Heat shock factor
HSP	Heat shock protein
IPCC	Intergovernmental Panel on Climate Change
JA	Jasmonic acid
JAZ	Jasmonate ZIM-domain
LC-MS	Liquid chromatography-mass spectrometry
LD	Linkage disequilibrium
LEA proteins	Late embryogenesis abundant proteins
LOX	Lipoxygenase
LTP	Lipid transfer protein
MCP	1-Methylcyclopropene
MDMV	Maize dwarf mosaic virus
MEOX derivative	Methoxime derivative
MQTL	Meta-quantitative trait locus
MV	Methyl viologen
NMR Spectroscopy	Nuclear magnetic resonance spectroscopy
NO	Nitric oxide
NO_2	Nitrogen dioxide
NO_x	Oxides of nitrogen
NOA	NO-associated protein
NR	Nitrate reductase
$O^{\bullet -}$	Activated oxygen atom
$O_2^{\bullet -}$	Superoxide radical
O_2	Atmospheric oxygen
O_3	Ozone
$^{\bullet}OH$	Hydroxyl radical

OPDA	12-Oxo-phytodienoic acid
OPR3	OPDA reductase 3
OTC	Open top chambers
PAMP	Pathogen-associated molecular patterns
P5CS	Delta 1-pyrroline-5-carboxylate synthetase
PC	Plastocyanin
PCA	Principal component analysis
PCR	Polymerase chain reaction
PMWaV-1	Pineapple mealybug wilt-associated virus-1
POD	Peroxidase
PPN	Plant parasitic nematodes
PR	Pathogenesis-related
PSI	Photosystem I
PSII	Photosystem II
PQ	Plastoquinone
QTL	Quantitative trait loci
RH	Relative humidity
RKN	Root knot nematode
RPF1	Ribosome production factor 1
ROS	Reactive oxygen species
RuBisCO	Ribulose-1,5-bisphosphate carboxylase
SACC	Screen aided CO_2 control
SAM analysis	Significant analysis of microarrays
SAR	Systemic acquired resistance
SAT	Semi-arid tropics
SCF	Skp, Cullin, F-box
SIPK	Salicylic acid-induced protein kinase
SLA	Specific leaf area
SO_x	Oxides of sulphur
SOD	Superoxide dismutase
SOS	Salt overly sensitive
T	Transpiration
T_{calc}	Calculated transpiration
T_{sla}	Specific leaf area based transpiration
T_{sim}	Simulated transpiration
TBARS	Thiobarbituric acid reactive substances
TC	Thermocouple
TCA cycle	Tricarboxylic acid cycle
TE	Transpiration efficiency
TMS	Trimethylsilyl derivative
TMTT	(*E, E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene
TMV	Tobacco mosaic virus
TSI1	Tobacco stress-induced1
TuMV	Turnip mosaic virus
VOC	Volatile organic compounds
VPD	Vapour pressure deficit
WIPK	Wound-induced protein kinase

Chapter 1
Consideration of Combined Stress: A Crucial Paradigm for Improving Multiple Stress Tolerance in Plants

Ramamurthy Mahalingam

1.1 Introduction

Food security is a major issue in the global policy agenda. In the next 40 years, demand for cereal production is predicted to increase by 60 % as the population rises from the current 6.6 to 8.7 billion by the year 2050 (Bengtsson et al. 2006). In a world where population growth exceeds food supply (Malthus 1817), a second green revolution is necessary. But the challenges in overcoming the constraints in food production are complex. The ongoing change in climate mostly due to anthropogenic activities causes increases in carbon dioxide (CO_2) emissions (Peters et al. 2011), further exacerbating the agricultural land deterioration due to increasing temperature (Kissoudis et al. 2014). Increasing temperature in turn leads to higher evapotranspiration, drought intensification, and increasing soil salinization (Munns and Tester 2008; Zhao and Running 2010). Though the existing data on the impact of climate change on pathogen spread are inconclusive, evidence points to increased reproductive potential and geographic expansion leading to interactions with more hosts and new virulent pathogenic strains (Garrett et al. 2006). An analysis of the natural disasters that resulted in more than a billion dollars in the USA in the past three decades clearly shows that both the frequency and intensity of these events are increasing (Fig. 1.1). Hence, the chances of plants encountering new combination of stresses in the future are likely to be higher. It thus behooves upon plant scientists working on stress resistance to consider the combination of stresses that are likely to co-occur under field conditions.

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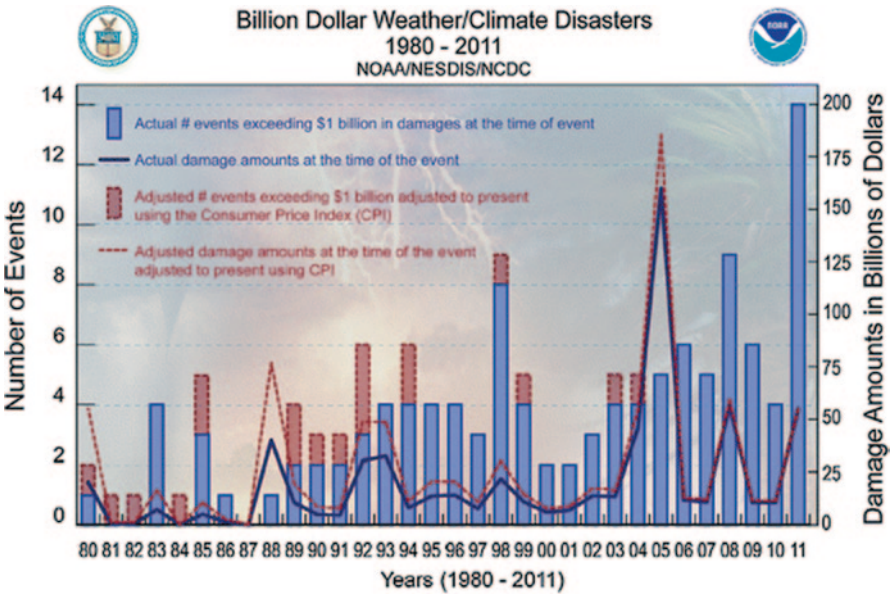

Fig. 1.1 The US billion-dollar weather and climate disaster time series from 1980 to 2011. (Adapted from reference Smith and Katz 2013)

1.2 Importance of Combined Stress

Literature is replete with studies on plant responses to stresses. PubMed search using keywords "stress" and "plants" in title and abstract field alone identified nearly 15,300 citations while "combined stress" and "plants" retrieved 480 citations. A closer inspection of the latter search revealed only around 180 original articles that actually dealt with the combination of two or more stresses in plants. A listing of primary research articles on combined stress in various plant species is given in Table 1.1.

The combined occurrence of drought and heat in the USA from 1980 to 2012 was shown to cause fivefold more damage when compared to drought alone (Fig. 1.2). Increase in global surface temperature is a major indicator of global warming (Van Vuuren et al. 2008). This rise in mean global temperature is attributed to increases in the greenhouse gases such as CO_2 and air pollutants such as ozone (O_3) that have been brought about by anthropogenic activities. For the first time in recorded history, the average level of CO_2 has topped 400 parts per million (ppm) for an entire month in April 2014 according to the Scripps Institution of Oceanography. Efforts to control CO_2 emissions on a global scale will be difficult to enforce given the political and economic implications surrounding such legislations. More than 400 ppm of CO_2 may thus be the new reality for crop plants in the future.

Table 1.1 Primary research studies of combined stresses in various plant species

Stress combination	Plant species (references)
Drought + heat	*Arabidopsis* (Koussevitzky et al. 2008; Rizhsky et al. 2004; Vile et al. 2012; Wolfe and Tonsor 2014), tobacco (Cvikrova et al. 2013; Rizhsky et al. 2002), wheat (Keles and Oncel 2002; Prasad et al. 2011; Rampino et al. 2012; Szucs et al. 2010; Yang et al. 2011), *Sorghum* (Johnson et al. 2014), *Carissa spinarum* (Zhang et al. 2010), lotus (Sainz et al. 2010), soybeans (Simon-Sarkadi et al. 2005), *Jatropha* (Silva et al. 2010); barley (Rollins et al. 2013); poplar (Centritto et al. 2011); prosopis (Delatorre et al. 2008)
Drought + chilling	Sugarcane (Sales et al. 2013), maize (Aroca 2003)
Drought + high light	*Arabidopsis* (Estavillo et al. 2011; Giraud et al. 2008), *Haberlea rhodopensis* (Georgieva et al. 2010), rice (Zhou et al. 2007), watermelon (Nanasato et al. 2005), pearl millet and *Sorghum* (Masojidek et al. 1991); *Nerium oleander* (Demmig et al. 1988)
Drought + heavy metals	Red maple (de Silva et al. 2012); *Populus cathayana* (Han et al. 2013); oak (Sardans and Penuelas 2007); Stackhousia tryonii (Bhatia et al. 2005)
Drought + ozone	Birch (Paakkonen et al. 1998), beech (Nunn et al. 2007), *Medicago truncatula* (Iyer et al. 2013), Quercus (Alonso et al. 2014), poplar (Bohler et al. 2013), *Dactylis glomerata*, and *Ranunculus acris* (Wagg et al. 2012); wheat (Biswas and Jiang 2011; Herbinger et al. 2002), spruce (Karlsson et al. 1997; Kivimaenpaa et al. 2003); *Pinus halepensis* (Manes et al. 2001; Fontaine et al. 2003)
Drought + salinity	Barley (Ahmed et al. 2013a, b, c); *Sesuvium portulacastrum* (Slama et al. 2008)
Drought + soil compaction	Tobacco (Alameda et al. 2012)
Drought + nutrients	Maize (Kandianis et al. 2013; Makumburage and Stapleton 2011); wheat (Wei et al. 2013); potato (Germ et al. 2007)
Drought + UV	Maize (Makumburage et al. 2013); wheat (Feng et al. 2007; Zhao et al. 2009); *Arabidopsis* (Comont et al. 2012; Schmidt et al. 2000); barley (Bandurska et al. 2012); peas (Nogues et al. 1998); *Populus cathayana* (Lu 2009); willows (Turtola 2006); soybeans (Sullivan and Teramura 1990)
Drought + high CO_2	Potato (Barnaby et al. 2014); maize (Sicher and Barnaby 2012); *Phaseolus vulgaris* (Medeiros and Ward 2013); *Viguiera discolor* (Oliveira et al. 2013); eucalyptus (Crous et al. 2012; Duursma et al. 2011; Lewis et al. 2013; Zeppel et al. 2011); maize and sorghum (Allen et al. 2011; Kakani et al. 2011; Leakey et al. 2006); pepper (del Amor et al. 2010); populus (Bobich et al. 2010); cucumber (Li et al. 2008); oak and pine (Schwanz et al. 1996)
Drought + pathogens/pest	*Arabidopsis* (Atkinson et al. 2013; Anderson et al. 2004); tobacco (Ramegowda et al. 2013); rice (Campo et al. 2012); *Alnus fruticosa* (Rohrs-Richey et al. 2011); beet and rice (Xu et al. 2008)
Salinity + heat	Tomato (Rivero et al. 2014); poplar (Behnke et al. 2013); *Artemisia* (Wen et al. 2005) *Swietenia macrophylla* (Rahman et al. 2013)

Table 1.1 (continued)

Stress combination	Plant species (references)
Salinity + ozone	Alfalfa (Maggio et al. 2009); chickpea (Welfare et al. 2002); rice (Welfare et al. 1996); wheat (Zheng et al. 2012)
Salinity + pathogens	Rice (Xiong and Yang 2003)
Salinity + nutrients	Barley (Talbi Zribi et al. 2011); *Hordeum maritimum* (Talbi Zribi et al. 2012); spinach (Kaya et al. 2001); soybeans (Grattan and Maas 1988); peanuts (Silberbush and Ben-Asher 1989); *Crithmum maritimum* (Labidi et al. 2011); broccoli (del Carmen Martinez-Ballesta 2008)
Salinity + high CO_2	*Arabidopsis* (Kanani et al. 2010); *Spartina maritima* (Mateos-Naranjo et al. 2010a); barley (Perez-Lopez et al. 2009, 2012); pepino (Chen et al. 1999); melon (Mavrogianopoulos et al. 1999); citrus (Garcia-Sanchez et al. 2006); olive (Melgar et al. 2008); aster (Geissler et al. 2009, 2010); tomato (Takagi et al. 2009); *Spartina densiflora* (Mateos-Naranjo et al. 2010b)
Heat + ozone	Birch (Kasurinen et al. 2012; Maenpaa et al. 2011; Riikonen et al. 2009, 2013); spruce (Riikonen et al. 2012); populus (Hartikainen et al. 2009); bean (Albertine and Manning 2009); radish (Kleier et al. 2001)
Heat + light	Sunflower (Hewezi et al. 2008); *Brassica* (Diaz et al. 2007), oats (Quiles 2006); seagrass (York et al. 2013); apple (Chen et al. 2008); grapes (Greer and Weedon 2012); *Arabidopsis* (Burgos et al. 2011; Lokhande et al. 2003; Vasseur et al. 2011); *Dunaliella salina* (Haghjou et al. 2009); *Phragmites australis* (Loreto et al. 2006); wheat (Monneveux et al. 2003); spruce (Mahoney et al. 1998)
Heat + UV	Wheat (Zheng et al. 2011); cucumber (Caldwell 1994)
Heat + high CO_2	Tomato (Li et al. 2014b); Kentucky bluegrass (Song et al. 2014); aspen (Sun et al. 2013); soybeans (Sicher 2013); rice (Madan et al. 2012); eucalyptus (Loveys et al. 2006); bell pepper (Aloni et al. 2001; Karni and Aloni 2002); *Abutilon theophrasti* (Ziska 2001); cotton and tobacco (Crafts-Brandner and Salvucci 2000)
Temperature + pathogens	*Arabidopsis* (Szittya et al. 2003; Yang and Hau 2004; Zhu et al. 2010); tomato (de Jong et al. 2002)
Ozone + high CO_2	Soybeans (Ainsworth et al. 2008; Gillespie et al. 2012); populus (Kets et al. 2010); wheat (Mishra et al. 2013)
Ozone + UV	Linseed (Tripathi and Agrawal 2013a, b); birch (Pliura et al. 2008); *Elymus athericus* (van de Staaij et al. 1997)
Ozone + pathogens	Tobacco (Ye et al. 2012); soybeans (Bilgin et al. 2008); Beech and spruce (Luedemann et al. 2005)
Chilling + high light	Tomato (Wang et al. 2008); cotton (Kornyeyev et al. 2001; Payton et al. 2001)
Chilling + pathogens	*Arabidopsis* (Yang et al. 2010)
UV + heavy metals	*Brassica campestris* (Shukla et al. 2008); *Pisum sativum* (Srivastava et al. 2012)
UV + pathogens	*Arabidopsis* (Kunz et al. 2006); tea (Gunasekera et al. 1997); cabbage (Brown et al. 2001); tobacco (Yalpani et al. 1994)

Table 1.1 (continued)

Stress combination	Plant species (references)
High CO_2 + high light	*Chlorella* (Kozlowska-Szerenos et al. 2004)
Nutrient + pathogens	*Arabidopsis* (Amtmann et al. 2008)
Drought + heat + high light	*Hibiscus* (Munoz and Quiles 2013); *Rosa meillandina* (Paredes and Quiles 2013); wheat (Sharma and Singhal 1993)
Drought + high light + UV	*Arabidopsis* (Poulson et al. 2006)
Drought + heat + virus	*Arabidopsis* (Prasch and Sonnewald 2013)
CO_2 + temperature + UV	Cowpea (Singh et al. 2010); soybeans (Koti et al. 2005); birch (Lavola et al. 2013)
Ozone + light	*Trifolium subterraneum* (Vollsnes et al. 2009)
CO_2 + temperature + drought	Eucalyptus (Roden and Ball 1996)
UV + nutrients	Vigna radiata (Agrawal et al. 2006); wheat (Shukla et al. 2002)
CO_2 + ozone + insects	Soybeans (Casteel et al. 2008)
CO_2 + temperature + insects	Soybeans (Niziolek et al. 2013)

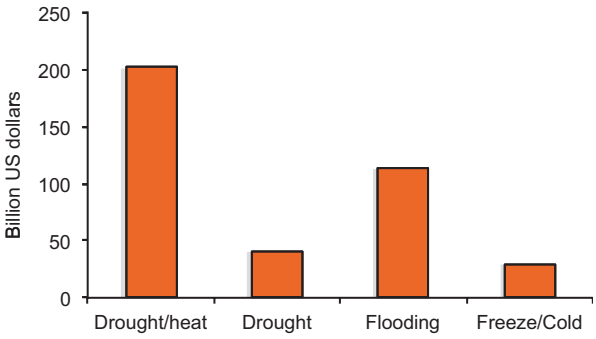

Fig. 1.2 A meta-analysis of naturally occurring disasters in the USA. Losses due to weather-related disasters (excluding tornadoes, hurricanes, and wildfires) occurring between 1980 and 2011 that exceeded more than a billion dollars were included in this analysis. Damage costs were normalized to the 2013 US dollar value. Raw data for this analysis were from reference (Smith and Katz 2013)

Here is an example to illustrate the importance of considering more than a single stress. A recent study indicated that rising CO_2 levels increased the estimated yield levels of soybeans during 2002–2006 by 4.34, 7.57, and 5.10%, in the USA, Brazil, and China, respectively (Sakurai et al. 2014). However, there are other studies using the free-air concentration enrichment (FACE) technology that consider the increasing levels of ozone, the most abundant air pollutant that will negate the fertilizing effects of CO_2 and predict a less-than-expected yield due to the increasing levels of CO_2 (Long et al. 2005, 2006).

1.3 Which Combination of Stresses to Study?

This begs the question which combination of stresses to study. As indicated earlier, plants are continually challenged by diverse array of biotic and abiotic agents from seed germination to senescence. We can envisage considering those stresses that are most likely to co-occur under field conditions and whose combined impact can adversely affect the final yield.

Stress Matrix Approach Mittler and coworkers have advocated the use of a stress matrix showing different combinations of potential environmental stresses that can affect crops. The use of colors to indicate potential positive and negative interactions provides a visually appealing schema for depicting combined stresses (Fig. 1.3). It

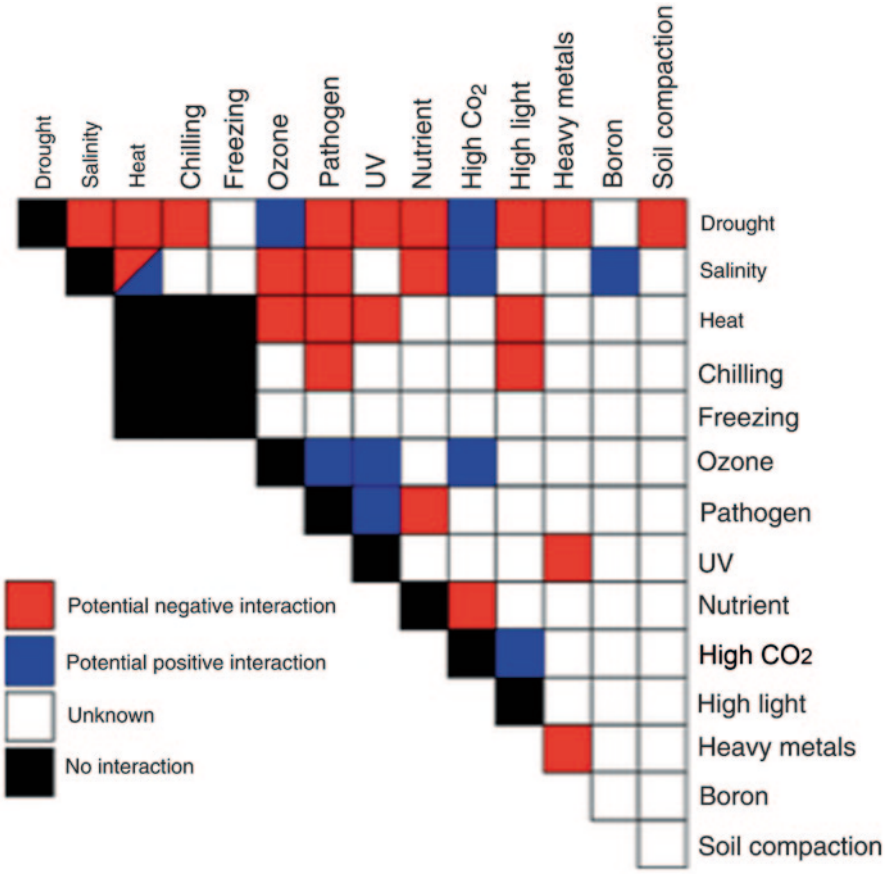

Fig. 1.3 The stress matrix. Different combinations of potential environmental stresses that can affect crops in the field are shown in the form of a matrix. The matrix is color-coded to indicate stress combinations that were studied with a range of crops and their overall effect on plant growth and yield. References for these studies are given in the text and in Table 1.1. (Adapted from Suzuki et al. (2014) and modified from Mittler (2006))

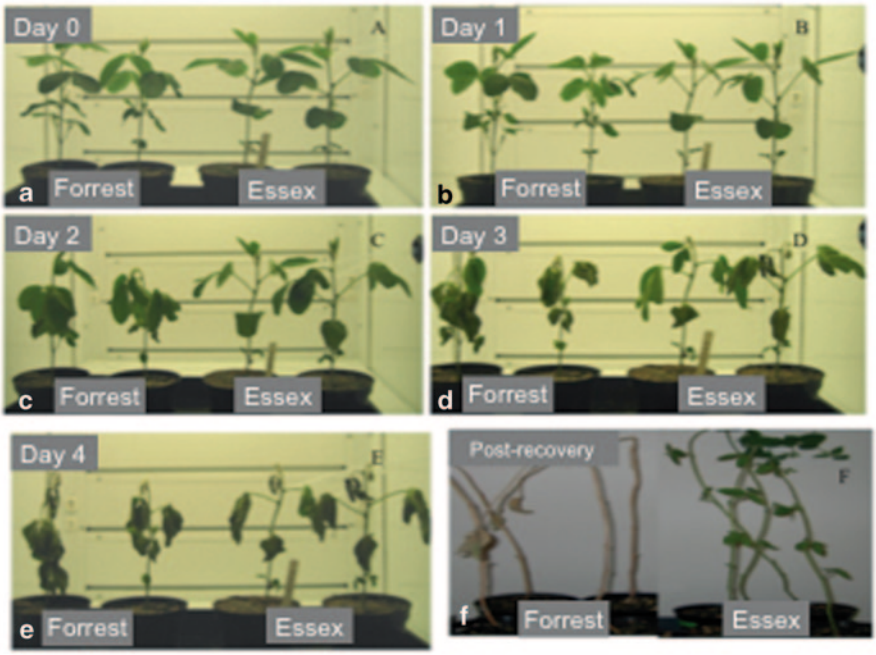

Fig. 1.4 Combined ozone and drought stress in two soybean cultivars. Cultivar Forrest (sensitive to ozone, sensitive to heat) and Essex (tolerant to ozone, tolerant to heat) were simultaneously exposed to 75–100 ppb of ozone and higher temperature of 37 °C for 3 h a day for 3 consecutive days. Photographs were taken at the end of the combined ozone and heat treatment on day 1 (A), day 2 (B), day 3 (C), 24 h after the end of the treatment (E), and 10 days of post-recovery in a growth chamber (F)

should be noted that this is a vast oversimplification of the complexity involved in combined stress scenarios. For example, Medicago truncatula cultivar Jemalong is sensitive to ozone and drought when the stresses were applied singly (Puckette et al. 2007). The combined application of drought and ozone in Jemalong evoked a very different transcriptome and metabolic response that manifested as a stress-tolerant phenotype (Iyer 2013). To test whether this observation can be extended to other legumes, we used the soybean cultivar Forrest that is sensitive to drought and ozone when applied singly. The combined application of drought and ozone for a period of 3 days was detrimental for Forrest plants (Fig. 1.4). Thus, based on single-case studies, it is naïve to interpret the interactions between stress combinations as positive or negative. Biswas and Jiang (2011) reported that, under conditions of combined ozone and drought stress, the ozone-sensitive modern winter wheat cultivar (*Triticum aestivum* L. cv. Xiaoyan 22) improved its tolerance against ozone, while the ozone-tolerant primitive wheat (*Turgidum* ssp. durum) lost ozone tolerance. Crops show wide variability in their phenotypic responses to stresses and this includes both the intra- and inter-specific variation (Biswas et al. 2008; Brosche et al. 2010).

Furthermore, it has been shown that the order in which the stress combinations are applied may evoke a different response. An early drought could lead to a decrease in stomatal conductance and a subsequent protection against a later ozone exposure while the appearance of drought during preexisting ozone stress would suffer under the appearing sluggishness of stomata, initially caused by ozone (Paoletti and Grulke 2010).

Plants can show varied responses to stresses depending on their developmental stage. This adds an additional layer of complexity in the analysis of plant stress studies. If a field is affected by stress at a very early stage of development (e.g., seedling stage), a farmer may be able to undertake second planting and still recover his losses. On the other hand, a severe stress in field during the reproductive stage of development will not be amenable for such amends. It has been reported that most plants of agronomic importance are gullible to abiotic stresses during reproductive stages with detrimental consequences to the yield (Barnabas et al. 2008). Interestingly, the consequences to yield in response to stresses are not considered in most studies involving model plants like *Arabidopsis*. The usefulness of model plants for understanding plant stress responses can be greatly increased by assessing impact of stress on seed yield and seed quality. From an agronomic perspective, the most important aspect of plant stress interactions will be to understand its impact on the final yield.

1.4 Omics of Combined Stress

A detailed review of the transcriptome studies on combined stresses in plants has been reported (Jambunathan et al. 2010). A few proteomic studies on the combined stresses have been reported. This includes drought and ozone in poplar (Bohler et al. 2013), drought, and heat in *Arabidopsis*, barley, *Carissa spinarum* (Koussevitzky et al. 2008; Rollins et al. 2013; Zhang et al. 2010), toxic compounds like mercury and salinity in *Suaeda salsa* (Liu et al. 2013), high temperature and humidity in *Portulaca oleracea* (Yang et al. 2012). Interestingly, transcriptomic and proteomic analysis of several different combined stresses in several different plant species converges on the antioxidant defense machinery as a key pathway. The observed higher antioxidant capacity and/or lower accumulation of the reactive oxygen species (ROS) seems to be a mechanism operative in plants tolerant to combined stresses (Iyer et al. 2013; Koussevitzky et al. 2008; Ahmed et al. 2013b; Perez-Lopez et al. 2009; Rivero et al. 2014; Sales et al. 2013). Omics approaches have also shown that there are unique transcription factors, hormone-responsive genes and osmolytes that are differentially expressed in response to different combined stresses (Iyer et al. 2013; Atkinson et al. 2013; Rasmussen et al. 2013; Rizhsky et al. 2004). An apparent gap in the knowledge is the lack of information on posttranscriptional gene regulation by microRNAs in response to combined stresses. In fact, a comprehensive analysis of transcriptome, proteome, metabolome, and miRNome even in response to a single stress has not been reported. Such integrated omics

studies of combined stresses imposed during reproductive stages of crop development are warranted.

DNA cytosine methylation and histone modifications such as methylation and acetylation affect transcription especially in response to changes in environment (Mirouze and Paszkowski 2011). Epigenetic modifications involving chromatin-regulated gene activation govern priming responses (Conrath 2011) and widespread alterations in DNA methylation have been reported in response to biotic and abiotic stresses (Bilichak et al. 2012; Dowen et al. 2012). The knowledge of epigenetic modifications in the wake of combined stresses is relatively unknown and is worthy of further investigations. It has been speculated that epigenetic modifications in response to a stress may predispose plants to a subsequent stress by either sensitizing or desensitizing. Such acclimation/predisposition may provide a novel avenue for preparing seeds for stressful environments (Kissoudis et al. 2014).

1.5 Phenotypic Responses to Stresses

From an agronomic point of view, the definition of plant sensitivity to stresses can be misleading. For example, crops can be sensitive to ozone with reference to visible foliar damage at early stages of growth but may not have a net impact on the grain yield during harvest. In rice and wheat, plants with least visible foliar symptoms showed maximum yield losses (Picchi et al. 2010; Sawada and Kohno 2009) and this was explained on the basis of stomatal closure response. Cultivars in which ozone causes stomatal closure prevent the influx of ozone and reduce the extent of foliar injury. Thus, based on the damage to leaves, these cultivars are resistant to ozone. However, prolonged stomatal closure affects carbon fixation and in turn the amount of assimilates required for grain filling. Thus, with reference to yield these cultivars are ozone sensitive. Other mechanisms for the negative effect of ozone could be due to the reduction of new growth (McKee and Long 2001), reduced root biomass (Grantz et al. 2006), reduced phloem translocation efficiency, or reduced carbon portioning to grains over synthesis of protective chemicals (Betzelberger et al. 2010).

It is important to understand the differences between sensitive and resistant responses that can differ depending on the stress. Let us consider the example of ozone exposure. The visible injury symptoms due to ozone are mostly assessed by damage to foliage. In sensitive plants, they appear as small chlorotic or necrotic lesions on leaves that can coalesce into larger patches of injured area, and such leaves usually senesce early. This reduces the effective biomass that in turn will take a toll on crop yields (Wilkinson et al. 2012). The same necrotic lesions on the foliage in response to avirulent pathogen infections are termed as hypersensitive response and the plant is considered to be resistant to the pathogen. The characterization of the same phenotype as being resistant with respect to one stress and as sensitive response to another stress is important to bear in mind while considering the combination of biotic and abiotic stresses.

1.6 Contrasts Between Laboratory and Field Studies

In several recent reviews, the limitations of single stress studies in controlled conditions compared to field conditions have been examined (Mittler and Blumwald 2010; Suzuki et al. 2014). The study of combined stresses in the laboratory is advocated so that the molecular pathways for tolerance to stresses that prevail under field condition can be identified. Most of the studies on combined stresses so far have been conducted under growth chamber or greenhouse conditions (Suzuki et al. 2014). Here, we have contrasted the combined stress studies in laboratory conditions versus the field conditions (Fig. 1.5). Combined stresses dealing with edaphic factors can be conducted effectively in greenhouse conditions. This includes the combinations of drought and nutrients, drought and salinity, drought and soil pathogen/pests such as nematodes. Combined stress experiments that involve interactions between climate change factors including CO_2, ozone, and temperature extremes (heat or cold) are ideal for growth-chamber studies. But the main constraint here is the number of large-sized plants that can be accommodated in such chambers. If greenhouse space and infrastructure for regulating gaseous mixtures (for example, CO_2 and ozone) are available, it provides an ideal platform for conducting controlled combined stress analysis of climate change variables and edaphic factors. Several reviews have examined the advantages and disadvantages of open-top chambers (OTCs), FACE systems, and screen-aided CO_2 control (SACC; Ainsworth et al. 2008; Li et al. 2007). Though FACE and OTCs provides an opportunity to examine the impact of climate change factors in actual field environment, it will be hard to use these facilities in combined stress scenarios such as drought or temperature stress. Rainout shelters can be constructed for studying drought in combination with other climate change factors in a FACE but may be expensive.

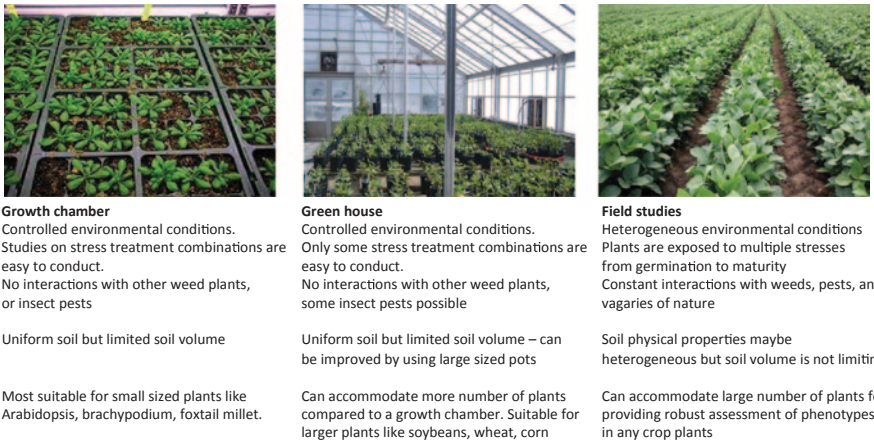

Fig. 1.5 Comparisons between growth chamber, green house, and field studies for analyzing the effects of combined stresses in plants

1.7 Advances in Phenomics

Following the enormous advances in the sequencing technologies, it has now become routine to sequence large collections of accessions or mapping populations in a plant species (Lam et al. 2010; Li et al. 2014a; Weigel and Mott 2009). The major bottleneck currently in utilizing the genome sequence deluge is the ability to procure reliable phenotype data. Over the past decade field, phenotyping has made rapid strides by utilizing remote-sensing technologies for crop monitoring (Furbank and Tester 2011). The field of phenomics described as a "high-throughput plant physiology" makes use of noninvasive imaging, infrared thermography, spectroscopy, robotics, image analysis, and high-performance computing. Several successful phenotyping screens for single stresses such as drought, UVB have been reported in model plant systems (Jansen et al. 2010; Woo et al. 2008) as well as in crop plants (Chapuis et al. 2012; Honsdorf et al. 2014; Sirault et al. 2009).

For UV stress and temperature extremes, the photosynthetic light-harvesting apparatus is often the first site of damage. UV stress can result in oxidative damage to the photosystems, perceived as a loss of efficiency of light harvesting, that can be exploited as a screening tool for tolerance to UVB exposure (Jansen et al. 2010). In the case of temperature extremes, the effects on photosynthesis and even changes in membrane lipid properties can lead to immediate effects on chlorophyll fluorescence (Armond et al. 1980).

Digital imaging in visible wavelength regions provides information on plant size, and also on the color of the plants. This information enables the quantification of senescence arising from nutrient deficiencies or toxicities, or pathogen infections. Germanium, a toxic analog of boron, was tested in a mapping population of barley to identify a Quantitative Trait Loci (QTL) at the same locus as previously identified for boron tolerance using a visual score of symptoms (Schnurbusch et al. 2010).

Near-surface reflectance spectroscopy was used to monitor the leaf nitrogen and chlorophyll content and epoxidation state of xanthophyll cycle pigments in field-grown soybean plants exposed to ozone (Ainsworth et al. 2014). This study shows that the leaf optical properties can be monitored using remote-sensing techniques to assess ozone damage and provide a promising tool for elucidating ozone tolerance in plants.

The examples mentioned above demonstrate the utility of the phenomics tools for precisely monitoring the physiological impacts of single stresses such as drought, salinity, nutrient deficiency, and air pollutants. It is conceivable that these tools will be harnessed for the analysis of combined stresses in the future.

1.8 Strategies for Improving Tolerance to Combined Stresses

Two major strategies can be envisaged for improving the tolerance to combined stresses (Fig. 1.6). First strategy involves the meta-analysis of whole genome expression studies in response to various biotic and abiotic stresses that can be

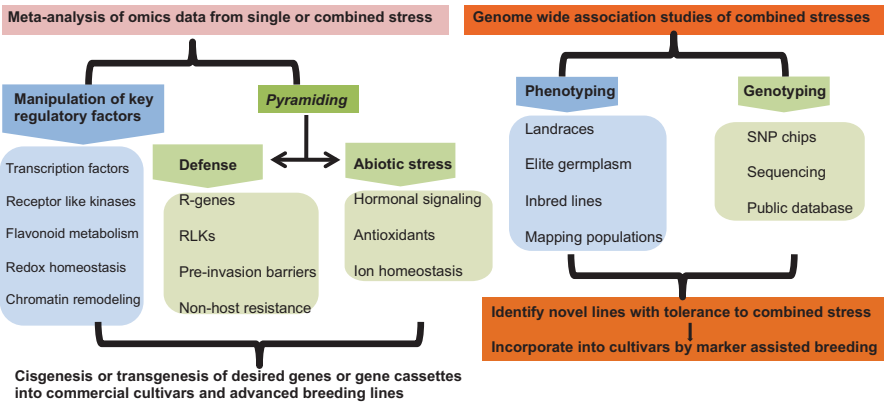

Fig. 1.6 Strategies for building tolerance to combined stresses in plants. A compendium approach for identifying key regulatory factors or by pyramiding key genes important in co-occurring stress scenarios that can be transferred into desired cultivars by genetic engineering. Another strategy will be to use genome-wide association mapping to identify novel germplasm containing alleles favorable for imparting tolerance to combined stresses and use naturally occurring variation for developing cultivars with improved resistance to multiple stresses via marker-assisted breeding

accessed through programs like Genevestigator (Zimmermann et al. 2004). Recent advances in computational tools such as co-expression modules and machine-learning approaches provide novel means for identifying the candidate genes for engineering broad-spectrum resistance based on gene expression data (Shaik and Ramakrishna 2013, 2014). Genetic components that potentially regulate the resistance to multiple stresses will be utilized for developing transgenic crops. Examples of genes for this strategy include stress-inducible transcription factors, receptor-like kinases, flavonoid metabolism, redox homeostasis, and chromatin modifications.

The same meta-analysis strategy can be adapted for gene pyramiding that has been successfully deployed for resistance to various plant pathogens (Joshi and Nayak 2010). In the case of combined biotic and abiotic stresses, the pyramided genes can be defense genes such as R-genes, pre-invasion defenses (such as callose deposition), nonhost resistance genes in combination with genes in the hormone signaling pathways, antioxidant defenses, or ion homeostasis (Fig. 1.6; Kissoudis et al. 2014).

A second strategy for improving plant tolerance to combined stresses involves the screening of large collections of germplasm in conjunction with genome-wide association mapping (Huang and Han 2014). In recent years, genotyping data for large collections of crop germplasms are becoming available in the public domain (Hao et al. 2012; Li et al. 2013; Song et al. 2013; Yu and Buckler 2006; Zhang et al. 2014). A reliable phenotypic evaluation of germplasm to various stress combinations of interest can be performed. The genotypic information from public domain can be exploited to precisely identify genomic regions associated with the traits of interest. The recent assembly and characterization of association mapping panels in various crop plants, development of improved statistical methods, user-friendly

tools for association mapping (e.g., GWAPP for *Arabidopsis*; TASSEL) and successful association of candidate genes have begun to realize the power of candidate-gene association mapping.

1.9 Conclusions/Perspectives

Studies of stress combinations that naturally occur under field conditions must be a priority for researchers working on abiotic and biotic stresses. Studies of such combined stresses should exploit the naturally occurring variation in the germplasm of crop plants to identify novel sources of resistance or tolerance. While imposing stress combinations, it is important to consider the plant developmental stages that can have the most detrimental agronomic consequences and conduct surveys of germplasm during these critical stages. Phenomic screening using noninvasive high-throughput phenotyping platforms will provide a wide spectrum of observations that span metabolic, physiological, and biochemical parameters. Though the initial costs are high for these setups, the long-term benefits are beyond comparison. Finally, integrating data from multiple omics platforms in conjunction with the phenotyping data will provide a cogent view of the responses to combined stresses in different genotypes. This is crucial for identifying the elite germplasm that can tolerate multiple stresses and provide maximum yields.

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Chapter 2
The Impact of Enhanced Atmospheric CO_2 Concentrations on the Responses of Maize and Soybean to Elevated Growth Temperatures

Richard C. Sicher and James A. Bunce

2.1 Introduction

The agricultural industry is uniquely dependent upon climate and a changing climate has the potential to alter crop productivity and affect economic returns to growers. Atmospheric CO_2 levels have risen about 40 % since the advent of the industrial revolution and this is largely due to fossil-fuel combustion and changes in land management (IPCC 2007). Because atmospheric CO_2 absorbs heat from the sun, global mean temperatures, over both land and water, increased to an average of 0.85 °C between 1880 and 2012 (IPCC 2013). Additional increases in the global mean temperature are likely to occur during the current century and this will have consequences for both mechanized and subsistence agriculture. The IPCC (2007) has concluded that global mean temperatures could increase by an additional 4 °C by the end of the current century, if mitigation measures are not enacted. Moreover, a report by Hatfield et al. (2008) predicts that agriculture will face a more variable, future climate with an increased frequency of extreme weather events including, prolonged drought, intense heat waves, and episodes of drenching rains. Above optimal temperatures decrease both the vegetative and reproductive growth of crop plants but this may be partially offset by greater rates of net photosynthesis due to CO_2 enrichment (Baker and Allen 1989; Boote et al. 2005). Several excellent reviews exist that discuss the effects of heat and/or abiotic stress (Vierling 1991; Wahid et al. 2007; Ahuja et al. 2010; Mittler et al. 2011) and of CO_2 enrichment (Kimball et al. 1993; Allen et al. 1996; Sicher and Kim 2011; Barnaby and Ziska 2012) on plants. The current chapter briefly discusses these subjects but principally focuses on how elevated temperatures and increased atmospheric CO_2 concentrations interact to affect the growth and harvestable yields of important crop plants.

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Our primary focus will be on soybean and maize but, where inadequate data are available, results for related legumes, tropical grass species, and specific crop plants also will be cited.

2.2 Positive Effects of CO_2 Enrichment on Plants

The carbon for plant growth is derived from CO_2 in the atmosphere and the light-dependent reactions of photosynthesis. The current atmospheric CO_2 concentration, i.e., 396 μmol mol^{-1}, does not saturate rates of photosynthesis for the majority of terrestrial plants that possess the C_3 pathway of photosynthesis (Stitt 1991). Many important agricultural crops, including rice, cotton, potato, wheat, and soybean, possess the C_3 pathway of photosynthesis. Supra-ambient CO_2 concentrations normally increase rates of photosynthesis, photoassimilate accumulation, and growth of most terrestrial plants. The conversion of carbon dioxide into organic products takes place in the chloroplast stroma and is catalyzed by the bifunctional enzyme, Rubisco. The concentration of CO_2 within the chloroplast is estimated to be 10 μM, which is close to the apparent Michaelis constant (K_m) for the CO_2 fixation reaction of Rubisco. Rubisco also functions as an oxygenase, that competitively inhibits the carboxylase activity of the enzyme, and the former reaction initiates the first step in photorespiratory metabolism. Therefore, an increase in atmospheric CO_2 concentration is capable of accelerating the rate of CO_2 fixation in the chloroplast by simultaneously enhancing the carboxylation and inhibiting the oxygenation reactions of Rubisco (Kobza and Edwards 1987). Stitt (1991) has argued that increasing the atmospheric CO_2 concentration from 396 to 700 μmol mol^{-1} should accelerate the net rate of photosynthesis of C_3 plants by 25–75 %.

Other plants, including maize, sorghum, and sugar cane, are dependent upon a second carboxylase enzyme, i.e., phospho(enol) pyruvate carboxylase (PEPCase), to catalyze the initial reactions of photosynthesis. The immediate products of the PEPCase reaction are C_4 acids, which are subsequently decarboxylated in the vicinity of Rubisco (Sage and Kubien 2003). This raises the intracellular CO_2 concentration in a manner that facilitates the carboxylase activity of Rubisco and almost completely inhibits the oxygenase activity. Unlike C_3 plants, photosynthetic rates of plants possessing the C_4 biochemical concentrating mechanism are effectively saturated at ambient atmospheric CO_2 levels. Therefore, rates of CO_2 fixation, whole plant growth rates, and harvestable yields of C_4 plants are not nearly as responsive to rising atmospheric CO_2 concentrations as that of C_3 plants. However, both C_3 and C_4 plants exhibit stomatal closure in response to elevated CO_2 and this has important consequences for plant–water relations (Bunce 2004). Because high concentrations of intracellular CO_2 are maintained, partial stomatal closure due to CO_2 enrichment normally does not inhibit photosynthetic rates of maize and other C_4 plants (Sage 1999). Therefore, growth rates of maize can be positively affected by CO_2 enrichment, in part, because of improved water relations. However, any growth enhancement of C_4 plants due to CO_2 enrichment is usually much smaller than that reported for C_3 plants (Kimball et al. 1993; Hatfield et al. 2011).

2.3 Negative Effects of CO_2 Enrichment on Plants

CO_2 enrichment is broadly beneficial for plant growth, although continuous exposure to elevated CO_2 can have a negative impact on plant development. It has been observed that the C/N ratio is frequently higher in plants grown in elevated than in ambient CO_2 (Baker et al. 1989; Foyer et al. 1994), which suggests that the uptake and assimilation of N, and possibly other nutrients from the soil, is not commensurate with the C gain due to CO_2 enrichment from the atmosphere. In some instances, plants grown in elevated CO_2 can become N deficient, which reduces tissue protein concentrations and decreases photosynthetic capacity (Stitt 1991). There are examples where photosynthetic rates of older leaves in the elevated CO_2 treatment were below that of comparable leaves in the ambient CO_2 treatment and this occurred when gas exchange rates were measured at the respective CO_2 concentrations used for plant growth (Sicher and Kremer 1996).

Increased leaf starch levels are almost always observed in leaves of CO_2-enriched plants and this may partly be due to low leaf N concentrations and to accelerated rates of net CO_2 assimilation (Stitt 1991). Some authors (Sasek et al. 1985) argue that excessive starch levels in the chloroplast can alter the structure of photosynthetic membranes and this physical disruption negatively impacts leaf photosynthetic rates. Leaves of plants grown in CO_2-enriched atmospheres can also become chlorotic, brittle, and malformed (Sasek et al. 1985; Sicher 1998). Low chlorophyll levels in CO_2-enriched tissues have been attributed to nitrogen insufficiency and to the onset of premature senescence (Sicher and Bunce 1998). Premature senescence as a result of CO_2 enrichment has been observed for cereal crops, such as wheat and barley, but this same treatment delays the onset of senescence in soybean (Rogers et al. 2004). Clearly, alterations in the timing of senescence affect the overall yield potential of annual crops. In some plant species, the initial stimulation of photosynthesis in response to CO_2 enrichment may be reversed over time as nitrogen becomes insufficient and chlorosis develops. This process is known as photosynthetic acclimation to CO_2 enrichment and photosynthetic rates can ultimately be below that of control plants grown with ambient CO_2 concentrations.

2.4 Elevated Temperature Effects on Plant Growth

The relationship between plant growth and temperature is complex. The variation between day and night temperatures and also mean annual or seasonal temperatures is an important determinant of plant growth rates. Also, the interaction of temperature with other environmental variables, such as irradiance, water availability, and atmospheric CO_2 levels, affects plant development. The growth of all plants is characterized by a number of critical temperatures that can be determined empirically. For example, all plants possess a minimum, maximum, and optimum temperature for growth (Luo 2011; Table 2.1). The minimum and maximum temperatures are the lowest and highest temperatures, respectively, that will sustain the growth of

Table 2.1 Responses of reproductive yields of major crop species to temperature. The optimum and maximum temperatures for reproductive yield (T_{opt} and T_{max}, respectively) are means of day and night values

Crop	T_{opt}, °C (yield)	T_{max}, °C, (yield)	Yield (T_{opt}) t ha^{-1}	Yield (28 °C), t ha^{-1}	Yield (32 °C), t ha^{-1}	% decrease (28–32 °C)
Rice	25	36	7.6	6.3	2.9	54
Soybean	26–28	39–40	3.4	3.4	3.1	10
Dry bean	22–24	32	2.9	1.4	0	100
Peanut	23–25	40	3.4	3.2	2.6	20
Sorghum	23–25	35	12.2	11.8	7.0	41
Maize	20–25	35	10.9	–	–	–

Temperature data are from Hatfield et al. (2011) and Luo (2011). Yield data are from Dr. V. R. Reddy (personal communication)

a given plant species. Agricultural crops have an optimum temperature for yield and this is normally below that of the temperature optimum for vegetative growth (Muchow et al. 1990; Luo 2011). The explanation for this is that lower temperatures usually extend the growing season, thereby maximizing light interception and enhancing crop yields. Temperatures above the vegetative and reproductive growth optima are deleterious, although plants do possess adaptive mechanisms that facilitate growth and successful reproduction under stress-inducing, elevated growth temperatures.

2.5 Heat Stress Responses of Plants

Exposing plants to high temperatures for the first time, even for a few hours, can cause heat stress, which is a dangerous condition that can result in cell damage or even death (Mittler et al. 2011). Because leaves are thin and have a low heat capacity, cellular injuries can occur within minutes when plants are exposed to acute heat stress (Sharkey 2005). Cellular damage also occurs at moderately high temperatures but only after longer periods of exposure. The heat stress response of plants is complex and involves many components including the following: susceptible proteins become inactivated or denaturated (Zhang et al. 2005), membrane integrity and function is compromised (Howarth 2005); metabolic pathways break down (Wahid et al. 2007); the assembly and elongation of microtubules is disrupted (Smertenko et al. 1997); ion fluxes decrease (Schöffl et al. 1999), toxic compounds and reactive oxygen species (ROS) accumulate and both RNA and protein synthesis become impaired (Schöffl et al. 1999; Howarth 2005). To cope with heat stress, plant cells completely reprogram metabolic networks and synthesize stress-related metabolites, proteins, and lipid constituents (Wahid et al. 2007). Plants that are pretreated with high temperatures normally have an improved ability to withstand

future heat stress episodes and this occurs by a process known as acquired thermotolerance. At the cellular level, acquired heat tolerance requires gene activation and specific changes to the metabolome and transcriptome. Low molecular weight metabolites accumulate that function as compatible solutes in the protection of cellular proteins and membranes (Kaplan et al. 2004). Conversely, processes involved in establishing a basal level of heat tolerance are not upregulated by stress pretreatments (Qin et al. 2008).

One of the most important and most thoroughly studied aspects of thermotolerance is the accumulation of heat shock proteins (HSP) in response to heat stress and related environmental stresses (Wang et al. 2004). Families of HSPs vary by molecular weight, i.e., Hsp60, Hsp70, Hsp90, Hsp100, and small or sHSP, and are synthesized within a few hours of acute heat stress in plants. These proteins function as molecular chaperones and are involved in stabilizing and resolubilizing proteins that have denatured due to heat stress. Specific HSPs can be found in the nucleus, chloroplast, mitochondria, and in other cellular compartments (Kotak et al. 2007). This suggests that HSPs are involved in protecting and sustaining numerous, vital processes throughout the cell.

It is also clear that the oxidative stress is a significant factor in the heat stress response of plants and of other species. Heat stress frequently induces the synthesis of highly reactive molecules including, singlet oxygen, the superoxide radical, hydrogen peroxide, and hydroxyl radicals (Wahid et al. 2007). One consequence of ROS is the peroxidation of membrane lipids, which can lead to membrane leakage and a loss of membrane integrity. Brief exposures to high temperatures also induce a burst of hydrogen peroxide in plant cells that may be derived from NADPH oxidase activity (Neill et al. 2002). It is believed that this burst of hydrogen peroxide is a signal for the induction of several heat stress-related genes. Various antioxidant molecules, including ascorbate and glutathione, can protect against ROS and controlling ROS is a crucial mechanism in minimizing damage due to heat stress.

2.6 Heat Stress Effects on Photosynthesis

There is broad agreement that photosynthetic reactions within the chloroplast are among the most highly sensitive to heat stress in higher plants (Berry and Bjorkman 1980; Sharkey 2005). Both light-driven electron transport reactions in the thylakoid membranes and enzymatic reactions promoting CO_2 fixation in the stroma are thought to be thermolabile (Weis and Berry 1988; Havaux and Gruszecki 1993). Various lines of evidence suggest that the oxidizing side of photosystem-II was impaired by heat stress (Havaux and Gruszecki 1993; Heckathorn et al. 1998). However, the reduction of plastoquinone by photosystem-II is relatively thermotolerant and cyclic electron flow involving photosystem-I actually increased with heat stress (Bukhov et al. 1999; Schrader et al. 2004). The above adjustments decrease linear electron flow and reduce rates of CO_2 fixation.

2.7 Effects of CO_2 Enrichment and Heat Stress on Photosynthesis

Soybean, which possesses C_3 photosynthesis, generally has a substantial, long-term increase in leaf photosynthesis when grown at elevated CO_2 (Sicher and Bunce 1998; Bunce 2014; Fig. 2.1a). Under field conditions, soybean exhibits little (Bernacchi et al. 2005) or no downregulation of photosynthesis at elevated CO_2 when measured at high light, except when the plants are under water stress (Sicher and Bunce 1998). This contrasts with the often substantial downregulation of photosynthesis observed at elevated CO_2 in this species when grown in controlled environment chambers (Sicher et al. 1995; Sims et al. 1998). However, during long-term growth experiments, single-leaf photosynthetic rates were not increased by CO_2 enrichment when measured at limiting light levels (Rogers et al. 2006; Bunce 2014). This finding suggested that long-term exposure to elevated CO_2 decreased the quantum efficiency of photosynthesis in soybean, similar to that observed for various other species (Bunce and Ziska 1999; Lewis et al. 1999; Takeuchi et al. 2001).

In plants with C_3 photosynthesis, such as soybean, the optimum temperature for photosynthesis increases with the carbon dioxide concentration, primarily because

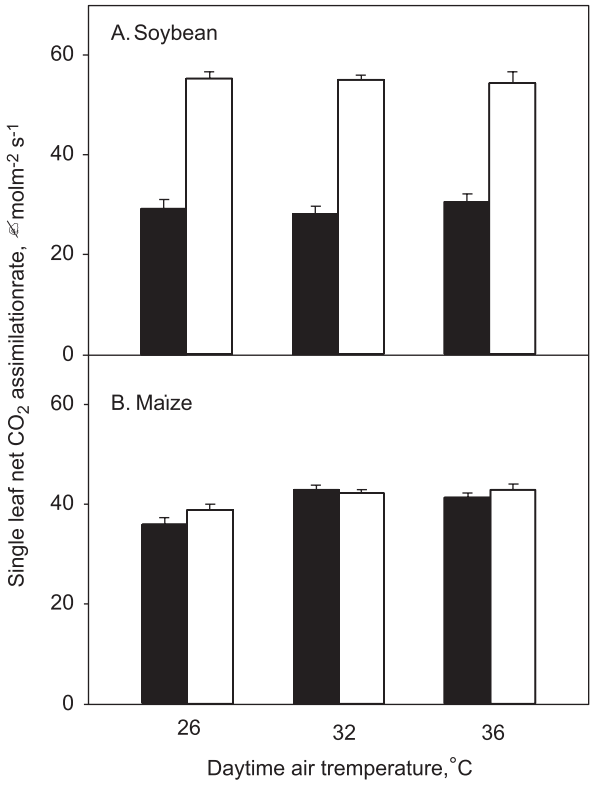

Fig. 2.1 Effects of elevated temperatures and CO_2 enrichment on single-leaf photosynthetic rates of maize and soybean. Plants were grown from seed in naturally sunlit, temperature-controlled enclosures at Beltsville, MD, and foliar photosynthetic rates were determined on sunny days shortly after canopy closure. Data are shown for ambient (*dark fill*) or twice ambient (*no fill*) CO_2 concentrations and are courtesy of Dr. V. R. Reddy

of the suppression of photorespiration and increased carboxylation rates due to CO_2 enrichment discussed above, i.e., due to changes of the V_c/V_o ratio (Long 1991; Kirschbaum 1994). This is true whether photosynthesis is light limited or light saturated. However, elevated temperatures can lower the ratio of the velocity of carboxylase to the velocity of oxygenase (V_c/V_o) (Jordan and Ogren 1984). Although a relative increase in photorespiration is a principal effect of elevated temperatures on photosynthesis, it is clear that other factors are also involved. The temperature at which the optimum rate of photosynthesis occurs largely depends upon the thermal stability of the RuBP-regeneration system, because the Rubisco protein itself is stable to at least 45 °C (Bjorkman et al. 1989; Devos et al. 1998). However, Crafts-Brandner and Salvucci (2000) and Ristic et al. (2009) observed that Rubisco became deactivated after the prolonged exposure of leaf tissue to acute heat stress. Briefly, in the inactivate state, the Rubisco enzyme tightly binds a substrate molecule to the active site, thereby blocking catalytic activity. A second protein, Rubisco activase, facilitates removal of the substrate from the active site and allows Rubisco to become activated and catalytically active. Both in vivo and in vitro evidence suggests that exposing leaf tissue to elevated temperatures can inactivate Rubisco activase. Therefore, one of the principal effects of elevated temperatures on photosynthesis is the conversion of Rubisco from an active to an inactive state. Lowering the Rubisco activation state decreases the carboxylation efficiency of photosynthesis and may lead to the production of excess energy that contributes to photo-oxidative stress (Ort and Baker 2002). However, Wise et al. (2004) and Kubien and Sage (2008) have argued that decreases in Rubisco activation state are a secondary effect caused by a reduction in electron transport rates. According to these authors, the deactivation of Rubisco at elevated temperatures functions naturally to restore the imbalance between electron transport rates and rates of CO_2 fixation.

The stimulation of photosynthesis by elevated CO_2 usually increases strongly and predictably with temperature (Long 1991). However, at excessively high temperatures, the CO_2-dependent stimulation of photosynthesis may be negated by low rates of Rubp-regeneration. When this situation occurs, the stimulation of photosynthesis by elevated CO_2 is highly insensitive to measurement temperatures (Bunce 2007; Ziska 2001; Yamori et al. 2005). Additionally, acclimation of photosynthesis to seasonal changes in temperature can result in the stimulation of photosynthesis by elevated CO_2 being nearly constant at different times of the year despite seasonal variations in temperature. This phenomenon has been attributed to thermal acclimation of the photosynthesis system (e.g., Bunce 1998, 2000; Tesky 1997; Tjoelker et al. 1998).

Above the optimum temperature of photosynthesis, photosynthetic rates may become unstable and decrease continuously with time. There is a critical temperature below which photosynthesis will completely recover after the plants are returned to ambient growth temperatures. However, above this critical temperature, irreversible damage occurs to the photosynthetic machinery of the leaf (Berry and Bjorkman 1980). This makes the assessment of CO_2 effects on responses of photosynthesis to extremely high temperatures difficult. Taub et al. (2000) found that for about 60 %

of the species they examined, cultivating plants in atmospheres containing elevated CO_2 resulted in about a 1 °C increase in the temperature required to damage photosystem II. This could also be due to decreased stomatal conductance during the growth at elevated CO_2 caused by leaves acclimating to warmer temperatures. A similar effect on photosynthetic thermal tolerance due to elevated CO_2 was reported in wheat (Gutierrez et al. 2009), birch, and aspen trees (Darbah et al. 2010). However, no effect of elevated CO_2 on the thermal tolerance of photosynthesis was observed with either creosote bush (Naumberg et al. 2004) or *Phillyrea angustifolium* (Vitale et al. 2008). Soybean photosynthesis has a relatively high temperature optimum (Harley et al. 1985) and photosynthesis was not damaged by exposures to temperatures up to 48 °C at either ambient or elevated CO_2 when plants were grown with a daytime temperature of 28 °C (Bunce, unpublished data). Thus, it is unlikely that soybean photosynthesis suffers from heat damage in any of the locations where it is currently grown.

As stated above, plants with C_4 photosynthetic metabolism, such as maize, generally exhibit little or no stimulation of leaf photosynthesis when grown at elevated CO_2 (Kim et al. 2007, Fig. 2.1b). However, maize plants in the field displayed episodic CO_2-dependent increases in photosynthetic rates during water stress events when stomatal conductance was reduced (Leakey et al. 2006). In maize, photosynthesis can be limited by PEP carboxylase (or C_4 cycle) activity, Rubisco activity, or by Rubp-regeneration capacity. Unlike Rubisco, PEP carboxylase activity is saturated by ambient atmospheric CO_2 concentrations. Therefore, photosynthesis rates of intact maize leaves are only limited by very low subambient CO_2 concentrations. Determining whether Rubisco activity or rates of Rubp-regeneration are limiting for photosynthesis in C_4 species often requires measuring light response curves, in addition to CO_2 response curves (Massad et al. 2007). Crafts-Brandner and Salvucci (2002) observed that photosynthesis rates of corn leaves decreased at temperatures above 38 °C. These authors attributed this to a reduced activation state of Rubisco rather than to either diminished C_4 cycle or electron transport activity (i.e., Rubp-regeneration). Because high intracellular CO_2 concentrations are available to Rubisco, C_4 species, in general, tend to have greater optimum temperatures for photosynthesis than do C_3 species (Pearcy and Ehleringer 1984). This is partly because rates of photorespiration are normally very low in C_4 species. Maize evolved at higher elevations in the tropics, so it is more heat sensitive than many closely related C_4 species. Qu et al. (2014) found that photosynthesis in corn leaves was inhibited by brief exposures to 45 °C and the temperature effect was more acute at elevated than at ambient CO_2 (Fig. 2.2). Hamilton et al. (2008) also found that elevated CO_2 decreased photosynthetic thermal tolerance in maize, as well as in *Amarathus retroflexus,* another C_4 species, although these earlier treatments were based on air temperature rather than leaf temperature.

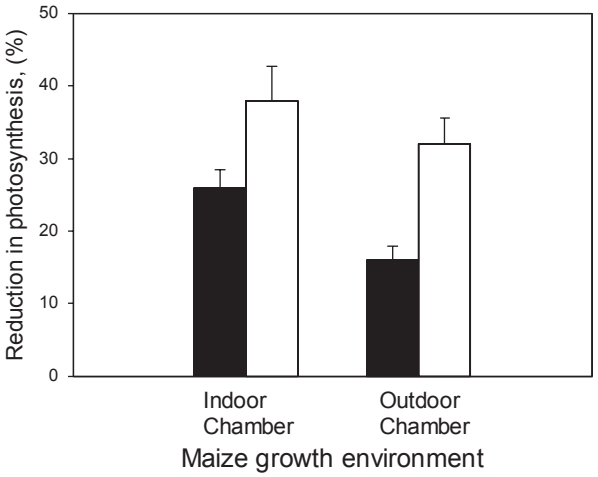

Fig. 2.2 Percentage reductions in single leaf rates of photosynthesis for *Zea mays* L. cv. Silver Queen, after leaf tissue was exposed to 45 °C for 2 h using plants grown in indoor or outdoor chambers. The "ambient" and "elevated" treatments were with 380 mmol mol^{-1} (*dark fill*) and 560 mmol mol^{-1} (*gray fill*) CO_2, respectively. In all cases, stomatal conductance was greater after heat treatment in comparison to the untreated controls. Data are unpublished results from Drs. M. Qu and J. Bunce

2.8 Effects of CO_2 Enrichment and Heat Stress on Leaf Components and Metabolism

Both CO_2 enrichment and supraoptimal temperatures affect a number of metabolic processes in plants including photosynthesis, photorespiration, and dark respiration. Consequently, these two environmental factors independently affect concentrations of primary and secondary metabolites in plant tissues (Kaplan et al. 2004; Prasad et al. 2004). As mentioned briefly above, CO_2 enrichment enhances the accumulation of carbon-containing compounds, such as starch, sucrose and hexoses, and may decrease levels of many nitrogen-containing metabolites, including soluble amino acids, photosynthetic proteins, such as Rubisco, and membrane-associated pigment-protein complexes. These conclusions are true for most C_3 plants, although soybean normally does not exhibit large changes of nitrogen metabolism in response to CO_2 enrichment (Campbell 1990; Sicher et al. 1995; Rogers et al. 2006).

As described above, heat stress affects the plant metabolome and leaf metabolites usually exhibit a greater response to heat stress than those found in other tissues on the plant (Rizhsky et al. 2004). Summarizing changes of plant metabolites due to elevated temperatures is complicated by the fact that two fundamentally different experimental approaches have been used. Some investigators examined metabolite changes in response to an acute heat shock treatment and other studies involved modified growth temperatures over longer period of time. These are two related but different approaches to studying heat stress that can have varying outcomes

(Kaplan et al. 2004). A second problem is that plants are usually adapted to specific cool or warm environments and this can affect the extent of thermal tolerance observed (Yu et al. 2012). Third, acute heat treatments when applied to plants can cause leaf tissues to lose water and become desiccated. This is a complication that can result in indirect treatment effects on foliar metabolite levels.

Although the total dataset is limited, the heat stress metabolome of *Arabidopsis* may be smaller than that for cold or drought stress. Kaplan et al. (2004) reported that 143 and 311 out of 497 real and putative compounds from *Arabidopsis* rosettes were affected by a heat and cold shock, respectively. Rizhsky et al. (2004) observed that 5 of 48 targeted metabolites in *Arabidopsis* rosettes differed from the controls after raising the growth temperature from 22 to 35 °C for 6 h. In the latter experiment, it also was observed that 17 of 48 metabolites were altered by water stress. To our knowledge, similar metabolite analyses from combined stress experiments have not been performed in other species.

Nonstructural Carbohydrates Elevated growth temperatures decreased partitioning to both transitory and storage starch (Geigenberger et al. 1998; Prasad et al. 2004). However, reports of changes of soluble nonstructural carbohydrates in response to elevated temperatures in plants have been variable. Sucrose, glucose, and fructose in leaves of specific crops and forage species frequently remained unchanged or decreased in response to elevated growth temperatures (Chatterton et al. 1987; Liu and Huang 2000; Sicher 2013). However, foliar sucrose levels also increased due to supraoptimal temperatures in reports by other authors (Kaplan et al. 2004; Yu et al. 2012). Sugar alcohols, or polyols, typically increased in soybean leaflets at elevated growth temperatures. Pinitol, which is a methylated derivative of inositiol, is particularly abundant in soybean leaves and it accumulates in response to elevated growth temperatures (Guo and Oosterhuis 1995; Sicher 2013). This result suggested there was a shift in metabolism from sucrose to pinitol synthesis in response to heat stress. Mannitol, myo-inositol, galactinol and raffinose have also been observed to accumulate in response to elevated temperatures (Kaplan et al. 2004; Sicher 2013). The former two compounds are polyols that likely function as osmolytes or compatible solutes that protect proteins and membranes from abiotic stress. Galactinol, raffinose, and myo-inositol also are involved in scavenging ROS (Loewus and Murthy 2000).

Organic Acids Organic acids are normally synthesized from soluble sugars, which are then converted to amino acids by transamination. In the *Arabidopsis* literature, changes of organic acids in response to heat shock were relatively minor. Rizhsky et al. (2004) reported that hydroxysuccinic acid and lactic acid increased with rising treatment temperatures. Hydroxysuccinic acid is another name for malic acid, which, surprisingly, did not respond to heat stress and lactic acid is normally synthesized during anaerobic metabolism. Kaplan et al. (2004) mentioned four organic acids and all increased with heat stress. These were quinic acid, citramalic acid, fumarate, and malate. Quinic acid is a cyclic polyol, citramalic or 2-methylmalic acid is involved in leucine synthesis and the latter two compounds are tricarboxylic acid (TCA) cycle intermediates with multiple cellular functions.

More recent investigations on crop species have suggested that organic acids may have a major role in the heat stress responses of plants. Yu et al. (2012) reported that oxalic, shikimic, malonic, threonic, glyceric and galacturic acids decreased from 55 to 85% in tall fescue leaves when the growth temperature was maintained at 10 °C above the optimum for plant growth. These same authors found that pyruvic and malic acid were unchanged and citric acid increased about twofold in response to elevated growth temperatures. Sicher (2013) observed that citrate, aconitate, succinate, fumarate, 2-oxoglutarate and malate decreased from 39 to 94% in soybean leaves when the average daytime growth temperature was increased from 28 to 36 °C (Fig. 2.3). All of these organic acids function in the TCA cycle and are important in respiratory metabolism, amino acid synthesis, ammonia detoxification, and nitrogen assimilation. The studies with tall fescue and soybean were longer-term growth studies using moderate increases in temperature, whereas the *Arabidopsis* experiments by Rizhsky et al. (2004), and Kaplan et al. (2004) employed acute heat shock experiments of 4 and 6 h duration.

Amines Soluble amino acids participate in nitrogen assimilation, protein synthesis and degradation, and in the manufacture of secondary metabolites. Prior studies with *Arabidopsis* and cowpea cells showed that alanine, β-alanine, asparagine, γ-amino butyric acid (GABA) and putrescine increased in response to heat shock

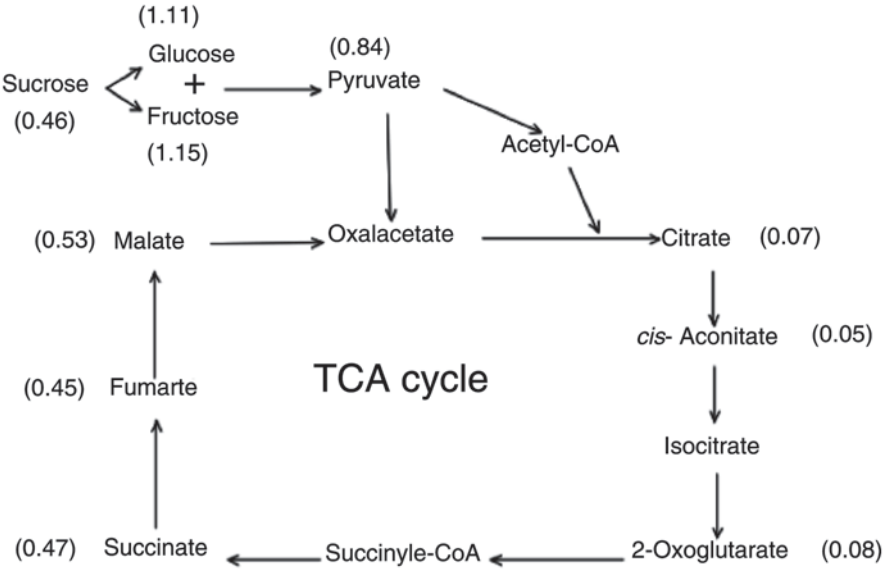

Fig. 2.3 Effects of heat stress on compounds involved in primary plant metabolism. Values in parentheses are ratios of metabolite concentrations from leaves of plants grown with 36/28 compared to 28/20 °C (day/night) temperatures. Experiments were performed with ambient (400 μmol mol^{-1}) CO_2 and observed changes in metabolite concentrations werenot observed when plants were grown with elevated (700 μmol mol^{-1}) CO_2. Data are based on results from Sicher (2013)

(Mayer et al. 1990; Kaplan et al. 2004; Rizhsky et al. 2004). Branched chain amino acids (BCAA), leucine, isoleucine, and valine, also accumulated in the prior heat shock studies by Mayer et al. (1990) and Kaplan et al. (2004). Alanine and asparagine can accumulate to very high levels in plant tissues and these two amino acids function as important storage forms of nitrogen during abiotic stress events. GABA is a nonprotein amino acid that accumulates, often in combination with alanine, in affected cells in response to abiotic and biotic stress (Bown and Shelp 1997). Mayer et al. (1990) argued that GABA accumulation was triggered by low cellular pH, a condition that is associated with Ca^{2+} buildup and the activation of glutamate decarboxylase, an enzyme involved in the synthesis of GABA from glutamate. Yu et al. (2012) and Sicher (2013) also observed that GABA increased in plants exposed to a moderate increase in growth temperature. The BCAAs accumulate in response to drought stress and these compounds are important precursors in the synthesis of secondary metabolites (Sicher and Barnaby 2012). Both Yu et al. (2004) and Sicher (2013) reported that glycine and serine decreased in leaves in response to elevated growth temperatures. This result was unexpected because elevated temperatures favor photorespiratory metabolism over CO_2 assimilation, and glycine and serine are important photorespiratory metabolites. However, both serine and glycine may be involved in other cellular processes that are inhibited by elevated temperatures (Sicher and Barnaby 2012). Overall, we can conclude that elevated temperatures cause large changes in amino acid metabolism.

Other Metabolites High temperature stress affects concentrations of phytohormones in various plant tissues and these are likely involved in regulating the growth and development of plants affected by abiotic stress (Wahid et al. 2007). Collectively, abscisic acid, ethylene, and salicylic acid have all been associated with temperature stress and brassinosteroid treatments reportedly improved the thermal tolerance of certain plant species (Dhaubhadel et al. 1999). Glycine betaine accumulates in many plant species in response to abiotic stress, and may be involved in the response to heat shock (Sakamoto and Murata 2002). This compound is a quaternary amine that likely functions as a compatible solute in the protection of stress-susceptible proteins. Additionally, elevated temperatures also affected products of lipid peroxidation, certain carotenoids, phenolics, and polyamines (Wahid et al. 2007).

2.9 CO_2 Enrichment Mitigates Metabolite Responses to Elevated Temperatures

The above-described metabolite changes in response to heat stress were measured using plants exposed to ambient CO_2. Again, the data are limited but there are strong indications that metabolite responses to moderate heat stress were partially to completely reversed by elevated CO_2 treatments. Yu et al. (2012) observed that the effects of elevated growth temperatures on six amino acids, two sugars, and

three amines were not observed when the CO_2 concentrations used for plant growth were increased from 400 to 800 μmol mol^{-1}. Similarly, Sicher (2013) working with soybean observed that 28 of 43 metabolites in soybean leaves were altered by increasing the growth temperature to 8 °C under ambient CO_2. Conversely, only three amines in soybean leaflets were affected by the same temperature treatment when experiments were performed at 700 μmol mol^{-1} CO_2. We are not aware of similar metabolite studies that have been performed on plants exposed to acute temperature stress during a heat shock. However, it is likely that CO_2 enrichment is capable of mitigating the effects of elevated temperature stress on plant metabolism.

2.10 Effects of CO_2 Enrichment and Heat Stress on Vegetative Growth

Atmospheric CO_2 concentrations and air temperatures are important determinants of plant growth and both of these environmental parameters are likely to be affected by climate change. As discussed above, fertilization with atmospheric CO_2 enhances photosynthetic rates and increases biomass formation of C_3 plants. Therefore, significant temperature by CO_2 interactions has been observed for many C_3 crop plants and observed growth responses to CO_2 enrichment are usually enhanced by moderate increases in air temperature (Boote et al. 2005). One additional reason that this would occur is that moderately warmer temperatures have the capacity to extend the length of the growing season (Hatfield et al. 2011). Although elevated temperatures normally enhance the CO_2 fertilization effect, there is a critical point at which temperature increases become deleterious to growth regardless of CO_2 concentrations. Idso et al. (1987) and Kimball et al. (2002) observed that the biomass growth modification ratio increased by 0.08/°C between 12 and 34 °C when the ambient CO_2 concentration was enhanced by 300 μmol mol^{-1}. In contrast to the above, Allen et al. (1996) observed that for soybean the season-long biomass growth modification ratio was −0.026 °C and he attributed this to a shortened grain filling period due to accelerated reproductive development at elevated temperatures. Allen et al. (1996) also observed that total biomass yields of soybean fell rapidly when day/night temperatures exceeded 44/34 °C.

The growth of maize normally does not respond to elevated atmospheric CO_2 concentrations except during periods of soil moisture deficits (Kim et al. 2006; Leakey et al. 2006). The latter authors reported that CO_2 enrichment increased photosynthetic rates of maize up to 41% in the field during periods of water stress. These authors proposed that CO_2 enrichment enhanced intercellular CO_2 concentrations and that this resulted in increased photosynthetic rates when the stomatal conductance was reduced. Kim et al. (2007) reported that biomass formation, photosynthesis, and leaf area of maize were unaffected by doubling the ambient CO_2 concentration and that this conclusion was maintained across a wide range of growth temperatures. These same authors observed that the total above-ground biomass and leaf area were negatively correlated with increasing growth temperatures

between 19/13 and 38.5/32.5 °C when experiments were performed using well watered plants in naturally sunlit, outdoor environmental chambers. The optimum temperature for maize leaf development was about 31 or 32 °C (Tollenaar et al. 1979; Kim et al. 2007), when determined with ambient or elevated CO_2.

2.11 Effects of CO_2 Enrichment and Heat Stress on Flowering/Reproductive Growth and Yield

Considerable research has been performed on predicting the effects of climate change on crop yields and broad agreement exists on the basic effects of elevated CO_2 and temperature on the yield parameters of various crop species (Table 2.1). However, there is widespread disagreement regarding the precise magnitude of the predicted responses of seed yield to carefully defined environmental parameters (Long et al. 2006). Crop yields are normally determined at numerous locations and data from each location are based on substantial land areas. It is not affordable to perform accurate yield determinations on a large scale using elevated CO_2 treatments. Therefore, all yield studies using elevated CO_2 treatments are based on a relatively small number of plants at a single location and are potentially subject to error.

Harvestable yields of soybean are consistently increased by CO_2 enrichment and changes of yield were commensurate with increased rates of net photosynthesis and total biomass production (Allen et al. 1996; Ainsworth et al. 2002). However, the harvest index, which is the ratio of seed mass to above-ground biomass, decreased in response to CO_2 enrichment (Baker et al. 1989; Ainsworth et al. 2002). This is an indication that the soybean plants have a greater capacity to synthesize biomass in response to elevated CO_2 than to utilize it for seed production. Allen and Boote (2000) reported that soybean yields were increased 34 % in a study based on a season-long doubling of ambient CO_2. Ainsworth et al. (2002) and Ziska et al. (2001) reported that mean soybean seed yields increased 38 and 40 %, respectively, in response to the same doubling of CO_2. In addition, Ziska et al. (2001) suggested that yield increases due to CO_2 enrichment varied widely among soybean genotypes, although genetic differences were not observed for single-leaf photosynthetic rates. Soybean yields in the USA have increased dramatically since 1924 and the rate of improvement has accelerated in the last four decades (Specht et al. 1999). Half of this yield improvement was attributed to genetic and technological advances but increased atmospheric CO_2 concentrations also were identified as a major contributor to enhanced soybean yields.

The temperature optimum for soybean seed yield is between 23 and 24 °C (Piper et al. 1998) and rising temperatures are expected to have a negative impact on harvestable yields. Diminished yields occur with increasing temperatures up to 40 °C, which is the point at which crop failure is possible (Allen et al. 1996). It should be pointed out that soybean is a moderately temperature tolerant species and significant yield losses have been observed when air temperatures exceeded 30 °C for

prolonged periods during the growing season. Yield losses due to heat stress can occur at any point in the growth cycle but temperature effects on yield are usually greatest during the reproductive growth. Hatfield et al. (2008) and Lobell and Field (2008) estimated that a 0.8–1.0 °C temperature increase across the Southeastern USA would result in a 1.3–2.4 % decrease in soybean seed yield. Single-leaf photosynthetic rates by soybean leaflets are fairly stable between 26 and 36 °C. Therefore, factors such as shortened grain-filling duration, poor seed set and decreased seed size are responsible for the yield decreases in soybean that occur at above optimum temperatures (Boote et al. 2005).

Baker et al. (1989) determined soybean seed yields (g plant^{-1}) using naturally sunlit controlled environment chambers set to provide 3-day/night temperatures and ambient or twice ambient CO_2 levels. Individual plants grown with 26/19 °C day/night temperatures and with 330 μmol mol^{-1} CO_2 yielded 9.0 g of seed plant^{-1}. This increased to 10.1 g seed plant^{-1} when the temperature was raised to 36/29 °C or to 13.1 g seed plant^{-1} when the CO_2 concentration was doubled to 660 μmol mol^{-1}. However, the same plants yielded 11.6 g seed plant^{-1} when grown at the higher temperature with double the ambient CO_2 concentration and intermediate results were observed at intermediate temperatures. The yield enhancement due to CO_2 enrichment was 45 and 15 % at the lower and higher growth temperatures, respectively. Therefore, the beneficial effects of CO_2 enrichment on soybean yields diminish at elevated growth temperatures and disappear at acutely high temperatures.

The effects of elevated temperatures on maize and soybean yields were basically similar. It is well recognized that elevated temperatures decreased the grain filling duration of maize and that this negatively affected crop yields (Muchow et al. 1990). Conversely, Tollenaar and Bruulsema (1988) showed that kernel dry matter accumulation only varied slightly between 10 and 25 °C. Commuri and Jones (2001) reported that heat stress decreased overall kernel dry weight and kernel density. Consequently, the reproductive growth of maize is generally more sensitive to heat stress than vegetative growth (Allen and Boote 2000; Reddy et al. 2000). Lobell et al. (2011) and Hawkins et al. (2013) used historical maize yield data to estimate yield losses due to excessive temperatures. The former paper studied maize production in southern Africa and determined that each day above 30 °C found reduced yields by 1.0–1.7 % depending upon water availability. The latter paper similarly found that maize yields in France decreased in proportion to the number of days during the growing season with temperatures above 32 °C.

High temperatures decrease maize yields primarily during the reproductive growth by inducing flower abortion, disrupting fertilization and inhibiting endosperm development. Herrero and Johnson (1980) showed that temperatures above 32.5 °C inhibited maize pollen germination and that this process was affected by the duration and severity of heat stress. There is also a possibility that maize pollen and silk become desiccated when exposed to elevated temperatures. Monjardino et al. (2005) reported that starch and protein synthesis in maize endosperm were inhibited by 4 days of heat treatment at 35 °C. These authors also observed that kernel sizes were smaller for the heat-treated samples in comparison with the controls.

Hatfield et al. (2011) summarized the effects of CO_2 enrichment on maize and concluded that seed yields would only increase 3–4 % on average in response to doubling CO_2 levels. The combined effects of CO_2 enrichment and elevated temperatures on maize yields have not been characterized adequately in field experiments. However, Prasad et al. (2008) demonstrated that elevated CO_2 treatments increased internal tissue temperatures of grain sorghum and this exacerbated the negative effects of elevated air temperatures on seed yields. Due to a lack of experimental data, estimating the combined effects of CO_2 and temperature on maize yields has relied, in part, on crop modeling approaches. Hatfield et al (2011) concluded that temperatures in the North American Corn Belt would increase to 0.8 °C in the next 30 years when atmospheric CO_2 concentrations could reach 440 μmol mol^{-1}. These authors suggested that these conditions would result in a minimum 2–3 % decrease in maize grain yields under water-sufficient conditions. Easterling et al. (2007) concluded that a 1–2 °C increase in global mean temperatures would increase maize yields by a few percent in the mid latitudes, that maize grown in the tropics would have major yield losses due to temperatures 3–5 °C above today's values and that the elevated atmospheric CO_2 concentrations would have negligible benefits for maize production.

2.12 Summary

CO_2 enrichment is capable of mitigating the effects of moderate heat stress on plants, such as soybean, that have the C_3 pathway of photosynthesis. Evidence for this was based on changes of net photosynthetic rate, primary metabolism, plant growth, and yield. However, the mitigation of heat stress by CO_2 enrichment diminishes in soybean and other species as temperatures elevate further and heat stress becomes more acute. Very high air temperatures, i.e., those that exceed 40.0–42.5 °C, frequently cause irreversible damage to plant tissues and may cause death or reproductive failure. Unlike soybean, the reversal of moderate heat stress by CO_2 enrichment is almost immeasurable for maize and other plants that possess the C_4 photosynthetic pathway. This is because maize has high internal CO_2 concentrations that almost completely saturate rates of photosynthesis in ambient air. Second, elevated CO_2 concentrations induce stomatal closure of many plant species and this decreases evapotranspiration rates from leaves. The resultant improved water status would certainly benefit maize and soybean in the field during prolonged exposures to heat stress. Note that acute air temperatures create a demand for lower leaf temperatures and this requires stomatal opening and increased evapotranspiration rates. Thus, very high temperatures negate the effects of CO_2 enrichment on stomatal aperture. Third, plant growth in elevated CO_2 is capable of accelerating or delaying the onset of senescence of several annual crops. Elevated growth temperatures accelerate plant development and this shortens the growing season and negatively affects crop production. Therefore, delaying the onset of senescence via CO_2 enrichment should mitigate the effects of a shortened growing

season due to elevated growth temperatures. Conversely, cereals, such as wheat, exhibit premature senescence in response to CO_2 enrichment and the combination of elevated temperatures and supra-ambient CO_2 levels would work synergistically to decrease yields.

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Chapter 3
Investigating the Effect of Elevated CO_2 in the Growth Environment of Salt-Stressed Plants Using Integrated Omic Analyses

Matthaios-Emmanouil P. Papadimitropoulos and Maria I. Klapa

3.1 Introduction

In an era, when the need for food of consistent and high quality throughout the globe is of great interest, while the changes in the environment, including the greenhouse effect, are altering the conditions of plant growth, furthering our understanding of how the plants respond to various stresses at the molecular level becomes a major objective for molecular plant physiologists, agricultural engineers, and the food industry. Major abiotic stress factors for plants that are under investigation individually but mainly in combination are the extreme cold or heat, the drought or flooding, the soil or water salinity, chemicals and pollutants like heavy metals and pesticides, the oxidative stress (i.e., the reactive oxygen species (ROS), the ozone), the nutrient deprivation in soil, and changes in the composition of the atmosphere, mainly the increase in the carbon dioxide (CO_2) concentration.

Among these, the investigation of the salinity effect on plant growth has intensified in the recent years, because high soil or water salinity is a major environmental stress and a substantial constraint to crop production. Increased salinization of arable land is expected to have devastating global effects, estimated to result in 50 % land loss by the middle of the twenty-first century (Wang et al. 2003). Hot and dry climates favor water evaporation, leading thus to an increase in the salt concentration. Heavy or low quality irrigation may also contribute to an increase in salinity. The problem

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is even greater in the coastal areas, where the seawater enters the aquifer, increasing thus the soil salinity in intensively cultivated areas (Mahajan and Tuteja 2005). As the growing of hydroponic cultures in greenhouses gains momentum as a means for consistent plant and product quality independently of the place of plant growth around the globe (Jones 2005), this trend has also contributed in the past decade to an increase in the studies about the effect of varying water salinity on plant growth. On the other hand, considering the elevation of the CO_2 concentration in the environment due to the greenhouse effect, which can drastically change the physiology of the plants and the quality of crop production in the future (Solomon et al. 2007), the particular stress has been the subject of molecular plant physiology studies for many years. This is also due to the fact that CO_2 is the major carbon source for the plants and its increase at moderate levels and for moderate durations has been shown to be beneficial for the plant growth, especially when the plants are also under the influence of other stresses, including salinity (Takagi et al. 2009; Geissler et al. 2010; Kanani et al. 2010; Perez-Lopez et al. 2012; Ratnakumar et al. 2013). Therefore, the combined effect of high soil, but mainly water, salinity, and elevated CO_2 on plants has been under investigation by agricultural engineers and plant physiologists not only in the context of the greenhouse effect but also for the development of plant growth optimization strategies in the presence of salinity stress.

In the system biology era, the investigation of the molecular mechanisms underlying plant growth and response under various stresses has been enhanced by the high-throughput biomolecular (i.e., omic) analyses. The latter enable the simultaneous quantification of the concentration of tens to hundreds to thousands of molecular quantities from the RNA to protein to small molecule (i.e., metabolic) level. However, these are new technologies, most at the stage of standardization, and the current number of omic analyses in plants is not extensive, especially in the case of integrated analyses at various molecular levels of cellular function. Moreover, the investigation of intact plants using omic analyses presents unique challenges over similar investigations in cell cultures or other biological systems, among which are the current lack of full genome sequence information for most plants, long life cycles, and poorly controlled conditions in field experiments. In this chapter, we present the transcriptomic and metabolomic studies of salinity and elevated CO_2 stresses in plants, applied individually or in combination, emphasizing on the integrated analyses of both levels of cellular function. The specifications of the experimental design for the plant growth and the omic analyses, the challenges of such experiments, the acquired results, and future directions for research and practice are also discussed.

3.2 Physiological Characteristics of the Plant Response to High Salinity and/or Elevated CO_2

3.2.1 High Soil and/or Water Salinity

High soil salinity can affect plants in multiple ways. High salt depositions in the soil generate low water potential in the root zone, making it difficult for the plants

to pump water. Thus, the physiology of high-salinity-stressed plants resembles the physiology of drought-stressed plants (Mahajan and Tuteja 2005). To avoid loss of water through osmosis, plants have to increase the osmotic pressure of their cells. In light of this need, under salinity stress plant cells tend to accumulate metabolites that act as osmolytes, e.g., proline (Delauney and Verma 1993; Ford 1984). The net effect of this metabolic "deviation" is that the plants have to use part of their resources towards the production of osmolytes, thus decreasing carbon flux towards their growth (Kanani et al. 2010). Moreover, under high-salinity stress plants tend to close their stomata to reduce water loss by transpiration. Carbon dioxide fixation through the Calvin cycle is then reduced and the photosynthesis rate declines (Chaves et al. 2009). Furthermore, reduced CO_2 in the chloroplasts combined with intense light enhances the photoproduction of ROS (Asada 2006). High levels of sodium may also have deleterious effect on the functioning of some of the enzymes (Niu et al. 1995). Lower photosynthesis and transpiration levels in combination with a lower flux towards the plant growth cause a decrease in the development and productivity of the salinity-stressed plants (Cuartero and Fernandez-Munoz 1999; Shannon and Grieve 1999). It is thus apparent that the salinity stress is a substantial constraint to crop production especially in the arid and semiarid climates (Wang et al. 2003). In greenhouses, the problem of high-salinity stress may be even more intense, especially in the case of poor quality water in combination with high temperatures (Ayers and Westcot 1985). In addition, in hydroponics, the salinity of the small volume nutrient solution can increase rapidly, especially in closed systems with nutrient solution recycling (Magan et al. 2008).

3.2.2 Elevated CO_2 in the Growth Environment of the Plants

Short-term enrichment of the CO_2 in the growth environment of the plants up to three times the current ambient (375 ppm) level has a positive impact in the plants as it stimulates photosynthesis and reduces stomatal conductance (Ainsworth and Rogers 2007). However, the long-term exposure of plants to elevated CO_2 leads to photosynthetic acclimation and reduced CO_2 uptake (Rogers and Ellsworth 2002). Positive responses to elevated CO_2 are mainly attributed to the competitive inhibition of the photorespiration by the carbon dioxide. Increase in the CO_2 levels in the growth environment of the plants increases carbon fixation. The elevated CO_2 conditions can also enhance growth through improved plant water relations, since the increased CO_2 slows down the transpiration by inducing the partial closure of stomatal guard cells of the leaves (Prior et al. 2011).

3.2.3 Combined Application of High Salinity and Elevated CO_2

Based on the observed physiological characteristics of the plants under short-term elevated CO_2 and high-salinity treatment, the former perturbation can be beneficial for the plant growth, while the latter initiates a series of negative physiological consequences on plants upon its application. Thus, it is of interest to investigate how

this physiological "divergence" between the effect of these stresses on the plants when they are applied separately, is finally manifested when these perturbations are combined on plants. Interestingly, physiological (Geissler et al. 2010; Perez-Lopez et al. 2009, 2012; Ratnakumar et al. 2013; Takagi et al. 2009) and high-throughput omic studies (Kanani et al. 2010) have shown that short-term application of elevated CO_2 in the growth environment of salt-stressed plants can alleviate the negative effect of high salinity on the plant growth. Different justifications have been provided for this observation, with more prevalent the one supporting that the additional CO_2 contributes to the maintenance of the redox homeostasis of the plants (Perez-Lopez et al. 2009). According to the presently single integrated transcriptomic and metabolomic analysis of the combined high-salinity and short-term elevated CO_2 perturbations on *Arabidopsis thaliana* plant liquid cultures presented below (Kanani et al. 2010), a major reason for the positive effect of the elevated CO_2 on the salt-stressed plants is the availability of additional carbon resources. The latter enable the plants to produce the required osmoprotectant metabolites while at the same time maintaining their normal growth rate.

3.3 Integrated High-Throughput Biomolecular Analyses in Plant Systems Biology

The technologies for high-throughput biomolecular analysis (omics) have revolutionized the way in which questions are approached in life sciences. Rather than examining a small number of genes and/or reactions at any one time, we can now begin to look at gene expression and protein activity in the context of networks and systems of interacting genes and gene products (Sussman et al. 2009). Because our knowledge of this domain is still not extensive, investigations are now routinely moving from being purely "hypothesis driven" to being largely "data driven" with analysis based on a search for biologically relevant patterns from which network structures could be inferred. Recent developments have shown that educated use of the existing biological knowledge in the application of data mining methods can indeed lead to the reconstruction of the active biomolecular networks at each level of molecular function that characterize a particular physiology ("knowledge"-driven approach). These technological advances have created enormous opportunities for accelerating the pace of science. One can now envision the possibility of obtaining a comprehensive picture of the mechanisms underlying the cellular function, its regulation, and the interactions of an organism with its environment. While the greatest attention to date has been paid to gene sequence and transcriptional expression analysis using mainly microarrays, it is becoming increasingly clear that these alone cannot be used to accurately determine cellular function and system physiology. Rather, a comprehensive analysis of biological systems requires the integration of all fingerprints of cellular function (Vidal 2009), i.e., genome sequence, transcriptional, proteomic, and metabolic profiles, and flux distributions. While each of these fingerprints has significant value on its own, the picture that emerges from any single approach is quite limited in nature. Gene transcription is a necessary but not

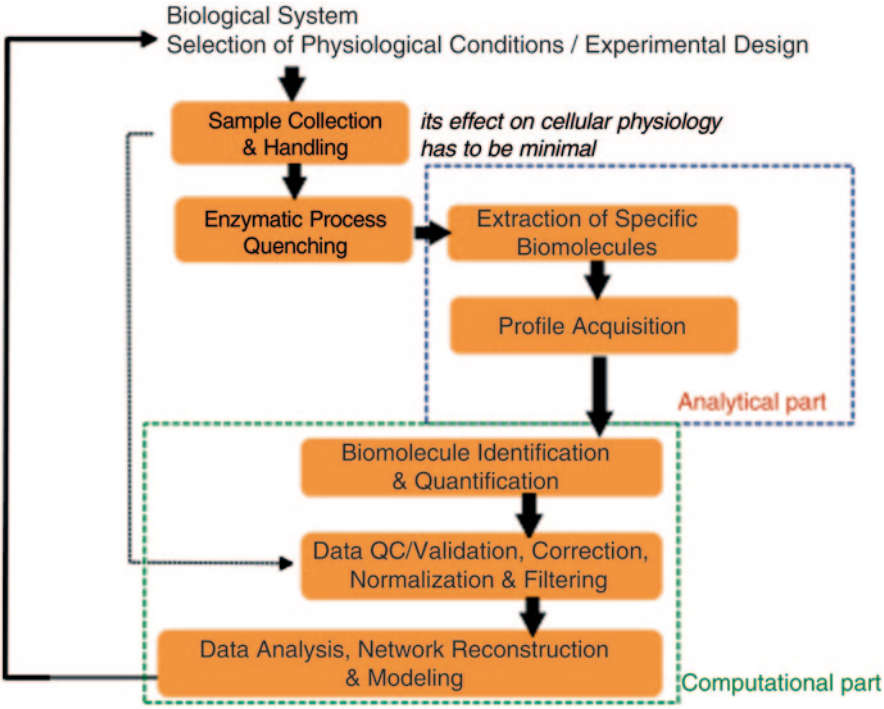

Fig. 3.1 Omic profiling analyses are multistep procedures with potential sources of systematic biases at any stage

a sufficient condition for high *in vivo* protein production. Regulation of translation, RNA and protein stability, and posttranslational modifications can alter the linear relationship between the message and the corresponding protein. Additionally, a protein could be present in high concentration, but it may lack the requisite conditions (substrate concentration, cofactors, etc.) for activity in the actual cellular environment. Moreover, in the context of the regulatory networks, a modification in the expression levels of a gene is not expected to alter only the concentration of the corresponding protein and the activity of the corresponding biochemical reaction, but it would also affect other parts of the cellular networks depending on the role of this gene in cellular function. Therefore, it is the integration of all of these molecular profiles for a systematically perturbed cellular system that can provide insight about the function of unknown genes, the relationship between gene and metabolic regulation, and even the reconstruction of the gene regulation network (Klapa and Quackenbush 2003; Vidal 2009). To succeed in the challenge of quantitative systems biology, however, major issues concerning the quantification capabilities and sources of biases of these multistep molecular analyses (Fig. 3.1) need to be thoroughly resolved for each level of molecular function and for the specific needs of each investigated biological system. They range from limitations in the available experimental protocols, to lack of data analysis and visualization techniques for upgrading the information content of the acquired measurements.

In comparison with the profiling analyses of other molecular levels of cellular function, the main asset of transcriptomics derives from the relative simplicity of its subject: mRNA is a polymer of only four different subunits, unlike proteins that are composed of 20 different amino acids and have various 3D structures and metabolites that have great chemical diversity. Thus, a single method of extraction and detection can theoretically identify and quantify every transcript in a tissue sample. As a result, transcriptomic studies tend to identify at least one order of magnitude more gene products than proteome studies (Baginsky 2009; Deyholos 2010). Moreover, the protein and metabolic levels are highly dynamic and environment sensitive. Currently, microarrays (Pease et al. 1994; Schena et al. 1995) have been the main platform used for transcriptomic studies in plants and in general in most biological systems. Microarrays have proven to be a reliable technological platform for the study of gene expression patterns, because of their relatively high sensitivity, specificity, accuracy, throughput, and cost-efficiency. However, array-based technologies are limited to the analysis of known transcripts. This limitation can be bypassed with transcriptome analysis based on the next-generation ("deep") sequencing platforms (Wang et al. 2009), which have not yet gained adequate momentum in plant physiology studies. Recently, Mizuno et al. (2010) conducted a study on the transcriptional effects of salinity stress on rice using both RNA deep sequencing and microarrays. RNA sequencing predicted the expression of more than 3000 transcripts not previously annotated by the Rice Annotation Project. Some of the unannotated genes were differentially expressed in response to salinity stress (Mizuno et al. 2010).

Metabolomic analyses provide the link between gene expression and the metabolic phenotype, the latter being very sensitive to the physiological responses caused by environmental perturbations on the plants. It has been estimated that about tens of thousand primary and secondary metabolism intermediates (metabolites) occur in the plant kingdom (Fiehn 2002). The metabolite concentration profile is affected and also affects the metabolic reaction rates, being thus a fingerprint of the metabolic state of the cells and tissues. Most metabolites act as regulatory molecules of protein functions and interactions, their accurate quantification being of additional importance for deciphering the molecular mechanisms that impose the physiology of the plants under specific conditions. Because of the chemical diversity of metabolites, metabolomic analysis is subject to analytical constraints that limit the number of metabolites that can be identified and quantified in a single sample. Currently, there is no extraction protocol and technological platform that can detect and quantify the total metabolome. Most often, extraction protocols of polar and semi-polar compounds are used in the metabolomics studies, as they capture a larger chemical diversity range. The most common technological platforms used for metabolome analysis are liquid or gas chromatography coupled with mass spectrometry (LC–MS or GC–MS), capillary electrophoresis coupled with mass spectrometry (CE–MS), and nuclear magnetic resonance (NMR) spectroscopy. Each platform has certain analytical limitations and a single platform can detect only a fraction of the total metabolome. The combined use of multiple analytical techniques, if available, can increase the fraction of the observable metabolome. Depending on the tissue, such a protocol and analytical technique will extract the components of the

primary metabolism, like sugars (monosaccharides and oligosaccharides), organic acids, amino acids, phosphate compounds, and amines, providing thus an extensive perspective of the primary (central carbon) metabolism. The primary metabolism is indicative of the energy, redox homeostasis, and growth demands of the plant cells, while it produces all the precursors of the cellular macromolecules and secondary metabolites. The changes in the primary metabolism reflect core perturbations in the metabolic physiology of the plants. Conserved among species metabolic responses to environmental stress acclimation should be observable within the primary metabolism. On the other hand, the secondary metabolism is more diverse among species and presumably reflects the successful adaptation of a species to particular environmental stresses through the acquisition of novel biosynthetic capacities of its primary metabolism (Sanchez et al. 2008a). Therefore, even if there are differences in the secondary metabolism, they can be inferred from the changes in the concentration profile of precursor molecules in the primary metabolism.

A major advantage of the high-throughput biomolecular analyses is that by observing a large number of molecular quantities at the same time, correlations between the activity of various molecular pathways can be determined, new knowledge can be extracted, and the biomolecular networks at different levels of cellular function (e.g., gene regulation, protein interaction, or metabolic networks) can be reconstructed. To this end, we are in search of multi-compound biomarker profiles and patterns of expression, rather than single molecules that can be sensitive sensors of changes in the physiology of the plants. Thus, the acquired datasets have to be analyzed with multivariate statistical methods, attempting to identify either clusters of genes or gene products that have similar expression or concentration, respectively, profiles among various physiological conditions, or physiological states that are of similar omic profiles. For this purpose, clustering, e.g., hierarchical clustering (HCL), analysis, and dataset dimension reduction and visualization, e.g., principal component analysis (PCA), methods are used. Customized multivariate significance analysis methods for omic data, like significance analysis for microarrays (SAM) (Tusher et al. 2001), have been developed enabling the identification of the genes or gene products, the change in the expression or concentration, respectively, of which is characteristic of the difference between two sets of physiological conditions. In the case of time-series experiments, particular modifications of PCA (Scholz et al. 2005) and SAM analyses (Dutta et al. 2007) have been proposed to take into consideration that the physiological states of the plants at the different time points are not independent, but rather part of the same physiological history.

3.4 Omic Analyses of Salinity Stress on Plants

3.4.1 Metabolomic Analyses

The effect of salt stress on plant metabolic physiology using metabolomic analytical platforms has been investigated in the context of maize (Gavaghan et al. 2011),

barley (Widodo et al. 2009), and grapevine (Cramer et al. 2007) in addition to the studies using the model organism *A. thaliana* (Kanani et al. 2010; Kim et al. 2007; Gong et al. 2005). Major aspects of each study that need to be considered when attempting to unify their results are the selected level of the salt stress and the duration of the treatment. Treatment durations can be categorized into: (a) short term, i.e., up to 24–30 h, (b) mid-term, i.e., from few days up to one week, and (c) long term, i.e., longer than one week up to few months.

Gavaghan et al. (2011) studied the mid-term responses of maize to high-salinity (i.e., 50 and 150 mM NaCl) stress using NMR spectroscopy. They observed a significantly increased concentration of sucrose, γ-aminobutyric acid (GABA), glycine-betaine, and free amino acids, including alanine, in the roots of the salt-stressed plants. The changes correlated with the salt concentration, suggesting thus a response mechanism for the plants to maintain osmotic balance. The concentrations of citrate, malate, succinate, and α-ketoglutarate declined in the shoot extracts in response to the salinization. The depletion of these tricarboxylic acid (TCA) cycle intermediates implies that the TCA cycle flux is reduced in the shoots as a result of the salt stress, hence the plant growth and energy metabolism is slowed down or arrested. Differences between the responses of salt tolerant and salt sensitive cultivars to salinity stress were observed in rice plants after long-term treatment with 100 mM NaCl (Zuther et al. 2007). Even the tolerant cultivars did not have common responses to salinity stress, but formed physiological response subgroups. One common response to salinity stress for most cultivars was the depletion of TCA cycle intermediates, in agreement with the results of the previously described maize study. Hence, both studies suggest that the acclimation to high salt concentrations has a high demand for energy, competing thus with the plant growth.

Lu et al. studied the mid-term response to the salinity (i.e., 100 mM NaCl) stress of two varieties of soybean using GC–MS and LC–MS metabolomics (Lu et al. 2013). In leaf samples from salt-stressed plants of both varieties, they observed a significant reduction in the concentration of alanine, sucrose, and TCA cycle intermediates and a significant increase in the concentration of abscisic acid (ABA), glycine, serine, and sugar alcohols, such as lactitol and maltitol, compared to the control conditions. ABA is a plant hormone that accumulates under drought stress and causes stomata closure. The ABA-induced stomata closure reduces transpiration, thus preventing further water loss from the leaves in times of low water availability (Steuer et al. 1988). Sugar alcohols and amino acids can act as osmolytes and their increase under salt stress is a response mechanism for the plants to maintain osmotic balance, balancing the decreased water potential associated with the sodium ion accumulation in the vacuoles and the extracellular volume, as stated above. The reduction in the concentration of sucrose and TCA cycle intermediates suggests the high energy cost for the acclimation to salinity stress that was observed in all relevant studies discussed so far. The accumulation of osmolytes under salinity stress has also been observed in grapevines after mid- and long-term treatment (Cramer et al. 2007). The shoot concentrations of fructose, glucose, proline, glycine, and malate increased in the salinized compared to the control plants. The observed increase in the malate concentration was consistent with the significant increase in the

transcripts of the glyoxysomal and chloroplastic malate dehydrogenases and the decreased abundance of transcripts of the cytoplasmic and mitochondrial malate dehydrogenases. Moreover, the accumulation of proline was consistent with an increase in the transcript abundance for delta 1-pyrroline-5-carboxylate synthetase (P5CS), the enzyme that catalyzes the first two steps in the proline biosynthetic pathway.

Kanani et al. (2010) observed that after a short-term (i.e., 30 h) continuous exposure to high salinity, *A. thaliana* plant liquid cultures accumulated fatty acids and sterols including tocopherol, a known antioxidant. A significant increase was also observed in the levels of homo-serine, β-alanine, methionine, glycine, *N*-acetyl-glutamate, allantoin, and the TCA cycle intermediates from citrate to fumarate throughout the treatment period. Homoserine and methionine are precursors of the *S*-adenosyl-methionine, which is required along with glycine for the biosynthesis of glycine-betaine, the main osmoprotectant in *A. thaliana*, and along with β-alanine for the production of β-alanine-betaine. Polyamines and betaines are known osmolytes in plants. As it was the case with the previously discussed studies, these observations are in accordance with the need of the plants to produce osmoprotectants and antioxidants to counteract the stress conditions. At the same time, the increased production of amino acids/amine group containing metabolites that are precursors of osmoprotectants and antioxidants was accompanied by a significant decrease in the concentration of metabolic intermediates that are required for plant growth. Moreover, based on their time-series analysis, Kanani et al. were able to observe a change in the metabolic physiology of the plants even from the first hour of the salinity treatment.

Sanchez et al. (2008a) studied comparatively the metabolic responses of *A. thaliana*, *Lotus japonicus*, and rice after long exposure and potential acclimation of the plants to salinity stress (i.e., 75, 150, and 100 mM NaCl for each plant species, respectively). They reported a salinity dose-dependent increase in the concentration of sucrose and amino acids like proline, glycine, serine, threonine, leucine, and valine, in all the three species. The TCA cycle intermediates, citrate, succinate, malate, and other organic acids, such as oxalic and maleic acids, which are direactly related with the TCA cycle flux, exhibited conserved reduction in their pool sizes in response to long-term salinity stress. Reduction was also observed in the concentrations of the glycolysis intermediates glucose, fructose, glucose-6-phosphate, and fructose-6-phosphate. The authors suggest that a reason for the reduced acid levels under salt stress may be their involvement in the compensation of the ionic imbalance. At physiological pH levels, organic acids exist as carboxylic anions and counterbalance inorganic anions, so a depletion of organic acids may actually reflect preferential uptake of anions compared to cations. Moreover, the increased amino acid biosynthesis may also serve the plants to absorb excess ammonium while producing osmolytes. Excess organic acids could be recruited from the TCA cycle and sequestered into the biosynthesis pathways of amino acids and amines. Thus, the maintenance of the charge balance, the ammonium detoxification, and the compatible solute accumulation could all be met by a common mechanism.

Gong et al. (2005) compared the short-term responses to salinity (i.e., 150 mM NaCl) stress of *A. thaliana* and *Thellungiella halophila*, a species related to

A. thaliana with extreme tolerance to a variety of abiotic stresses, including low humidity, freezing, and high salinity. As expected, metabolites that act as osmolytes, i.e., proline, galatinol, and glycine, increased under salt stress in both species. It has to be noted that, at the control conditions, the concentration of several compounds that have protective functions was much higher in *T. halophila* than in the *A. thaliana* plants. Maybe, this concentration profile could partly justify the *T. halophila* surviving mechanisms under extreme salt concentrations. Widodo et al. (2009) studied the long-term responses of two barley cultivars to salinity (i.e., 100 mM NaCl) stress using GC–MS. After three weeks of high-salinity treatment, the more sensitive cultivar ceased growing, while the tolerant resumed similar growth to the control plants. At the metabolic level, the sensitive cultivar exhibited an increase in the levels of proline, GABA, and the polyamine putrescine, most in accordance with the previous salinity studies. They suggested, however, that the observed increase in these metabolites is not an adaptive response to salinity but an indication of slower growth or tissue necrosis. On the other hand, in the tolerant plants, the levels of TCA cycle intermediates and hexose phosphates increased in response to salt. However, the response of each cultivar to salinity stress depended heavily on the duration of its exposure to high salinity.

3.4.2 Transcriptomic Analyses

Many DNA microarray transcriptomic studies of the plant response to high salinity have been reported in the literature (Sanchez et al. 2008b, 2011; Beritognolo et al. 2011; Bazakos et al. 2012; Kanani et al. 2010; Legay et al. 2009; Jankangram et al. 2011; Gong et al. 2005; Chao et al. 2005; Evers et al. 2012; Wang et al. 2013; Cramer et al. 2007). Main common observations of these studies are: (a) the significant decrease in the transcripts related to photosynthesis, i.e., the photosystem I and II subunits, Calvin cycle enzymes, RuBisCO subunits and the RuBisCO activase, protein synthesis and energy metabolism pathways (Beritognolo et al. 2011; Kanani et al. 2010; Legay et al. 2009; Gong et al. 2005; Chao et al. 2005; Evers et al. 2012; Wang et al. 2013) and (b) the simultaneous significant increase in the abundance of transcripts related to signaling, membrane transporters, and the synthesis of osmoprotectants and antioxidants (Deyholos 2010). These observations are in agreement with the known decrease in the photosynthesis rate of the salinized plants based on physiological studies (Chaves et al. 2009) while providing molecular insights about this decrease. Interestingly, however, Cramer et al. report an increase in the transcript levels of the photosystem I and II subunits and the RuBisCO activase after long exposure of grapevines to progressive salinity stress (Cramer et al. 2007). This could be a secondary response of the specific species after long exposure to salinity stress that ensures the survival of the plant. It also underlines the significance of considering all parameters of the experimental design, including the treatment duration and strength, when trying to integrate the results among different studies. In the salt-stressed plants, the abundance of transcripts encoding proteins related to cellular growth like histones (Kanani et al. 2010; Gong et al. 2005) and the as primary metabolism (Beritognolo et al. 2011; Legay et al. 2009; Evers et al. 2012)

was significantly decreased, suggesting that the salinity stress affects in a negative way the plant-growth-related pathways at the transcriptional level.

The levels of transcripts encoding late embryogenesis abundant (LEA) (Sanchez et al. 2011; Legay et al. 2009; Chao et al. 2005; Wang et al. 2013; Cramer et al. 2007) and heat shock proteins (HSP) (Beritognolo et al. 2011; Legay et al. 2009) tend to increase after exposure of plants to high salinity. LEA proteins are small hydrophilic, largely unstructured, and thermostable proteins that are synthesized in the seeds during maturation. It is believed that they play a protective role against desiccation through multiple functions, including ion binding, hydration buffering, and membrane and protein stabilization (Battaglia et al. 2008). Most HSPs have been shown to act as molecular chaperones, which are responsible for protein synthesis, targeting, maturation, stabilization, refolding under stress conditions, and degradation in a broad array of normal cellular processes. Moreover, the HSPs participate in the membrane stabilization under stress conditions (Wang et al. 2003).

Consistent with the findings from metabolomics, most transcriptomic studies of the salinity effect on plants record increased the abundance of gene transcripts involved in the biosynthesis of osmolytes (Sanchez et al. 2011; Legay et al. 2009; Gong et al. 2005; Chao et al. 2005; Evers et al. 2012). The abundance of transcripts related to the ROS scavenging and detoxification has in some studies been reported as increasing (Beritognolo et al. 2011; Gong et al. 2005; Chao et al. 2005; Cramer et al. 2007) and in some others as decreasing (Legay et al. 2009; Evers et al. 2012; Wang et al. 2013), after the plants are exposed to salinity stress. This discrepancy could be an indication that in some cases ROS act as signaling molecules for the salinity stress and have thus to attain high concentrations to trigger other reactions, or it could just be a consequence of different durations of plant exposure to stress. Transcripts that encode ion and amino acid transporters also accumulate in the plants after exposure to the salinity stress (Beritognolo et al. 2011; Kanani et al. 2010; Legay et al. 2009; Gong et al. 2005).

The activity of the salt overly sensitive (SOS) signaling pathway is of particular interest regarding the response of the plants to the salt stress. This pathway is responsible for the extracellular and vacuolar sequestration of the Na^+ ions with H^+/Na^+ antiporters, a process of high significance for the ion homeostasis of the plants (Zhu 2002). The Na^+ ion increase caused by the salt stress could be detrimental to the plants, causing membrane disorganization, impaired nutrient and water acquisition, metabolic toxicity, inhibition of photosynthesis, and the production of ROS (Niu et al. 1995). In a transcriptomic analysis of *A. thaliana* plant liquid cultures under salt stress, the activity of the SOS pathway was indeed observed as significantly increased at the transcriptional level (Kanani et al. 2010).

3.4.3 Results of Integrated Metabolomic and Transcriptomic Analyses

Some of the above-mentioned studies carried out both transcriptomic and metabolomic analyses on the same set of plants, in an effort to comprehensively investigate the changes in the physiology of the plants due to the high-salinity stress

at the two molecular levels of cellular function. Some observations were consistent between the transcriptional and metabolic level, indicating regulation of the relevant response mechanisms at the transcriptional level, which are then by consequence reflected at the metabolic level too. For example, Cramer et al. (2007) and Gong et al. (Gong et al. 2005) reported that the proline accumulation after salinity treatment was consistent with the observed increase in the abundance of transcripts encoding enzymes in the proline biosynthesis pathway (Cramer et al. 2007; Gong et al. 2005). However, other results at the metabolic level would not have been directly predictable if only the transcriptomic information had been available, indicating thus regulatory mechanisms that are active at the metabolic level. There are also processes that are not directly involved in metabolism and cannot thus be directly observable through the metabolic profiles, but only through the transcriptomic profiles, like photosynthesis, ethylene signaling, and others. Integrated omic analyses at multiple molecular levels are thus required for the comprehensive understanding of all physiological changes due to a particular stress.

3.5 Omic Analyses of Elevated CO_2 Stress on Plants

The effect of the elevated CO_2 concentration in the growth environment of the plants has been extensively studied with both physiological and high-throughput biomolecular analysis studies at the transcriptional, protein, and metabolic levels, mainly in the context of long-term (i.e., 1–2 weeklong) adaptation to high CO_2 environments. The main reason for these studies has been to investigate how the plants will change their physiology in response to the greenhouse effect. Li et al. (2008) conducted a free-air CO_2 enrichment (FACE) experiment to study the metabolic and transcriptional effects of elevated CO_2 (i.e., 550 ppm) in the growth environment of two *A. thaliana* ecotypes. At the metabolic level, they observed an increase in the concentration of sugars, like maltose, glucose, fructose, and galactose, and of TCA cycle organic acid intermediates, along with a decrease in the levels of most amino acids, with the exception of the aromatic amino acids tryptophan and phenylalanine, the concentration of which increased under elevated CO_2. In accordance with the metabolomic results, transcriptomic analysis indicated an increase in the concentrations of transcripts related to the cell wall formation and metabolic processes like the glycolysis, the TCA cycle, and the anthocyanin and flavonoid biosynthesis. Moreover, transcripts related to the amino acid biosynthesis were downregulated or did not change, with the exception of those involved in the tryptophan and phenylalanine biosynthesis. The abundance of transcripts related to photosynthesis, like the photosystem I and II subunits, as well as Calvin cycle enzymes, was reduced in plants treated with elevated CO_2 for long durations. The amount of transcripts encoding chloroplast-localized proteins unrelated to light capture and fixation functions also declined significantly. The authors suggested that these changes reflect nitrogen deprivation. Increased photosynthetic CO_2 fixation altered the apparent C:N balance. The findings of Miyagi et al. (2011) were consistent with this hypothesis.

After four weeks of growth in 1000 ppm of CO_2, *Rumex obtusifolius* plants had increased levels of TCA cycle intermediates, especially citrate and fumarate, while the amino acid levels, apart from phenylalanine and tryptophan, decreased. On the other hand, plants that were grown under elevated CO_2 in a medium rich in nitrogen exhibited increased levels of TCA cycle intermediates and amino acids compared to the control or just nitrogen-rich conditions. In contrast with the above findings, Kaplan et al. (2012) reported decreased levels of TCA cycle intermediates and glycine and increased levels of sugars in *A. thaliana* plants after long-time exposure to 1200 and 4000 ppm of CO_2. In the same plants, the concentration of transcripts related to starch synthesis and catabolism increased, with a simultaneous decrease in transcripts related to photosynthesis, like the photosystem and RuBisCO subunits. The amount of transcripts for genes that are inducible by ABA and jasmonic acid was also increased by elevated CO_2. The authors suggested that the elevated CO_2 conditions reduce respiration and act as a stressor for plants. All these discrepancies in the findings from the discussed studies underline the importance of carefully examining the physiological conditions to which each study refers with respect to the duration and severity of treatment, the plant species that is investigated, the tissue or cell type analyzed, and the type of plant culture (hydroponic or other), to accurately interpret and potentially generalize the observed results.

Dutta et al. (2009) examined the responses of *A. thaliana* plant liquid cultures to elevated CO_2 (i.e., 10,000 ppm) over a short period of 30 h in a time-series experiment using integrated metabolomic and transcriptomic analyses. It was observed that the plants which were grown in the elevated CO_2 environment had decreased pools of all the three organic acids (glycerate, glyoxylate, glycolate) and serine in the photorespiration pathway and decreased expression of the photorespiratory pathway genes at most of the examined time points. Interestingly, differences were observed between the responses of the plants at the earlier compared to the later time points of the experiment. Specifically, during the first six hours of the experiment, the levels of most amino acids (i.e., glutamine, asparagine, aspartate, arginine, valine, isoleucine, glycine, methionine, lysine, and GABA) increased. An increase was also observed in the levels of the TCA cycle intermediates citrate and isocitrate. However, beyond twelve hours of continuous exposure to elevated CO_2 conditions, the levels of almost all amino acids decreased. The transcriptomic analysis showed that at the early time points, the abundance of transcripts associated with the ribosomes decreased, whereas at the later time points many of the transcripts related with photosynthesis had a reduced abundance in response to the elevated CO_2, implying thus potential closure of stomata after a twelve hour exposure to elevated CO_2.

The above-mentioned data suggest that after a particular duration of growth under elevated CO_2 conditions, the plants seem to acclimate to the particular environment and the expression of genes related to photosynthesis declines. However, carbon fixation remains higher than in the ambient CO_2 conditions. Thus, after a certain duration of exposure to elevated CO_2, the carbon to nitrogen ratio increases and the nitrogen becomes the limiting factor for the plant growth. Therefore, the levels of amino acids are expected to decrease after a long-time exposure to elevated CO_2.

3.6 Omic Analysis of Plant Response to Combined High-Salinity and Elevated CO_2 Perturbations

Despite the fact that physiological measurements in different plants and trees have indicated that the elevated CO_2 conditions can alleviate the negative effect of salinity stress in plants at least for short-term treatments (Geissler et al. 2010; Perez-Lopez et al. 2012; Perez-Lopez et al. 2009; Ratnakumar et al. 2013; Takagi et al. 2009), to the best of our knowledge, there has currently been only one study, which has monitored the molecular response of the plants to combined salinity stress and elevated CO_2, using the high-throughput biomolecular (omic) analyses. Kanani et al. (2010) integrated GC-MS metabolomics and DNA microarray transcriptomics to study the growth of *A. thaliana* plant liquid cultures in a high-salinity (i.e., 50 mM NaCl) medium and elevated CO_2 (10,000 ppm) environment, for the first 30 h of continuous treatment in a time-series experiment. The plants had grown under constant light, temperature, and humidity and the same conditions were maintained throughout the treatment period. The authors support this setup, as it minimizes any contributions to the observed physiological changes from any other parameter but the two investigated factors. The authors report that the effect of the salinity stress was stronger than that of the elevated CO_2 conditions at both the transcriptional and metabolic levels. Interestingly, there was a strong similarity over time between the transcriptomic responses of the plants exposed to high salinity and those exposed to the combined stress. This similarity suggests that the early transcriptional response of the plant cultures to the salinity stress is robustly active independently of the co-occurrence of the elevated CO_2 conditions. For example, the SOS signaling pathway is upregulated at the transcriptional level under both high-salinity and the combined perturbation conditions. The major finding of this analysis, however, was that the observed physiological consequences of the combined stress at the metabolic level was different from what would have been expected based only on the transcriptomic profiles. Specifically, the combinatorial effect of the elevated CO_2 conditions and the salinity stress on the metabolic physiology of the plants was milder than that of the salinity stress alone, implying that the elevated CO_2 conditions are an alleviating factor for the salt-stressed samples. The analysis of the metabolomic profiles indicated that this beneficiary role of the elevated CO_2 can be primarily attributed to the provision of additional resources to the salt-stressed plants. Using these additional resources the plants can activate their response machinery against high salinity and produce osmoprotectants and antioxidants, without having, however, to sacrifice substrates needed for plant growth. This conclusion was based on the fact that, under the combined stress, the concentrations of the TCA cycle intermediates citrate, aconitate, and isocitrate, and the amino acids alanine, valine, lysine, and asparagine, which contribute to protein synthesis, were observed at similar values as in the control metabolic state. At the same time, metabolic precursors of osmoprotectants that exhibited increased concentration in the salt-stressed plants (i.e., *S*-adenosyl-methionine and glycine, which are precursors of glycine–betaine and β-alanine, which is a precursor of β-alanine-betaine) retained their concentrations in the plants subjected to the combined high-salinity and elevated CO_2 perturbation.

The alleviating role of the elevated CO_2 in the growth environment of salt-stressed plants was also supported by the downregulation of transcripts related to the ethylene signaling, a pathway that is characteristically upregulated at the transcriptional level in plants exposed to salt stress. These observations suggest that the controlled use of the CO_2 in greenhouses could offer a pragmatic solution for counteracting the negative effect of high soil or water salinity and lead to plant crops of consistent quality and yield.

3.7 Experimental Design Specifications of Integrated Omic Analyses of Combined Salinity and Elevated CO_2 Stresses

A controlled study of the combined effect of high salinity and elevated CO_2 on the plants should include at least four plant groups: plants grown in a control medium or soil and the ambient CO_2 concentration (i.e., the control group), plants grown in a high-salinity medium or soil and the ambient CO_2 concentration (i.e., the high-salinity group), plants grown in a control medium or soil and an elevated CO_2 concentration (i.e., the elevated CO_2 group), and plants grown in a high-salinity medium or soil and an elevated CO_2 concentration (i.e., the combined perturbation group). In this type of studies, hydroponic cultures provide a more controlled system over the soil-grown plants, as the effect of any perturbations in other growth parameters, e.g., the nutrient composition of the soil, are minimized. Comparison between the four measured physiological states can provide information about changes in the physiology of the plants due to stresses that are not directly measured. For example, the comparison between the omic profile of the combined perturbation and the high-salinity groups can provide information about the effect of the elevated CO_2 on the salt-stressed plants, even when this experiment has not been carried out. This is how the alleviating role of the elevated CO_2 on salt-stressed plants was identified in Kanani et al. (2010). Depending on the investigated species (or cultivars or ecotypes), the imposed salinity should be high enough to act as stressor for the plants, but not too high to cause tissue necrosis. In the high-salinity experiments, the utilized salt concentration usually ranges from 50 to 150 mM, reaching 250 mM in some studies of halophile species. In most reported studies, elevated CO_2 conditions are characterized by concentrations between 500 and 1500 ppm to simulate the plant responses to the predicted increase in the ambient CO_2 due to the greenhouse effect (Kaplan et al. 2012; Li et al. 2008; Miyagi et al. 2011). However, in some studies, a much greater CO_2 concentration has been used to ensure changes in the physiology of the plants due to this perturbation (Kaplan et al. 2012; Dutta et al. 2009). Apart from growth chambers, FACE facilities have also been used to study the effect of the elevated CO_2 on the plants (Li et al. 2008). At these facilities, horizontal or vertical pipes are placed in a 1 m to 30 m diameter circle around the experimental plot, and emit CO_2-enriched air around the plants (Ainsworth and Long 2005).

As discussed above, the responses of the plants to various perturbations depend also on the duration of the treatment. Time-series experiments are preferable to gain deeper insight into the physiology of the plants as a function of the exposure duration to stress. The first sampling should be carried out just before the perturbation/treatment is applied to monitor the physiological state of the plants at the time zero of the experiment. The rest of the samplings should be scheduled in such a way to allow for the determination of the short-, mid-, and long-term responses of the particular plant species (or cultivar or ecotype) to the applied perturbation/treatment. When scheduling the sampling points, the circadian rhythm of the plants and the difference in the timescale of the response between the transcriptional and the metabolic processes should also be taken into consideration. Cramer et al. conducted an interesting time-series experiment to study the metabolic responses of the grapevine to high salinity and drought (Cramer et al. 2007). Instead of using one fixed concentration of NaCl for the high-salinity "perturbed" plants, they started the experiment with zero salt concentration in the irrigating solution and increased it gradually over time. This experimental design enabled them to make the separation between the plant responses due to the water-deficit effects and those arising from ionic effects within the plants.

Three to six plants per group and per time point usually provide an adequate number of biological replicates for the extraction of accurate results from omic analyses. In most of the studies cited above, leaves were sampled. Leaves are the main photosynthetic organs of plants and their reaction to elevated CO_2 and the salt stress is of great interest. However, the first tissues that experience the salinity of the medium or the soil are the roots, so it would be of value for the roots to be sampled and their response to the applied perturbation(s) to be studied in comparison with that of the leaves. Immediately after sampling, the collected samples should be frozen in liquid nitrogen and kept at −80 °C until further processing. Freezing with liquid nitrogen is essential to stop all the enzymatic processes in the samples and to protect thermosensitive molecules like sugar phosphates and mRNA molecules from degradation. If the collected amount permits, the same sample should be divided and used for the extraction of mRNAs, proteins, and metabolites for integrated omic analyses.

Great care should be paid to eliminate or correct for various systematic biases introduced at various stages of the multistep omic analyses (see Fig. 3.1). For example, in a typical metabolomic analysis, injection errors or unequal division of a sample into replicates could affect the metabolic profiles. These types of errors affect equally all metabolites detected in a profile. To account for these biases, internal standard normalization is required. The selected internal standard should not be produced by the plant species of interest. Ribitol or isotopes of known metabolites are commonly used as internal standards in plant MS metabolomics (Fiehn et al. 2001; Roessner et al. 2000). Errors that affect specific metabolites may also occur. In the case of GC–MS metabolomics, the metabolites have to be derivatized so that they are converted to their volatile, nonpolar, and thermostable derivatives. The most common method for derivatization involves the conversion of the original metabolites to their methoxime (MEOX) and trimethylsilyl (TMS) derivatives (Roessner et al. 2000). However, some metabolites produce more than one

derivative with relative concentrations depending on the derivatization conditions and time. Thus, the derivatization step itself can introduce biases affecting each metabolite in a different way depending on its structure, concentration, and relative affinity for the derivatizing agent. If these biases are not identified and properly accounted for, they will skew the measured metabolite concentration profile providing a faulty perspective of the metabolic state of the samples. Kanani and Klapa (2007) proposed a normalization method for this type of biases. Proper normalization of the omic profiles at each molecular level before data analysis is quite crucial to establish comparability between the samples and avoid assigning biological meaning to experimental biases.

In addition, in integrated transcriptomic and metabolomic analysis of (combined) stresses in plants, the final interpretation of the results should take into consideration the different specifics of the two analyses. Transcriptomic analyses are based on the comparison of the concentration profile of mRNA transcripts in an equal amount of total mRNA between for all samples. It is considered that the cells of the same species produce equal amount of total mRNA independently of their physiological state. Thus, the transcriptomic analysis of a biological sample among different physiological states provide a measure of the change in the composition of the total mRNA of this sample among different physiological conditions. On the other hand, the amount of the acquired metabolite extract of a biological sample can change between states. The internal standard is added to allow for sample normalization per unit of mass of the investigated biological system. In this way, it is mainly the change in the amounts and to a lesser extent the change in the relative concentrations of the metabolites in the extract that governs the observed differences among the metabolic profiles of the various samples (Kanani et al. 2010).

3.8 Conclusions

In the post-genomic era, high-throughput biomolecular (omic) analyses have been used to gain insight into the molecular response of the plants to abiotic stresses. Among the mostly investigated abiotic stresses are the high soil or water salinity and the elevated CO_2 in the growth environment of the plants. The most popular omic analyses for this type of stresses have been the MS metabolomics and the transcriptomics based on DNA microarrays. While physiological studies have indicated that the elevated CO_2 can alleviate the negative effect of the salinity stress in plants, only one time-series omic analysis study has been reported so far for the combined implementation of elevated CO_2 and salinity stresses on plants. The particular study on *A. thaliana* plant liquid cultures indicated that the elevated CO_2 provides additional resources to the plants allowing them to produce the required osmoprotectants to counteract the salinity stress without having to sacrifice their growth (Kanani et al. 2010). However, for the insights garnered from this model system study to be directly applicable in crop improvement and production, species and cultivars of commercial value for the food industry and/or agro-biotechnology

should be studied. Plants such as tomato or pepper are of great interest because they are mainly cultivated in greenhouses where the levels of CO_2 could be adjusted. The identification of molecular biomarkers for the salinity stress in plants could help monitoring the progress of its negative effect on the plant growth and yield of salt-stressed plants. The latter can further our understanding of the underlying molecular mechanisms and assist us in devising methods for the educated use of elevated CO_2 conditions to alleviate the salinity impact, supporting plant cultivation processes of consistent quality and yield.

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Chapter 4
Combination of Elevated CO_2 Levels and Soil Contaminants' Stress in Wheat and Rice

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4.1 Introduction

With increasing global industrialization, the atmospheric CO_2 concentration has risen from approximately 280 mmol mol^{-1} in preindustrial times to approximately 380 mmol mol^{-1} now, and it is expected to continue increasing in the future (IPCC 2007). Elevated atmospheric CO_2 levels can stimulate photosynthesis (Zhang et al. 2008), enhance carbon deposition in soil (Hill et al. 2007), and change the rhizosphere conditions of the plant, leading to increases in biomass and yields of crops (Delucia et al. 1997; Lieffering et al. 2004; Liu et al. 2008).

Some industrial, mining, and agricultural activities have contaminated soils with heavy metals, and such pollution is increasingly becoming a serious environmental problem. In China, more than 2.0×10^7 ha of agricultural land is reportedly

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Reprinted (adapted) with permission from elevated CO_2 levels affects the concentrations of copper and cadmium in crops grown in soil contaminated with heavy metals under fully open-air field conditions. Copyright (2011) American Chemical Society.

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contaminated with heavy metals (Huang et al. 2009). Heavy metals are toxic to plants, animals, and humans at different concentrations, and are known to cause significant environmental damage and human health problems (Huang et al. 2009; Mulligan et al. 2001; Nahmani et al. 2005; Maksymiec 2007). Of the heavy metals, copper (Cu) is an essential micronutrient for plants and animals. However, when in excess, Cu can interfere with numerous physiological processes, resulting in cell toxicity. Cadmium (Cd) is a toxic trace element in the environment that can be easily taken up by plants and subsequently transferred to humans through food chains.

In the future, further increases in global CO_2 levels and contamination with heavy metals are likely. More research is needed to investigate the response of crops grown in soils contaminated with metals under elevated CO_2 levels. In the few reports available, Duval et al. (Duval et al. 2011) indicated that CO_2 alters the distribution of contaminant elements in ecosystems; Wu et al. (2009) showed that elevated CO_2 level increases cesium (Cs) concentrations in rice shoots and roots; Li et al. (2010) reported that elevated CO_2 levels decrease or barely affect Cu concentrations in six rice varieties grown in contaminated soils, but increase Cd levels in three rice varieties. These studies highlight the need for a better understanding of the mechanisms by which CO_2 and heavy metals jointly affect crop growth and uptake of metals, especially from the viewpoint of food safety. One also must consider that these studies were conducted in open-top chambers (OTCs). Long et al. (2006) reported that the effects on plants grown in OTCs are often greater than on plants grown under open air. Therefore, the results of such studies cannot be extrapolated to address the effect of long-term, more realistic CO_2 fumigation on plants. One way to approach such a study is to use free-air CO_2 enrichment (FACE). FACE experiments are conducted in open fields, allow the best simulation of elevated CO_2 environments (Long et al. 2006), and have been carried out in many countries (Lieffering et al. 2004; Andrews and Schlesinger 2001; Hoosbeek et al. 2007).

With the aim of predicting future food safety and the combined stress of CO_2 and soil pollution, we used a full-size (14 m diameter) FACE system in farm fields in Jiangsu Province, China, to investigate the effect of elevated atmospheric CO_2 on Cd and Cu levels in two important crops worldwide, rice and wheat.

4.2 Materials and Methods

4.2.1 Experimental Site

The FACE system was established in the town of Xiaoji, Jiangdu County, Jiangsu Province, China (119°42’E, 32°35’N). Here, rice–wheat rotation system is practiced. This region lies within the northern subtropical monsoon climate. The annual mean temperature is 14–16 °C, and the mean annual precipitation is 980 mm. The annual length of the nonfrost period is approximately 220 days. The soil is Shajiang-Aquic Cambosols with a sandy–loamy texture.

4.2.2 FACE System

The FACE system has been described in detail by Liu et al. (2002) and Okada et al. (2001). In brief, the FACE system consists of octagonal plots located in different paddies having similar soils and agronomic histories. The plots have either elevated CO_2 levels (hereafter called FACE plots) or ambient CO_2 conditions (hereafter called ambient plots). Each plot is ca. 80 m^2. In the FACE plots, plants were exposed to elevated CO_2 levels within rings 12.5 m in diameter that emitted pure CO_2 from the periphery toward the center through emission tubes located about 50–60 cm above the canopy. In the ambient plots, plants were grown under ambient CO_2 conditions. To minimize the CO_2 contamination, ambient plots were at least 90 m away from the nearest FACE ring. The season-long average CO_2 concentration of the ambient plots was about 370 μmol mol^{-1}. The CO_2 concentration in the FACE plots was constantly controlled at about 200 μmol mol^{-1} higher than in the ambient plots.

4.2.3 Crop Cultivation and Sample Preparation

Rice (*Oryza sativa* L. cv. Wu Xiang jing 14) and wheat (*Triticum aestivum* L. cv. Yangmai 14) plants were grown in plastic pots in soils collected from a local farm. The properties of the soils are shown in Table 4.1. Fresh soil was sieved through a 3-mm sieve and kept in the dark until used. Soils were spiked with either Cu or Cd; control soil was not spiked. Specified amounts of Cd in the form of a dissolved solution of $CdCl_2 \cdot 2H_2O$ were added and thoroughly mixed into the soil as 0, 0.5, and 2.0 mg kg^{-1}. Specified amounts of Cu in the form of a dissolved solution of $CuSO_4 \cdot 5H_2O$ were added and thoroughly mixed into the soil as 0, 50, and 400 mg kg^{-1}. The spiked and unspiked soils were then watered to field water capacity and kept in the dark for 6 months. Prepared soil was placed in plastic pots (5 kg soil per pot; 20 cm in diameter, 35 cm in height). Two FACE plots and two ambient plots were used in this experiment; plants treated with Cu were grown on one FACE plot and one ambient plot, and plants treated with Cd were grown on another FACE plot and another ambient plot. Each treatment consisted of three replicate pots.

The experiments were conducted from June 2006 to October 2008. Rice–wheat rotation was used. The first rice seeds were planted in June 2006, and the plants were harvested in October 2006. The first wheat sowing was in November 2006, with harvest in May 2007. The second rice sowing was in June 2007, with harvest in October 2007. The second wheat sowing was in November 2007, with harvest in May 2008. The third rice sowing was in June 2008.

Table 4.1 Physical and chemical characteristics of soils used in this study

Soil spiked with	Organic matter (g kg^{-1})	Sand (%)	Silt (%)	Clay (%)	pH	Total Cu (mg kg^{-1})	Total Cd (mg kg^{-1})
Cu	17.3	57.8	28.5	13.7	6.92	21.7	0.15
Cd	18.4	56.1	29.6	14.3	7.21	18.9	0.11

The rice and wheat plants from the first sowing were sampled at the mid-tillering, panicle-initiation, and grain-maturity growth stages, and those from the second sowing were only sampled at grain maturity. Leaves for enzyme assays were frozen in liquid nitrogen when sampling and stored at −80 °C. Soils were sampled at the rice grain-maturity growth stage of the second sowing (October 2007) for pH analysis and metal fractionations. Rice roots were sampled at the panicle-initiation stage of the third sowing and stored at −40 °C for microscopy (August 2008).

4.2.4 Analysis of Antioxidant Enzyme Activities in Leaves

The preparation method for crude enzyme referred to the method proposed by Cho and Seo (2005). The activities of superoxide dismutase (SOD) were measured by nitroblue tetrazolium (NBT) photoreduction method (Dhindsa et al. 1981). The methods for determining the activities of catalase (CAT), peroxidase (POD), and ascorbate peroxidase (APX) were as described earlier (Cakmak and Horst 1991; Cakmak et al. 1993; Asada 1984).

4.2.5 Determination of Cd and Cu

The sampled plants were thoroughly washed with tap water and then with deionized water, and were then oven-dried to a constant weight at 70 °C. The dried samples were ground, weighed, and digested with concentrated HNO_3/$HClO_4$ (4:1 v/v; Li et al. 2001). The Cd and Cu concentrations in the digested solution were analyzed by atomic absorption spectroscopy (AAS; Thermo Solaar M6, USA).

4.2.6 Determination of Soil pH and Sequential Extraction of Soil

The pH of the soil was measured in a 0.01 M $CaCl_2$ solution at a 1:2.5 ratio of soil to solution (w/v) using a pH meter. Cu and Cd fractionation in soil was determined by sequential extraction using the method of the Commission of the European Communities (Community Bureau of Reference; BCR). The method is described in detail by Quevauviller et al. (1997).

4.2.7 Scanning Electron Microscopy

Fresh rice roots were thoroughly washed with deionized water. The first 1 cm of each root tip was cut and coated with gold (ca. 1 nm thickness) for 60 s using a sputter coater (HITACHI E-1010, Japan). The samples were viewed with a scanning electron microscope (SEM; S-3400N II, Hitachi, Japan).

4.2.8 Statistics

Data were expressed as means ± standard deviation ($n = 3$; n represents three replicate pots) and analyzed statistically using the SPSS software program (SPSS Inc., USA, version 16.0). The data were analyzed with a two-way analysis of variance (ANOVA) approach, i.e., Cd or Cu treatment (three levels) and CO_2 treatment (two levels). The mean values from the ambient plots were compared to the mean values from the FACE plots on each day of measurement using Tukey's test. The unilateral t test was also performed to distinguish among enzyme activities. The difference between the means was considered significant at $p < 0.05$.

4.3 Results and Discussion

4.3.1 Changes in Enzyme Activities

The changes in the activities of enzymes in the leaves of rice at mid-tillering growth stage are shown in Figs. 4.1 and 4.2. In Cu treatment group, the activity of CAT in leaves of rice grown on FACE plots with 50 and 400 mg kg^{-1} Cu in the soil was 41.9 and 40.0% lower, respectively, than that in leaves of rice grown on ambient plots (Fig. 4.1a). The activity of APX in leaves of rice grown on FACE plots with 400 mg kg^{-1} Cu was 18.9% lower than that in leaves of rice grown on ambient plots (Fig. 4.1b). The activity of POD in leaves of rice grown on FACE plots with 0 (control) mg kg^{-1} Cu added was 39.3% higher than that in leaves of rice grown on ambient plots, while no significant differences were found between the FACE and ambient plots either 50 or 400 mg kg^{-1} Cu treatment group (Fig. 4.1c). The activity of SOD in leaves of rice grown on FACE plots with 0 (control) mg kg^{-1} Cu was 30.9% higher than that in leaves of rice grown on ambient plots. No significant differences were found between the FACE and ambient plots either 50 or 400 mg kg^{-1} Cu treatment group (Fig. 4.1d). In Cd treatment group, the activity of CAT in leaves of rice grown on FACE plots with 0.5 mg kg^{-1} Cd was 34.0% lower than that in leaves of rice grown on ambient plots (Fig. 4.2a). The activity of APX in leaves of rice grown on FACE plots with 0.5 mg kg^{-1} Cd was 18.9% lower than that in leaves of rice grown on ambient plots (Fig. 4.2b). No significant differences on the activity of POD were found between the FACE and ambient plots in each Cd treatment group (Fig. 4.2c). The activity of SOD in leaves of rice grown on FACE plots with 0 (control) mg kg^{-1} Cd added was 46.3% higher than that in leaves of rice grown on ambient plots, while that was 50.4% lower in 0.5 mg kg^{-1} Cd treatment group (Fig. 4.2d).

In Cu treatment groups, the changes in the activities of enzymes in the leaves of wheat at mid-tillering and panicle-initiation growth stages are shown in Fig. 4.3. No significant differences in CAT activities were found between the FACE and ambient plots in each Cd treatment group at mid-tillering growth stage (Fig. 4.3a).

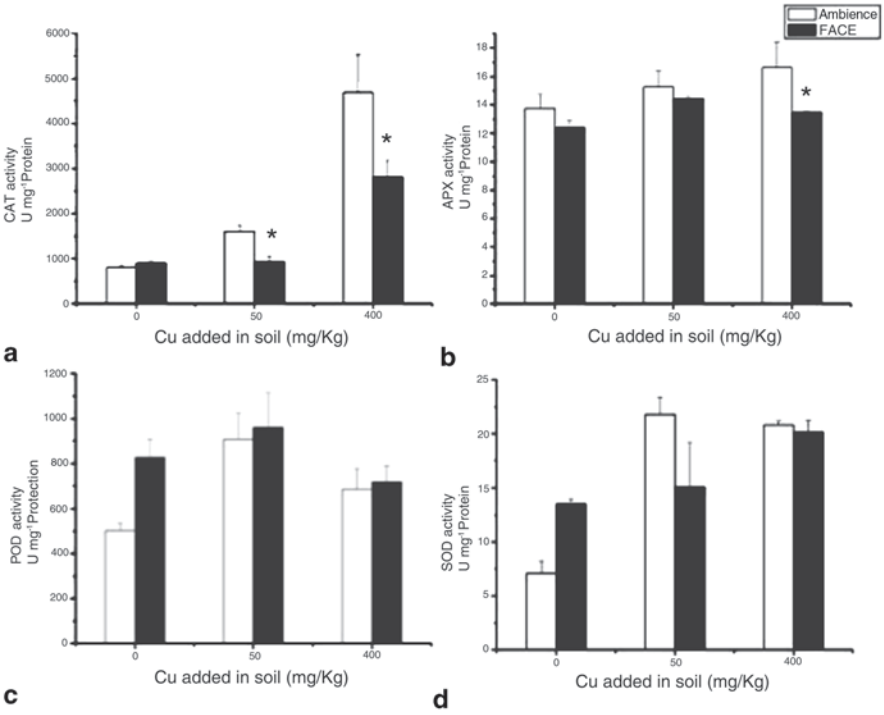

Fig. 4.1 Changes in the activities of CAT (**a**), APX (**b**), POD (**c**), SOD (**d**) in the leaves of rice at mid-tillering growth stage. Rice plants grown in soil with 0, 50, or 400 mg kg^{-1} Cu, under either ambient CO_2 levels or elevated CO_2 levels (*FACE*). Values represent means ± SD. An asterisk indicates a significant difference between FACE and ambient conditions ($p < 0.05$). *CAT* catalase, *APX* ascorbate peroxidase, *POD* peroxidase, *SOD* superoxide dismutase, *FACE* free-air CO_2 enrichment

At panicle-initiation growth stage, the activity of CAT in leaves of wheat grown on FACE plots with 50 mg kg^{-1} Cu was 24.8 % higher than that in leaves of wheat grown on ambient plots, while that was 35.3 % lower in 400 mg kg^{-1} Cu treatment group (Fig. 4.3b). No significant changes in the activity of APX in the leaves of wheat at mid-tillering and panicle-initiation growth stages were observed (Fig. 4.3c, d). The activity of POD in leaves of wheat grown on FACE plots with 400 mg kg^{-1} Cu added was lower than that in leaves of wheat grown on ambient plots at mid-tillering growth stage, as well as panicle-initiation growth stage (Fig. 4.3e, f). In all Cu treatment groups, the activities of SOD in leaves of wheat grown on FACE plots were lower than that in leaves of wheat grown on ambient plots (Fig. 4.3g, h). The changes in the activities of enzymes in the leaves of wheat at mid-tillering and panicle-initiation growth stages in response to Cd treatment are shown in Fig. 4.4. At mid-tillering growth stage, the activity of CAT in leaves of wheat grown on FACE plots with added Cd was higher than that in leaves of wheat grown on ambient plots.

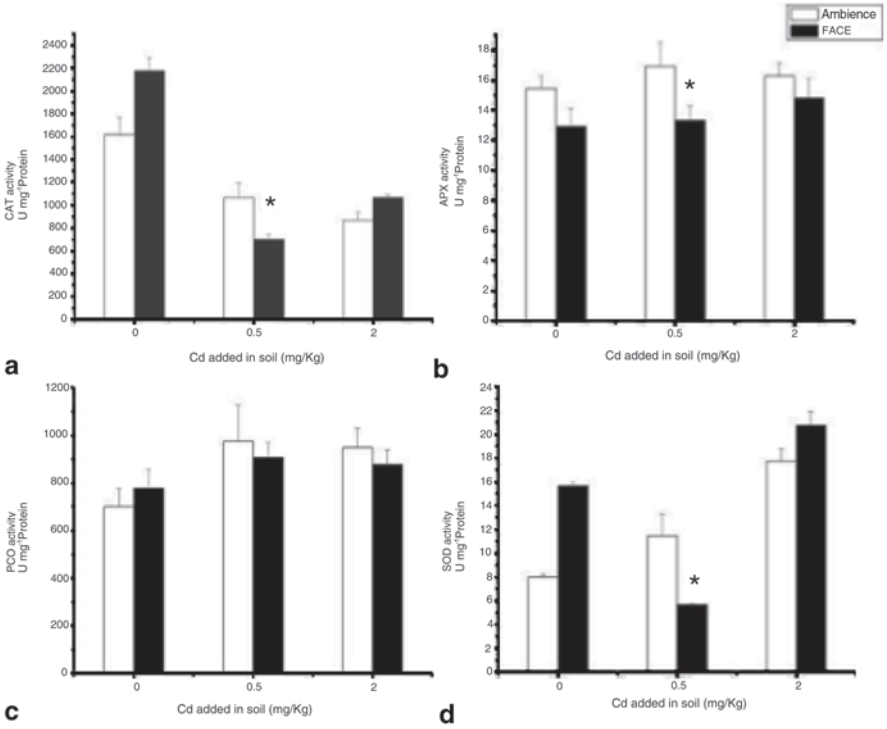

Fig. 4.2 Changes in the activities of CAT (**a**), APX (**b**), POD (**c**), SOD (**d**) in the leaves of rice at mid-tillering growth stage. Rice plants grown in soil with 0, 0.5, or 2 mg kg^{-1} Cd, under either ambient CO_2 levels or elevated CO_2 levels (*FACE*). Values represent means ± SD. An asterisk indicates a significant difference between FACE and ambient conditions ($p < 0.05$). *CAT* catalase, *APX* ascorbate peroxidase, *POD* peroxidase, *SOD* superoxide dismutase, *FACE* free-air CO_2 enrichment

Interestingly, CAT activity was lower in 2 mg kg^{-1} Cd treatment group at panicle-initiation growth stage (Fig. 4.4a, b). In all Cd treatment groups, the activities of APX, POD, and SOD in leaves of wheat grown on FACE plots were higher than that in leaves of wheat grown on ambient plots (Fig. 4.4c, h).

Studies have shown that metals such as Cu and Cd exhibit the ability to produce reactive oxygen species (ROS) such as superoxide ion, hydrogen peroxide, and hydroxyl radical, resulting in lipid peroxidation (Stohs and Bagchi 1995; Skorzynska-Polit et al. 2006; Schraudner et al. 1997). The excessive production of ROS in chloroplasts of plants has proven to be the main cause of oxidative damage in leaves (Foyer and Noctor 2003). Plants have evolved an antioxidant defense system including enzyme system and nonenzymatic system to avoid damage caused by ROS. The antioxidant defense system plays an important role in the ROS removal and protective defense reaction (Hernandez et al. 2001). SOD is a major scavenger of superoxide ion, its enzymatic action results in the formation of hydrogen

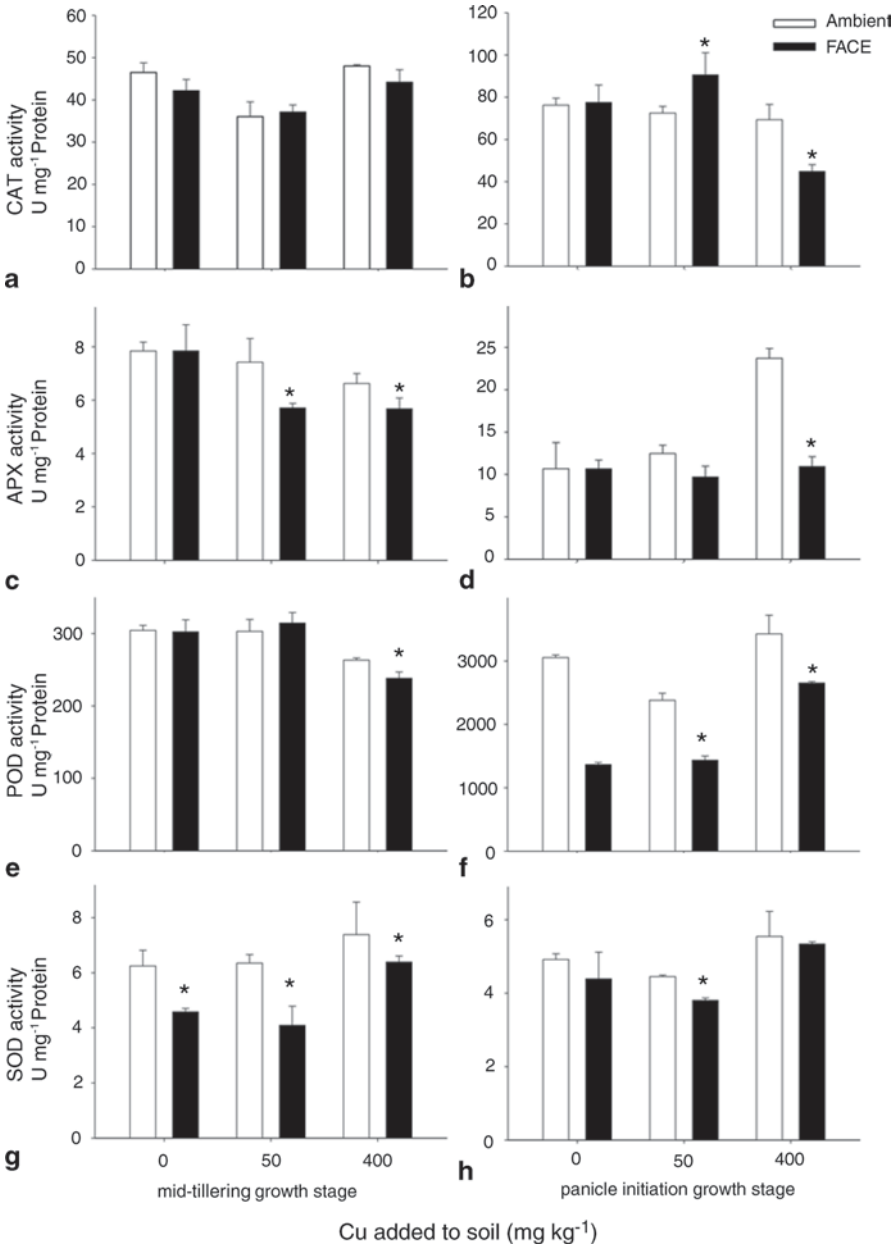

Fig. 4.3 Changes in the activities of CAT (**a**), APX (**c**), POD (**e**), SOD (**g**) in the leaves of wheat at mid-tillering growth stage and changes in the activities of CAT (**b**), APX (**d**), POD (**f**), SOD (**h**) in the leaves of wheat at panicle-initiation growth stage. Wheat plants grown in soil with 0, 50, or 400 mg kg^{-1} Cu, under either ambient CO_2 levels or elevated CO_2 levels (*FACE*). Values represent means ± SD. An asterisk indicates a significant difference between FACE and ambient conditions ($p < 0.05$). *CAT* catalase, *APX* ascorbate peroxidase, *POD* peroxidase, *SOD* superoxide dismutase, *FACE* free-air CO_2 enrichment

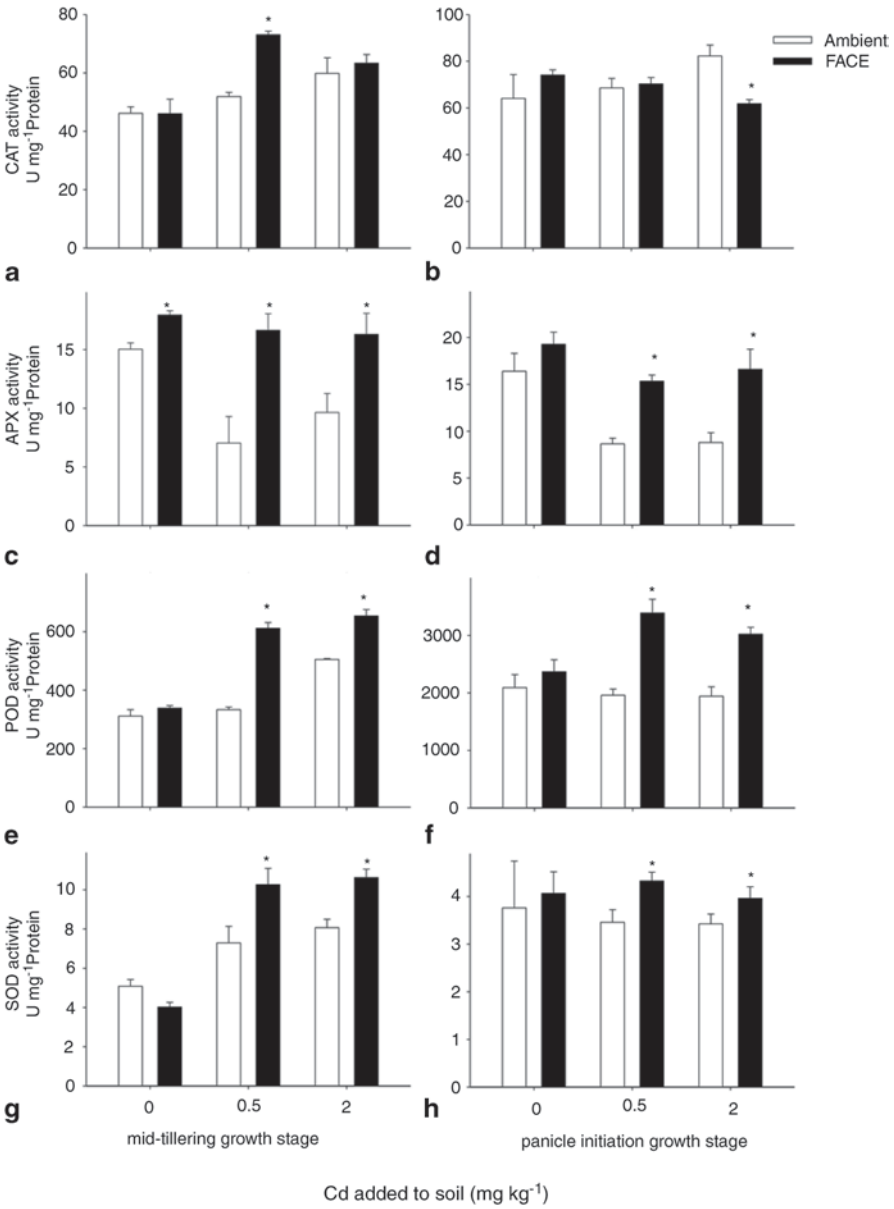

Fig. 4.4 Changes in the activities of CAT (**a**), APX (**c**), POD (**e**), SOD (**g**) in the leaves of wheat at mid-tillering growth stage and changes in the activities of CAT (**b**), APX (**d**), POD (**f**), SOD (**h**) in the leaves of wheat at panicle-initiation growth stage. Wheat plants grown in soil with 0, 0.5, or 2 mg kg^{-1} Cd, under either ambient CO_2 levels or elevated CO_2 levels (*FACE*). Values represent means ± SD. An asterisk indicates a significant difference between FACE and ambient conditions ($p < 0.05$). *CAT* catalase, *APX* ascorbate peroxidase, *POD* peroxidase, *SOD* superoxide dismutase, *FACE* free-air CO_2 enrichment

peroxide and molecular oxygen, while CAT decomposes hydrogen peroxide into water (Dionisio-Sese and Tobita 1998). For higher plants, the induction of POD activity is a comprehensive reaction to harmful stress, which may be associated with oxidation reactions of membrane (Lin and Kao 2002). The most important reducing substrate for hydrogen peroxide detoxification is ascorbate, APX uses two molecules of ascorbate to reduce hydrogen peroxide to water (Noctor and Foyer 1998).

In this study, at mid-tillering growth stage, the activities of enzymes (CAT, APX, POD, and SOD) in leaves of rice grown on FACE plots with Cu and Cd added groups were lower than that in leaves of rice grown on ambient plots. We speculate that elevated CO_2 levels might alleviate oxidative stress in leaves of rice polluted by Cu and Cd. Some research suggested that elevated concentrations of CO_2 caused a significant reduction in the activities of SOD and CAT in leaves of plant, and the oxidative stress of plant was alleviated to a certain extent (Polle et al. 1993; Schwanz et al. 1996). It was suggested that the activity of SOD in leaves of beech (*Fagus sylvatica L.*) was inhibited with elevating CO_2 levels as a result of increase of NADPH which was the intermediate of photosynthesis and the activity of CAT decreased with elevating CO_2 levels because the respiration rate of plant slowed down and the concentration of hydrogen peroxide which was the product of respiration decreased (Polle et al. 1997).

In Cu treatment groups, the activities of enzymes in the leaves of wheat grown on FACE plots at mid-tillering and panicle-initiation growth stages were lower than that in leaves of wheat grown on ambient plots, while the trend was opposite in Cd treatment groups. We surmise that the absorption of Cu and Cd was different in wheat under different atmospheric conditions. Elevated CO_2 levels increased the absorption of Cd, resulted in the increase of oxidative stress. Increase in ROS probably served as an inciting factor that increased the activities of antioxidant enzymes.

4.3.2 Copper Concentration in Plants

In this 2-year study, elevated CO_2 levels significantly led to lower Cu concentration in both rice and wheat (Figs. 4.5 and 4.6). At the mid-tillering stage of the first rice season, the Cu concentrations in shoots of rice grown on FACE plots with 50 and 400 mg kg^{-1} Cu in the soil were 23.0 and 22.9% lower, respectively, than in shoots of rice grown on ambient plots (Fig. 4.5a, $p < 0.05$). At the panicle-initiation stage, the Cu concentration in shoots of rice grown on FACE plots with 50 mg kg^{-1} Cu was 22.2% lower than that in shoots of rice grown on ambient plots (Fig. 4.5b, $p < 0.05$). At grain maturity during the first rice season, the Cu concentration in the shoots of rice grown on FACE plots was 34.1, 16.1, and 19.7% lower (Fig. 4.5c, $p < 0.05$) than their counterparts grown on ambient plots with 0 (control), 50, and 400 mg kg^{-1} Cu, respectively, and the Cu concentration in the grains of rice grown on FACE plots with 400 mg kg^{-1} Cu (Fig. 4.5d, $p < 0.05$) was 8.8% lower than in grains of rice grown on ambient plots. A similar trend was detected in samples from the second year. At grain maturity during the second rice season, the Cu concentration in the

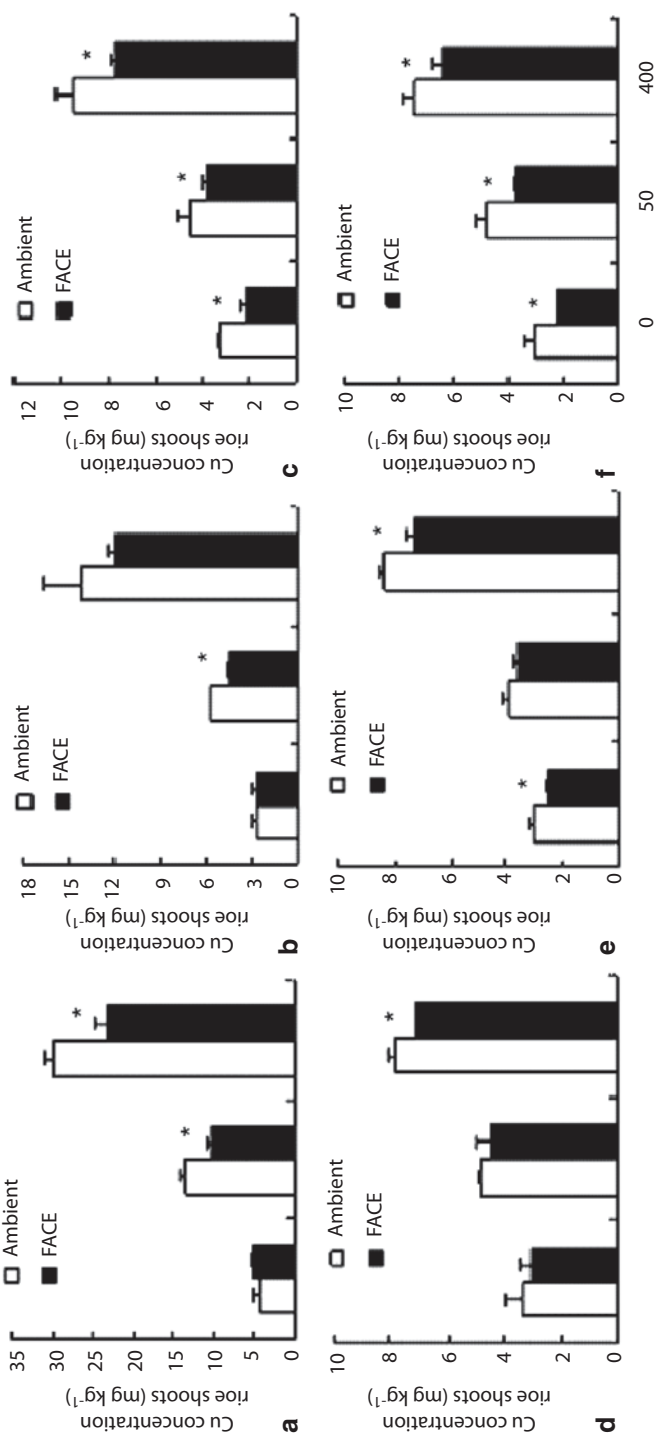

Fig. 4.5 Copper concentrations in tissues of rice plants grown in soil with 0, 50, or 400 mg kg^{-1} Cu, under either ambient CO_2 levels or elevated CO_2 levels (*FACE*). **a** Rice shoots at the mid-tillering growth stage of the first rice sowing, **b** rice shoots at the panicle-initiation growth stage of the first rice sowing, **c** rice shoots at grain maturity of the first sowing, **d** rice seeds at grain maturity of the first season, **e** rice shoots at grain maturity of the second season, and **f** rice seeds at grain maturity of the second season. Values represent means ± SD. An asterisk indicates a significant difference between FACE and ambient conditions ($p < 0.05$). *FACE* free-air CO_2 enrichment

shoots of rice grown on FACE plots with 0 (control) and 400 mg kg^{-1} Cu was 18.6 and 12.6% (Fig. 4.5e, $p<0.05$) lower than shoots of rice grown on ambient plots, and the Cu concentration in the grains of rice grown on FACE plots with 0 (control), 50, and 400 mg kg^{-1} Cu was 25.5, 20.3, and 14.2% lower than in grains of rice grown on ambient plots (Fig. 4.5f, $p<0.05$). Similar results were observed for wheat (Fig. 4.6).

In the previous studies of uncontaminated soils, Manderscheid et al. (1995) found that elevated CO_2 levels led to lower concentrations of Ca, S, Mg, Fe, and Zn in the wheat grain. Fangmeier et al. (1999) reported that elevated CO_2 levels resulted in lower Ca, S, and Fe concentrations in spring wheat. Yang et al. (2007) showed that the Cu content of milled rice grain under elevated CO_2 levels was 20% lower than that of ambient atmosphere. In an OTC experiment with contaminated soils and elevated CO_2 levels, Li et al. (2010) reported that the Cu concentration in rice grains was significantly lower than that of ambient atmosphere. In the short term, lower Cu concentrations in crops probably alleviate the Cu toxicity and have important positive implications for the food quality of crops harvested from soils contaminated with Cu. In this study, the SEM images of rice roots showed that exposure to elevated CO_2 levels alleviated Cu stress and increased the root hair density. When the plants were grown with 2 mg kg^{-1} Cd on either FACE or ambient plots, the root hair density of rice was low (Fig. 4.7). However, when the plants were grown with 400 mg kg^{-1} Cu on FACE plots, the root hair density was markedly higher than that of plants grown on ambient plots with 400 mg kg^{-1} Cu (Fig. 4.7). In the long term, depending on the magnitude of the effect, Cu deficiency in crops has the potential to contribute to health problems.

4.3.3 Cadmium Concentration in Plants

Elevated CO_2 levels resulted in higher Cd concentrations in the tissues of both wheat and rice, especially in those plants grown in soils contaminated with high levels of Cd (Figs. 4.8 and 4.9). At the mid-tillering stage of the first rice season (Fig. 4.8a), the Cd concentration in shoots of rice grown on the FACE and ambient plots did not differ significantly. At the panicle-initiation stage, the Cd concentrations in shoots of rice grown on FACE plots with 0.5 and 2 mg kg^{-1} Cd were 55.7 and 7.8% higher, respectively, than in shoots of rice grown on ambient plots (Fig. 4.8b, $p<0.05$). At grain maturity of both the first and second rice season, the Cd concentration in shoots of rice grown on FACE plots with 2 mg kg^{-1} Cd was 11.3 and 21.5% higher ($p<0.05$), respectively, than in shoots of rice grown on ambient plots. But the Cd concentration in shoots of rice grown on FACE and ambient plots with 0 and 0.5 mg kg^{-1} Cd did not significantly differ (Fig. 4.8c, e). The Cd concentration in seeds was not significantly affected by elevated CO_2 levels in the first rice season (Fig. 4.8d). In the second rice season, the Cd concentration in seeds of plants grown on FACE plots with 2 mg kg^{-1} Cd was 38.8% higher than in seeds of plants grown on ambient plots ($p<0.05$), but the Cd concentration in seeds of rice grown

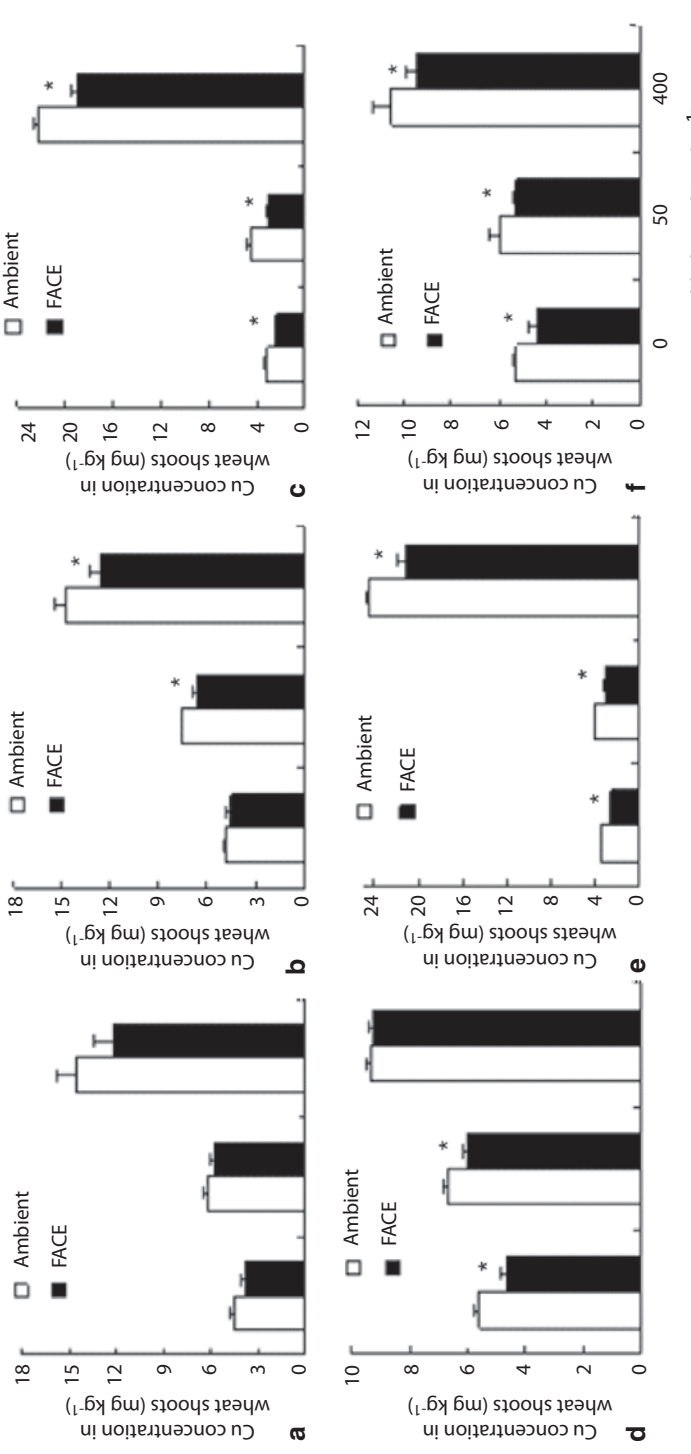

Fig. 4.6 Copper concentrations in tissues of wheat plants grown in soil with 0, 50, or 400 mg kg^{-1} Cu added, under either ambient CO_2 levels or elevated CO_2 levels (*FACE*). **a** Wheat shoots at the mid-tillering growth stage of the first season, **b** wheat shoots at the panicle-initiation growth stage of the first season, **c** wheat shoots at grain maturity of the first season, **d** wheat seeds at grain maturity of the first season, **e** wheat shoots at grain maturity of the second season, and **f** wheat seeds at grain maturity of the second season. Values represent means ± SD. An *asterisk* indicates a significant difference between FACE and ambient conditions ($p < 0.05$). *FACE* free-air CO_2 enrichment

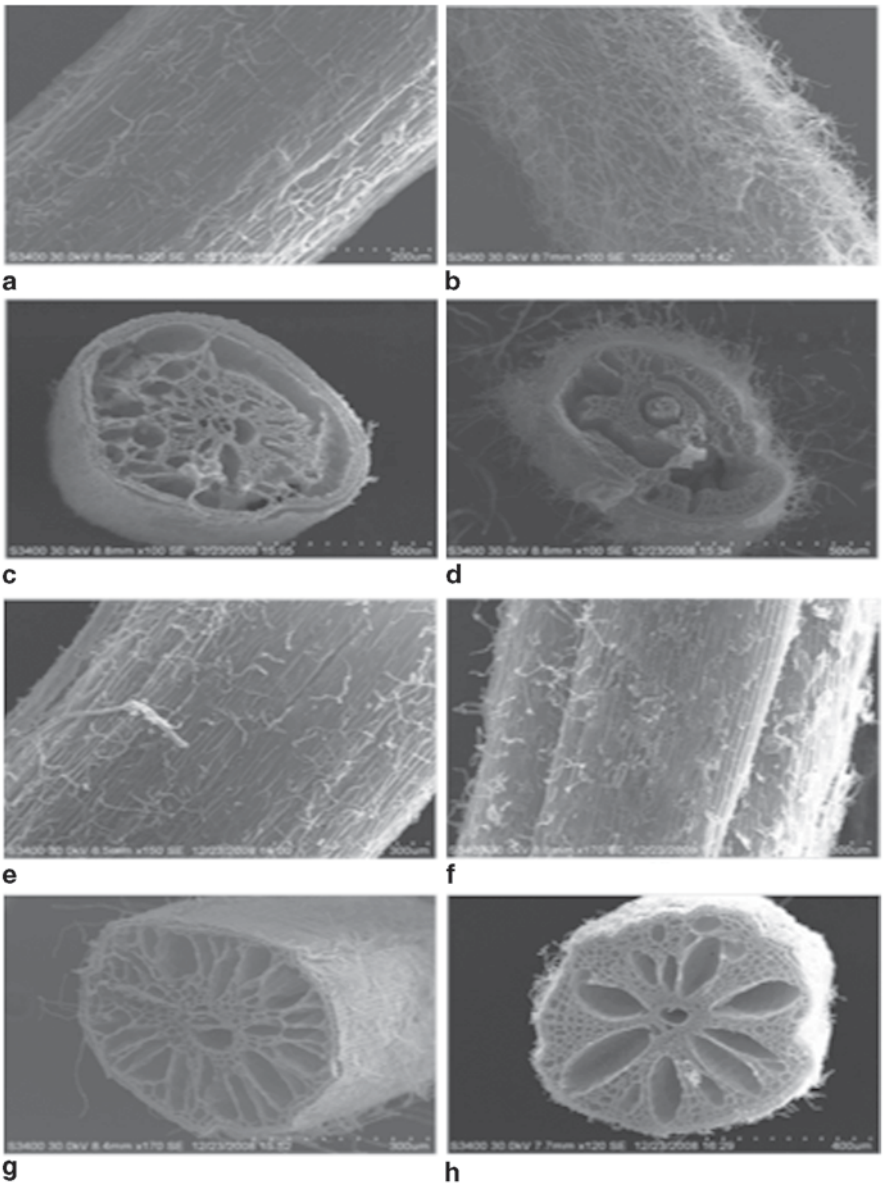

Fig. 4.7 Scanning electron microscopy images of rice root tips at the panicle-initiation growth stage. Plants were grown in soil with (**a**) and (**c**) 400 mg kg^{-1} Cu on ambient plots, (**b**) and (**d**) 400 mg kg^{-1} Cu on FACE plots, (**e**) and (**g**) 2 mg kg^{-1} Cd on ambient plots, and (**f**) and (**h**) 2 mg kg^{-1} Cd on FACE plots. (**a**), (**b**), (**e**), and (**f**) cross section; (**c**), (**d**), (**g**), and (**h**) longitudinal section

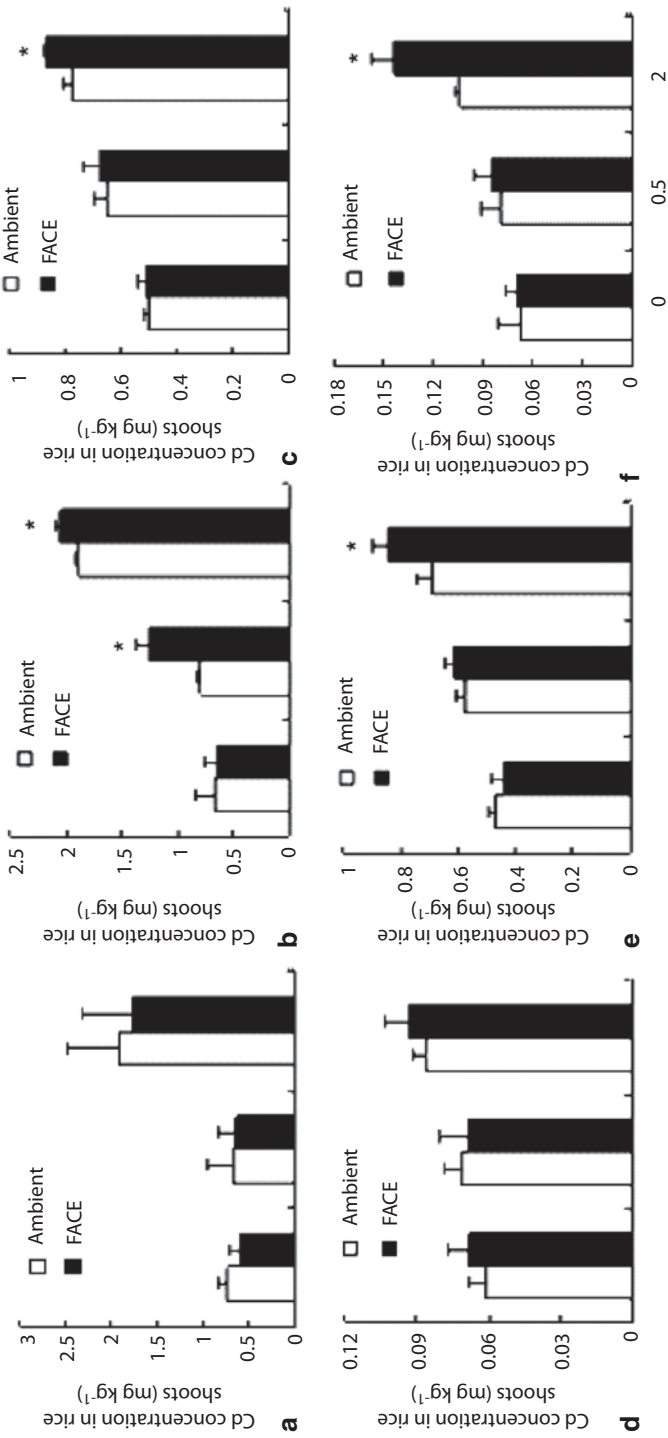

Fig. 4.8 Cadmium concentrations in tissues of rice plants grown in soil with 0, 0.5, or 2.0 mg kg^{-1} Cd, under either ambient CO_2 levels or elevated CO_2 levels (*FACE*). **a** Rice shoots at the mid-tillering growth stage of the first season, **b** rice shoots at the panicle-initiation growth stage of the first season, **c** rice shoots at grain maturity of the first season, **d** rice seeds at grain maturity of the first season, **e** rice shoots at grain maturity of the second season, and **f** rice seeds at grain maturity of the second season. Values represent means ± SD. An asterisk indicates a significant difference between FACE and ambient conditions ($p < 0.05$). *FACE* free-air CO_2 enrichment

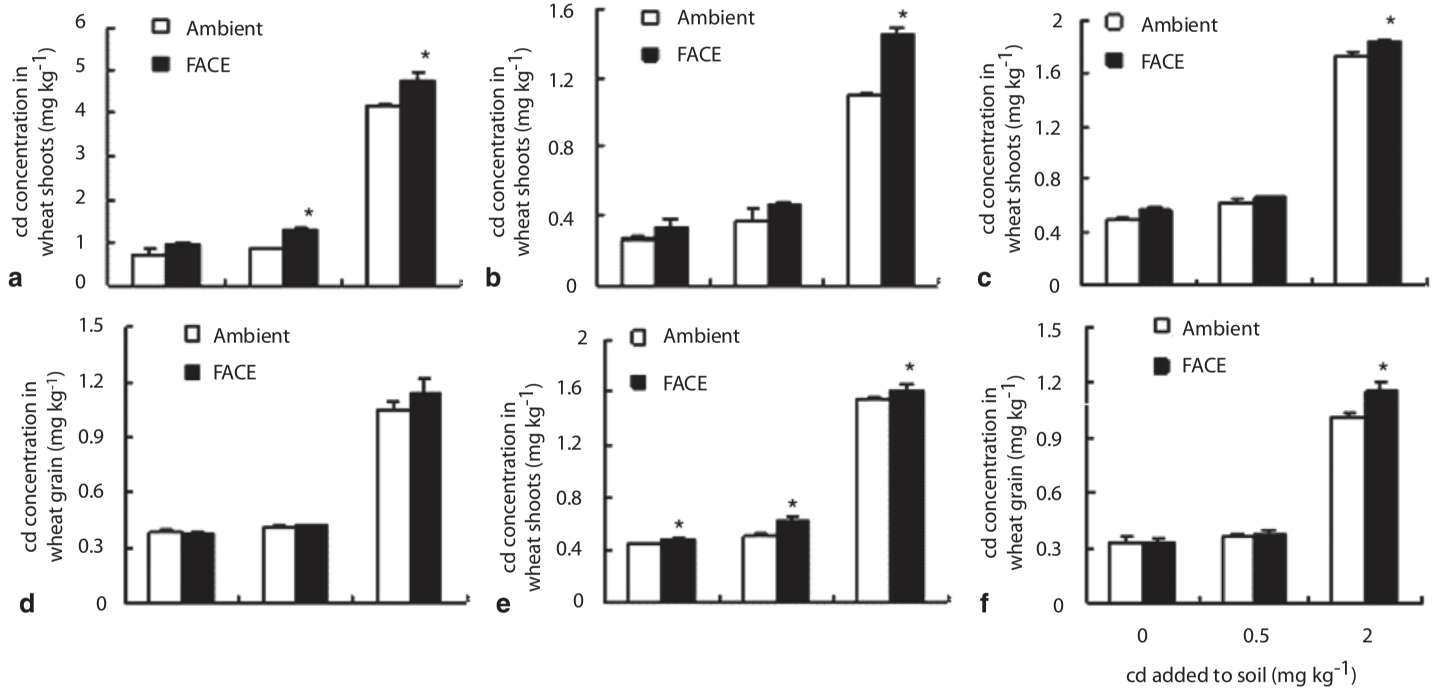

Fig. 4.9 Cadmium concentrations in tissues of wheat plants grown in soil with 0, 0.5, or 2.0 mg kg^{-1} Cd, under either ambient CO_2 levels or elevated CO_2 levels (*FACE*). **a** Rice shoots at the mid-tillering growth stage of the first season, **b** rice shoots at the panicle-initiation growth stage of the first season, **c** rice shoots at grain maturity of the first season, **d** rice seeds at grain maturity of the first season, **e** rice shoots at grain maturity of the second season, and **f** rice seeds at grain maturity of the second season. Values represent means ± SD. An asterisk indicates a significant difference between FACE and ambient conditions ($p < 0.05$). *FACE* free-air CO_2 enrichment

on FACE and ambient plots with 0 and 0.5 mg kg^{-1} Cd did not significantly differ (Fig. 4.8f). Similar results were observed for wheat (Fig. 4.9).

In this study, the Cd concentration in wheat grains of all samples far exceeded the legal limits (wheat flour: 0.1 mg kg^{-1}; Ministry of Health 2005). The Cd concentration in rice seeds of the first and second seasons from plants grown on either FACE or ambient plots was below the legal limits (rice: 0.2 mg kg^{-1}; Ministry of Health 2005). But after exposure to elevated CO_2 level, the Cd concentration in rice seeds of second season is more close to the legal limits than that of the first season. Such increasing trends of Cd concentrations in rice seeds under elevated CO_2 suggest that the levels of these toxic metals could exceed the legal limit in the future. Cadmium can accumulate in the human body and damage kidneys, bones, and reproductive system (Jarup and Akesson 2009). To keep the Cd levels in creatinine in urine below 1 μg Cd g^{-1} in 95% of the population by age 50, the European Food Safety Authority (EFSA 2009) has suggested that the average daily dietary Cd intake should not exceed 0.36 μgCd/kg body weight, which corresponds to a weekly dietary intake of 2.52 μg Cd/kg body weight (EFSA 2009). For an average adult of 60 kg with a daily intake of 261.1 g rice or wheat (Pan et al. 2007), this estimated weekly dietary intake the levels of Cd far exceeds the levels suggested by EFSA in all of the wheat samples from this study grown in control and contaminated soils and in rice samples grown in highly contaminated soil and elevated CO_2 levels. Li et al. (2010)also found significantly higher Cd concentrations in three rice varieties grown on contaminated soils under elevated CO_2 levels. In China, farmland polluted by Cd has reached 20×10^4 ha and produces 14.6×10^8 kg of agricultural products annually (Li et al. 2003). Since almost the entire population in China currently depends on rice and wheat as staple foods, the high, toxic concentrations of Cd accumulated in crops threaten food quality and safety. This threat will increase as the CO_2 levels increase in the future.

4.3.4 Variations in Soil pH, and Cu and Cd Fractionation in Soil

After the second rice harvest (October 2007), the pH of the soil was slightly but significantly lower in the FACE plots than in the ambient plots (Table 4.2). Elevated CO_2 levels also led to the changes in the available Cu and Cd in the soil. Compared to soil from ambient plots, the acid-extractable fraction of Cu in soil from FACE plots with 50 and 400 mg kg^{-1} Cu was 10.5 and 16.4% higher ($p < 0.05$), and the reducible fraction of Cu in soil from FACE plots was 3.9 and 7.9% lower ($p < 0.05$), respectively. Compared to the soil from ambient plots, the acid-extractable and reducible fractions of Cd in soil from FACE plots with 2 mg kg^{-1} Cd were 4.7 and 6.9% higher ($p < 0.05$), and the oxidizable and residual fractions of Cd were 45.9 and 7.1% lower ($p < 0.05$), respectively.

Several studies have indicated that elevated CO_2 levels lower the pH of rhizosphere soils, favor the release of elements into soil solution, and as a result, help the plant to take up more elements. DeLucia et al. (1997) reported that elevated

Table 4.2 pH of soil after the second harvest of rice (November 2007) from FACE and ambient plots to which Cu or Cd was added

Heavy metal	pH	
	Ambient plots	FACE plots
Cu (0 mg kg^{-1}) (control)	7.06 ± 0.02	6.85 ± 0.03*
Cu (50 mg kg^{-1})	7.04 ± 0.03	7.11 ± 0.03
Cu (200 mg kg^{-1})	6.93 ± 0.06	6.80 ± 0.01*
Cd (0 mg kg^{-1}) (control)	7.48 ± 0.11	7.38 ± 0.03*
Cd (0.5 mg kg^{-1})	7.36 ± 0.01	7.31 ± 0.02*
Cd (2 mg kg^{-1})	7.35 ± 0.01	7.06 ± 0.07*

Values represent means ± SD. An asterisk indicates a significant difference in pH between FACE and ambient conditions ($p < 0.05$)
FACE free-air CO_2 enrichment

CO_2 levels increased the concentration of oxalate in the soil, and that this low molecular weight organic acid solubilized inorganic phosphorus, making it available for uptake by the plant. Andrews and Schlesinger (2001) observed an increase in cation concentration in the deep soil (200 cm) in the third year of CO_2 fumigation, and proposed that the observed increase in cation availability was caused by the increased organic acid content. Wu et al. (2009) showed that elevated CO_2 levels lowered the pH by 0.2–0.4 units compared to ambient CO_2 levels, which implies that the lower pH in the rhizosphere zone could help the plants take up more Cs. Li et al. (2010) reported that the decrease in pH of 0.04–0.15 in the rhizosphere soil of rice was due to elevated CO_2 levels, and considered that this slightly decreasing trend might be linked to higher Cd concentrations in rice. Cheng et al. (2010) reported that elevated CO_2 levels significantly increased the concentration of Ca^{2+} and Mg^{2+} in soil solution and reduced the solution pH, and total cations in plant biomass were also significantly higher under elevated CO_2 levels. In this study after the second rice harvest, especially for heavy-metal-contaminated soils, the pH of the soil also exhibited a decreasing trend and the acid-extractable fraction of metals in soils exhibited an increasing trend at elevated CO_2 levels. It is known that the mobility and bioavailability of heavy metals in the acid-extractable form are greater than that of other fractions (Mulligan et al. 2001). These changes can link elevated CO_2 levels to the increasing phytoavailability of heavy metals and are probably sufficient to explain the higher Cd concentrations in rice and wheat in this study. Thus, we propose that at elevated CO_2 levels, the exudation of low molecular weight organic compounds by the roots of plants lowers the pH of rhizosphere soils, facilitates metal solubility and bioavailability, and increases the uptake of metal by plants. But if the soils are contaminated with little or no Cd under elevated CO_2 conditions, the slight decrease in the pH of the soil will not lead to a significant uptake of Cd by crops.

Since in this study the bioavailability of both Cu and Cd increased under elevated CO_2 levels, we were surprised that Cd concentrations in the crops increased, but Cu concentrations decreased. There are a few possible explanations for these

contrasting results (IPCC 2007). Many studies have shown that elevated CO_2 levels increase plant growth and yields (Liu et al. 2008; Ziska et al. 1996; Moya et al. 1998; Kim et al. 2003), including a study using a same FACE system that reported that elevated CO_2 levels enhance hybrid rice grain yield by 34 % (Liu et al. 2008). Recently, Duval et al. (2011) indicated that CO_2 alters the distribution of contaminant elements in ecosystems, with contaminant elements accumulating in plants and declining in soil, both likely explained by the CO_2 stimulation of plant biomass. Li et al. (2010) reported that although higher Cd concentrations and lower Cu concentration in rice grown on contaminated soils under elevated CO_2 were detected, elevated CO_2 still significantly increased the total uptake of Cu and Cd owing to the change in biomass. Similarly, the higher concentrations of Cd and lower concentrations of Cu in crops observed in this study were probably due to the change in biomass under elevated CO_2 conditions (Zhang et al. 2008). As reported, elevated CO_2 levels increased the exudation of low molecular weight organic compounds by the roots of plants (Delucia et al. 1997; Andrews and Schlesinger 2001), but the binding strength of Cd and Cu to organic compounds differs (Groenenberg et al. 2010). Cu has a relatively high binding affinity to organic matter, whereas Cd has a relatively weak affinity. This could have an influence on the uptake of Cd and Cu under elevated CO_2 levels, leading to differences (Hill et al. 2007). Cations, such as Ca^{2+}, Mg^{2+}, Na^{+}, and K^{+}, interfere with the heavy metal bioavailability and alleviate metal toxicity owing to cation competition (Voigt et al. 2006; Luo et al. 2008; Wang et al. 2008; Li et al. 2009). Kinraide et al. (2004) reported that the addition of Ca^{2+} and Mg^{2+} alleviates metal toxicity, but the relative ameliorative effectiveness of Ca^{2+} and Mg^{2+} depends upon the metal. Cheng et al. (2010), who used a similar FACE system, reported that elevated CO_2 levels significantly stimulate Ca^{2+} and Mg^{2+} release from soil. In this study, increased Ca^{2+} and Mg^{2+} in solution in soil could have decreased both Cu and Cd uptake owing to cation competition, but the relative effectiveness for Cu and Cd could differ based on the plant species. Experimental evidences supporting the above explanations are lacking. Additional research is needed to investigate the relationship between elevated CO_2 levels and the increased phytoavailability of heavy metals and to elucidate the different mechanisms of the uptake of these two metals. The data presented here were obtained from crops grown in artificially contaminated soils in pots. More data need to be collected from crops grown under a wide range of soil conditions and realistic field conditions to make better predictions on the combined effects of elevated CO_2 levels and multimetal-contaminated soils on the metal uptake by crops and thereby on their contribution to food quality and safety.

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Chapter 5
Tolerance to Combined Stress of Drought and Salinity in Barley

Imrul Mosaddek Ahmed, Umme Aktari Nadira, Noreen Bibi, Guoping Zhang and Feibo Wu

5.1 Introduction

Drought and salinity stresses occur naturally (Dai 2011), and have been expanding worldwide due to human activities such as deforestations, salt mining (Ghassemi et al. 1995), poor irrigation water (Marcum and Pessarakli 2006), and escalating emissions of greenhouse gases (IPCC 2000). Currently, more than 800 million hectares (ha) of land are affected by salinity (Munns 2005), and about one third of the world's arable land has experienced yield reduction due to cyclical or unpredictable drought (Chaves and Oliveira 2004), which are causing a great threat to crop production. For example, China, India, and the USA, the world's three major grain producers and exporters, have been suffering serious water shortages in many major agricultural regions. In China, according to the survey by the Ministry of Water Resources, over 25.67 million ha of farmland was annually affected by drought stress during the 15th 5-year plan, which caused production reduction of 3.5×10^{10} kg and economic losses of more than 230 billion Chinese Yuan (http://mt.china-papers.com/1/?p=185213).

Generally, the co-occurrence of several abiotic stresses, rather than an individual stress condition, is even worse for crop production (Mittler 2006). For example, the combined effects of salinity and drought on yield are more detrimental than the effects of each stress alone, as observed in potato (Levy et al. 2013), wheat (Yousfi et al. 2012), and barley (Yousfi et al. 2010). However, most studies to date have addressed the effects of single stresses on plant (Zhao et al. 2010; Wu et al. 2013), and little is known about the physiological and molecular mechanisms underlying the acclimation of plants to a combination of salinity and drought (Mittler 2006). Recent studies have revealed that the response of plants to a combination of different abiotic stresses is unique and cannot be directly extrapolated from the response of plants

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to each of the different stresses individually (Rollins et al. 2013; Iyer et al. 2013). Breeding of stress-tolerant crops is the most efficient strategy to maintain yield in stress-prone marginal land. It is thus important to identify genetic resources with high tolerance to abiotic stresses, especially those co-occurring in the field, such as salinity and drought, and to understand its mechanisms.

Barley (*H. vulgare* L.) is the fourth most important cereal crop in the world in terms of production. For its versatile properties, it has been used for animal feed, human food, and beverage (Koornneef et al. 1997). Barley as a staple food is attracting renewed attention, especially in Asia and northern Africa, because of its nutritional value (Baik and Ullrich 2008). In addition to its agricultural importance, barley is a genetic model for other crops. However, much of the genetic variation for improving abiotic stress tolerance has been lost during the process of domestication, selection, and modern breeding (Zhao et al. 2010). Even more, barley has a wider ecological range than any other cereals and is widespread in temperate, subtropical, and arctic areas, from sea level to heights of more than 4500 m in the Andes and Himalayas (Bothmer et al. 1995). Barley can be grown on soils unsuitable for wheat, and at altitudes unsuitable for wheat or oats. Because of its salt and drought tolerance, barley thrives in nearly every corner of the earth, including extremely dry areas near deserts. Barley is a short-season, early-maturing, diploid, and self-pollinating crop, thus it is also an ideal model plant for genetic study of drought and salinity tolerance (Li et al. 2007). Several papers have summarized research on barley abiotic stress tolerance including drought and salinity tolerance (Zhao et al. 2010; Wu et al. 2013). In this chapter, we review the impact of salinity and drought stress applied singly and in combination in barley through morphological, physiological, biochemical, molecular, cellular, and ultrastructural approaches.

5.2 Drought Stress and Tolerance

Drought is a meteorological term and is commonly defined as a period without significant rainfall or a deficiency of water supply. Generally, drought stress occurs when the available water in the soil is reduced and atmospheric conditions cause continuous loss of water by transpiration or evaporation. Hence, a continuous shortfall in precipitation (meteorological drought) coupled with higher evapotranspiration demand leads to agricultural drought (Mishra and Cherkauer 2010). Agricultural drought is the lack of ample moisture required for normal plant growth and development to complete the life cycle (Manivannan et al. 2008). Although droughts can persist for several years, even a short, intense drought can cause significant damage and harm the local economy. Drought is a worldwide problem, constraining global crop production and quality seriously and recent global climate change has made this situation more serious (Apel and Hirt 2004; Forster et al. 2004; Zhao et al. 2010; Budak et al. 2013).

Drought stress is also considered to be a moderate loss of water, which leads to stomatal closure and limitation of gas exchange. Desiccation is a much more exten-

sive loss of water that can potentially lead to gross disruption of metabolism and cell structure and eventually to the cessation of enzyme-catalyzing reactions. Drought is characterized by the reduction of water content, turgor, total water potential, wilting, closure of stomata, and decrease in cell enlargement and growth. Barley is one of the most important cereal crops grown in many developing countries, where it is often subject to extreme drought stress that significantly affects production (Ceccarelli et al. 2007). Investigating the drought-tolerance mechanisms in barley could facilitate a better understanding of the genetic bases of drought tolerance, and facilitate the effective use of genetic and genomic approaches for crop improvement.

5.3 Salinity Stress and Tolerance

Salinity-affected soils are classified into two types: saline and sodic soils. Sometimes, a third type can be categorized as saline-sodic soils. Salt's negative effects on plant growth have initially been associated with the osmotic stress component caused by decreases in soil water potential and, consequently, restriction of water uptake by roots.

In agriculture, salt stress severely affects the growth and economic yield of many important crops (Maas and Hoffman 1977). Compared with other cereal crops, including wheat, rice, rye, and oat, barley is highly tolerant to salinity, thus offering a means for efficient utilization of saline soil and improvement of productivity in these environments. However, barley still suffers from salt toxicity in many areas of the world. On the other hand, dramatic differences can be found among and within the barley species, providing the potential for developing cultivars with improved salt tolerance. It is predicted that the genetic improvement of salt tolerance will be an important aspect of barley breeding in the future.

5.4 Overlap Between Salinity and Drought Stresses

Salinity and drought stress show a high degree of similarity with respect to physiological, biochemical, molecular, and genetic effects (Sairam and Tyagi 2004). Physiological drought occurs when soluble salt levels in the soil solution are high enough to limit water uptake due to low water potential, thereby inducing drought stress (Lee et al. 2004). The major difference between the low-water-potential environments caused by salinity versus drought is the total amount of water available. During drought, a finite amount of water can be obtained from the soil profile by the plant, causing ever-decreasing soil water potential. In most saline environments, a large amount of water is at a constant, but under low water potential. Plants have a chance to adjust their osmotic potential, which prevent loss of turgor and generate a lower water potential that allows plants to access water in the soil solution for growth (Taiz and Zeiger 2006).

Both stresses lead to cellular dehydration, which causes osmotic stress and removal of water from the cytoplasm into the intracellular space resulting in a reduction of the cytosolic and vacuolar volumes. Early responses to water and salt stress are largely identical except for the ionic component in the cells of plants under salt stress. These similarities include metabolic processes, e.g., a decrease of photosynthesis or increase in the levels of the plant hormonal processes, such as abscisic acid (ABA). High intracellular concentrations of sodium and chloride ions are an additional problem of salinity stress (Bartels and Sunkar 2005). Plants use common pathways and components in response to stresses, a concept known as cross-tolerance, which allows plants to acclimate to a range of different stresses after exposure to one specific stress (Pastori and Foyer 2002; Tuteja et al. 2007). Thus, a salinity-tolerant species could also be drought tolerant or vice versa, and has similar mechanisms to cope with those stresses (Ashraf and O'Leary 1996).

5.5 Mechanisms of Acclimation or Adaptation to Drought and Salinity Stress

Drought and soil salinity are among the most damaging abiotic stresses affecting today's agriculture. It is understandable that plants are under periodic water stress because of the unpredictable nature of rainfall. Salt stress is often observed in irrigated areas, hydraulic lifting of salty underground water, or spread of seawater in coastal areas. Plants have evolved mechanisms to perceive the incoming stresses and to cope with them by rapid regulation of their physiology and metabolism. Very often, such regulations and responses include feed-forward mechanisms for stress reduction that are in addition to the responses that are seen after stresses have caused irreversible damage to physiological functions. A good example of such a feed-forward mechanism is the ability of plants to regulate their water loss through partial closure of stomata and/or reduced leaf development, long before there is a substantial loss of their leaf turgor or some irreversible damage to inner membrane systems (Zhang et al. 2006a). The physiological responses of plants to survive under water stress include leaf wilting, a reduction in leaf area, leaf abscission, and the stimulation of root growth by directing nutrients to the underground parts of the plants. Besides, the effects of water deficit become more detrimental during reproductive stages of the plant (flowering and seed development), as the translocation of photosynthetic assimilates from leaf to root is reduced which cannot grow more deep in search of water and nutrients. In addition, ABA, the plant stress hormone, induces the closure of leaf stomata, thereby reducing water loss through transpiration, and decreasing the rate of photosynthesis. These responses improve the water-use efficiency of the plant on the short term (Muhammad and Asghar 2012).

5.6 Effects of Drought and Salinity Stress on Plant Morphology and Yield

5.6.1 Growth and Development

Plant responses to drought and salinity are complex and involve adaptive changes and/or deleterious effects. The decrease in the water potential occurring in both abiotic stresses results in reduced cell growth, root growth, and shoot growth and also causes inhibition of cell expansion and reduction in cell wall synthesis (Chaitanya et al. 2003). According to these authors, drought (likely to salinity) affects the regular metabolism of the cell such as carbon-reduction cycle, light reactions, energy charge, and proton pumping and leads to the production of toxic molecules. Literature has affirmed that plant responses to salt and water stress have much in common. For example, according to Munns (2002), salinity brings a decrease in water uptake by plants as the osmotic potential in the root vicinity will become high and a kind of exosmosis may occur. This will slow down the growth rate, along with a suite of metabolic changes identical to those caused by water stress. Ahmed et al. (2013a) observed that barley plants treated with single or combined stress of salinity (S) and drought (D) showed a significant decrease in plant height, shoot, and root dry/fresh weights, with the largest reduction in the combined stress (D + S). Therefore, most mechanisms to tolerate abiotic stresses like drought and salinity are detrimental to plant development (Fig. 5.1).

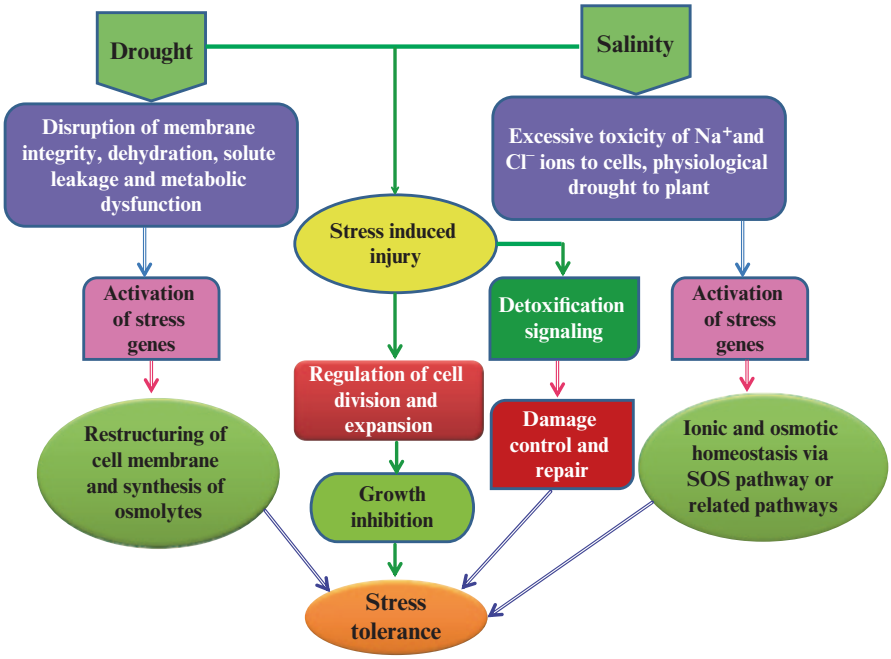

Fig. 5.1 Possible drought and salt stress tolerance mechanisms in barley plants

5.6.2 Yield

Many yield-determining physiological processes in plants respond to water stress. Yield is a quantitative trait and many physiological processes are involved. For water stress, severity, duration and timing of stress, as well as responses of plants after stress removal, and interaction between stress and other factors are extremely important (Plaut 2003). For instance, water stress applied at preanthesis reduced time to anthesis, while at postanthesis it shortened the grain-filling period in triticale genotypes (Estrada-Campuzano et al. 2008). In barley (*H. vulgare*), drought stress reduces grain yield by decreasing the number of tillers, spikes, and grains per plant and individual grain weight. Postanthesis drought stress was detrimental to grain yield regardless of the stress severity (Samarah 2005). In maize, water stress reduced yield by delaying silking, thus increasing the anthesis-to-silking interval. This trait was highly correlated with grain yield, specifically ear and kernel number per plant (Cattivelli et al. 2008). Following heading, drought had little effect on the rate of kernel filling in wheat, but its duration (time from fertilization to maturity) was shortened and dry weight reduced at maturity (Wardlaw and Willenbrink 2000).

Crop growth in saline medium is severely affected at different stages of the plant life cycle. It was suggested by Shannon et al. (1994), that overall plant response depends upon the concentration of salts in the tissue, composition of salts, the exposure time, and climatic conditions as well. The commonly observed adverse effects of salinity on Brassica species include the reduction in plant height, yield, as well as deterioration of the quality of the product (Kumar 1995). In barley and wheat, salinity stress lowered grain yield by reducing grain number and individual grain size (Harris et al. 2010). The plasticity of grain number and stability of grain size was found in another study in response to salinity (Sadras 2007). Ahmed et al (2013b) observed that the reduction in spike length was noticeably less in Tibetan wild barley than cultivated barley treated with single or combined stress of salinity and drought. Moreover, the 1000-grain yield and the filled grains per spike measurements were correlated, which may explain the yield loss in cultivated barley compared to Tibetan wild barley under combined drought and salinity during the anthesis stage. The decline in yield decline was possibly associated with the reduction in spikelet fertility and grain filling (Ahmed et al. 2013b).

In summary, prevailing drought and salinity reduce the plant growth and development, increase flower abscission, reduce grain size due to poor grain filling which arises due to the reduction in the partitioning of photosynthetic assimilate, and decrease carbohydrate metabolism.

5.7 Physiological and Biochemical Bases for Drought and Salinity Tolerance in Barley

5.7.1 Plant Water Relations

Leaf water potential, relative water content (RWC), stomatal movements, transpiration, leaf and canopy temperatures are the important characteristics that influence plant water relations. RWC represents plant water status including water uptake by the roots as well as water loss by transpiration, thus reflect the metabolic activity in plant tissue, and hence used as a most meaningful index for water stress tolerance. A decrease in the RWC in response to drought stress has been noted in a wide variety of plants (Nayyar and Gupta 2006). Furthermore, an exposure of plants to drought stress substantially decreased the leaf water potential, RWC, and transpiration rate, with a concomitant increase in leaf temperature as documented in the previous study (Siddique et al. 2001). Although the components of plant water relations are affected by reduced availability of water, stomatal opening and closing are more strongly affected. In barley, the application of the different watering regimes decreased the RWC, midday leaf water potential (ψ_w), and leaf osmotic potential (ψ_o) (Robredo et al. 2010).

Osmotic effects of salt on plants are due to the lowered soil water potential in the root zone and thus resemble drought stress by affecting the ability of plants to extract water from the soil and to maintain turgor (Sohan et al. 1999). However, at low or moderate salt concentrations (higher soil water potential), plants accumulate solutes and maintain a potential gradient for the influx of water. Under such conditions, Shannon et al. (1984) reported that growth may be moderated, but unlike drought stress, the plant is not water deficient. Several authors found that water potential and osmotic potential of plants became more negative with an increase in salinity, whereas the turgor pressure increased (Meloni et al. 2001; Gulzar et al. 2003). Vysotskaya et al. (2010) reported a similar decrease in leaf water potential with increasing salt concentration in wild barley species ("20–45" and T-1). At 75 mM NaCl, "20–45" plants were characterized by less inhibition of leaf area, root fresh weight, leaf water content, and leaf water potentials than T-1 species and were, therefore, considered more tolerant to salt stress. According to Vysotskaya et al. (2010), these investigators, it was concluded that, under high salt concentration, plants (1) sequester more NaCl in the leaf that lower the osmotic potential and (2) reduce the root hydraulic conductance causing water stress in the leaf tissue. The combined stress of drought and salinity depressed water potential, RWC in cultivated barley, but was unchanged in Tibetan wild barley relative to control (Ahmed et al. 2013a).

5.7.2 Photosynthesis

Photosynthesis, together with cell growth, is among the primary processes to be affected by drought (Chaves 1991) or by salinity (Munns et al. 2006). The effects can be direct, as the decreased CO_2 availability caused by diffusion limitations through

the stomata and the mesophyll (Flexas et al. 2007) or the alterations of photosynthetic metabolism (Lawlor and Cornic 2002) or they can arise as secondary effects, namely oxidative stress. Anjum et al. (2011) indicated that drought stress in maize led to considerable decline in net photosynthesis, transpiration rate, stomatal conductance, water-use efficiency, intrinsic water-use efficiency, and intercellular CO_2 as compared to well-watered control.

Suppression of the photosynthetic capacity by salinity stress has been reported in a number of plant species (Robinson et al. 1983; Ball and Farquhar 1984; Perez-Lopez et al. 2012) and might be due to lower stomatal conductance, depression in specific metabolic processes in carbon uptake, inhibition in photochemical capacity, or a combination of these (Dubey 1997). Tavakkoli et al. (2011) reported specific ion toxicities of Na^+ and Cl^- reducing the growth of four barley genotypes grown in varying salinity treatments. High Na^+, Cl^-, and NaCl separately reduced the growth of barley; however, the reductions in growth and photosynthesis were greatest under NaCl stress and were mainly additive of the effects of Na^+ and Cl^- stress. High concentrations of Na^+ reduced photosynthesis mainly by reducing stomatal conductance. Salt-tolerant species, Barque73, had significantly greater photosynthetic rate and water-use efficiency than those of Sahara, Clipper, and Tadmor. It was concluded that high salt tolerance of the Barque73 was associated with a high CO_2 assimilation rate, and water-use efficiency.

5.7.3 Chlorophyll Contents

Chlorophyll is one of the major components of photosynthesis, and decrease in chlorophyll content under drought stress has been considered as a peculiar symptom of oxidative stress and may be the result of pigment photooxidation and chlorophyll degradation. Drought stress caused a large decline in chlorophyll a content, chlorophyll b content, and total chlorophyll content in different sunflower varieties (Manivannan et al. 2007). Barley plants grown under drought showed inhibition of chlorophyll synthesis as demonstrated by reduced SPAD (soil-plant analyses development analyses, based on chlorophyll meter readings) values (Zhao et al. 2010). Guo et al. (2009) reported that, after 13 days of drought stress, Martin and HS41-1 (drought tolerant) had much higher chlorophyll contents than Moroc9-75 (drought sensitive).

The chlorophyll contents of leaves decrease in general under salt stress. The oldest leaves start to develop chlorosis and drop-off with prolonged period of salt stress (Hernandez et al. 1995; Gadallah 1999; Agastian et al. 2000). However, Wang and Nil (2000) have reported that chlorophyll content increases under conditions of salinity in *Amaranthus*. Salinity causes significant decreases in Chl-*a,* Chl-*b*, and carotenoid in leaves of barley (Vysotskaya et al. 2010). Ahmed et al. (2013b) reported that barley plants grown under combined drought and salinity treatment showed a marked reduction in chlorophyll content (Chl-*a,* Chl-*b*, and carotenoids), accompanied by a sharp decrease in net photosynthesis (Pn), stomatal conductance (gs), and transpiration rate (Tr). These results indicate that photosynthetic inhibition was caused by stomatal factors and by chlorophyll synthesis inhibition.

5.7.4 Chlorophyll Fluorescence

Chlorophyll fluorescence analysis has proven to be a sensitive method for the detection and quantification of changes induced in the photosynthetic apparatus. The chlorophyll fluorescence is based on the measurement of fluorescence signal of dark-adapted plants exposed to continuous light (Govindjee 1995). The dark-adapted samples show characteristic changes in the intensity of chlorophyll fluorescence during the illumination by continuous lights and this effect is called fluorescence induction of Kautsky's effect. When barley plants are exposed to drought, the values of maximal quantum yield of PSII (*Fv*/*Fm*) decrease, which is a reliable sign of photoinhibition (Guo et al. 2009).

Salt stress leads to a decrease in the efficiency of photosynthesis and is known to influence the chlorophyll content and chlorophyll a fluorescence of barley leaves (Fedina et al. 2003). Chlorophyll a fluorescence parameters have been used to study high salt-induced damage to PSII. By measuring 77 K fluorescence emission spectra in dark grown wheat leaves under high salt conditions, it was shown that salt stress inhibits the chlorophyll accumulation by restraining several steps in porphyrin formation (Abdelkader et al. 2008). Delayed fluorescence measurements in *Arabidopsis thaliana* seedlings have also proved to be useful as a marker for detecting damage caused by salt stress (Zhang et al. 2008). A significant decrease in Fv/Fm by combined drought and salinity (D + S) suggested a possible inhibition of PSII photochemistry, which could be due to insufficient energy transfer from light harvesting chlorophyll complex to the reaction center. Compared with Tibetan wild barley (XZ5), greater decrease in *Fv*/*Fm* in cultivated barley (CM72) indicated that PSII of the latter was more sensitive to D + S, suggesting that a higher protective capacity for PSII could be an important tolerance mechanism for barley genotypes (Ahmed et al., 2013a).

5.7.5 Plant Nutrition

Decreasing water availability under drought generally results in limited total nutrient uptake and their diminished tissue concentrations in crop plants. An important effect of water deficit is on the acquisition of nutrients by the root and their transport to shoots (Farooq et al. 2009). In general, moisture stress induces an increase in N, a definitive decline in P and no definitive effects on K (Garg 2003). Influence of drought on plant nutrition may also be related to limited availability of energy for the assimilation of NO_3^- / NH_4^+, PO_4^{3-}, and SO_4^{2-} : they must be converted in energy-dependent processes before these ions can be used for growth and development of plants (Grossman and Takahashi 2001). As nutrient and water requirements are closely related, fertilizer application is likely to increase the efficiency of crops in utilizing available water. This indicates a significant interaction between soil moisture deficits and nutrient acquisition. It was shown that N and K uptake was hampered under drought stress in cotton (McWilliams 2003). Likewise, P and PO_4^{3-}

contents in the plant tissues diminished under drought, possibly because of lowered PO_4^{3-} mobility as a result of low moisture availability (Peuke and Rennenberg 2004). In general, drought stress reduces the availability, uptake, translocation, and metabolism of nutrients. A reduced transpiration rate due to water deficit reduces the nutrient absorption and efficiency of their utilization (Farooq et al. 2009).

Salinity hampers the uptake of macro- and micronutrients and the concentrations of sodium (Na^+) and chloride (Cl^-) in the plant increase, and the concentrations of potassium (K^+) and calcium (Ca^+) are reduced (Mansour et al. 2005). This together result in inhibition of plant growth due to limitation in the absorption of other ions and nutrients required for growth. It has also been reported that the accumulation of Na^+ and Cl^- in both cellular and extracellular compartments competes with K^+, Ca^+, magnesium (Mg^{2+}), and manganese (Mn^{2+}), whereas Cl^- restricts the absorption of nitrate, phosphate, and sulfate ions (Termaat and Munns 1986; Romero and Maranon 1994) and ultimately limits plant growth. Further, high levels of salinity may also affect the transport of Cl^- and Na^+ by inhibiting the specific transport systems of these ions (Maathuis 2006). Ahmed et al. (2013) reported that combined stress (D + S) resulted in higher increase in Ca, Mn, and Fe concentrations in shoots of wild barley (XZ5) than that of cultivated barley (CM72). Concerning root mineral concentrations, drought or salinity stress alone and in combination significantly increased Ca concentrations in both genotypes, while no significant effect on Zn and Cu concentrations was observed. Drought alone and D + S markedly increased Mn concentration in XZ5, but had no effect on CM72 under salinity and D + S treatments. Maintaining higher translocation of Ca, Mn, and Fe maybe an important way to reduce D + S stress or beneficial to improve plant tolerance to drought and salinity stress (Ahmed et al. 2013a).

5.7.6 Oxidative Stress and Enzymatic Regulation

The generation of reactive oxygen species (ROS) is one of the earliest biochemical responses of eukaryotic cells to biotic and abiotic stresses (Apel and Hirt 2004). The production of ROS in plants acts as a secondary messenger to trigger subsequent defense reactions in plants. The most common ROS are hydrogen peroxide (H_2O_2), superoxide, the hydroxyl radical, and singlet oxygen that formed as a natural by-product of the normal metabolism of oxygen and is crucial in cell signaling. The overproduction of ROS leads to oxidative stress and can cause damage to cellular components.

To minimize the impact of oxidative stress, plants have evolved a complex system of enzymatic antioxidants, superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), glutathione reductase (GR), and ascorbate peroxidase (APX), and nonenzymatic antioxidants, ascorbic acid, α-tocopherol, reduced glutathione, β-carotene, Polyamines (PAs), salicylates, compatible solutes such as proline (Pro), glycine betaine (GB), and zeaxanthin that accumulate in higher plants under drought and salinity stress (Ozkur et al. 2009).

Plants enhance the production of antioxidants in order to minimize the detrimental effects of oxidative stress to normalize their metabolic activities under drought- and

salinity-induced oxidative stress (Fig. 5.2). Different antioxidants have roles in protecting cells in specific compartments and in particular conditions. It is generally accepted that O_2^- might be converted to H_2O_2 and then metabolized to water by APX and GR in plants to maintain membrane structures (Foyer and Fletcher 2001). Likewise, several other antioxidant enzyme molecules are responsible to counteract the deleterious effects of ROS. Initially, SOD catalyzes the conversion of O_2^- to H_2O_2 that is further reduced to water by APX by using ascorbate as an electron donor (Scandalios 2005). Elevated accumulation of antioxidant enzymes such as SOD, CAT, GR, APX, and POD is involved in lowering oxidative injury in caper bush seedlings under drought stress (Ozkur et al. 2009). Yang et al. (2009) reported an increase in the activity of CAT, SOD, POD, APX, and GR at 25% field capacity as compared with 100% field capacity. Seckin et al. (2010) observed the opposite patterns in the activities of SOD, CAT, POD, APX, and GR enzymes in response to NaCl stress in *H. marinum* and *H. vulgare*. Thus, the antioxidant system of *H. marinum*

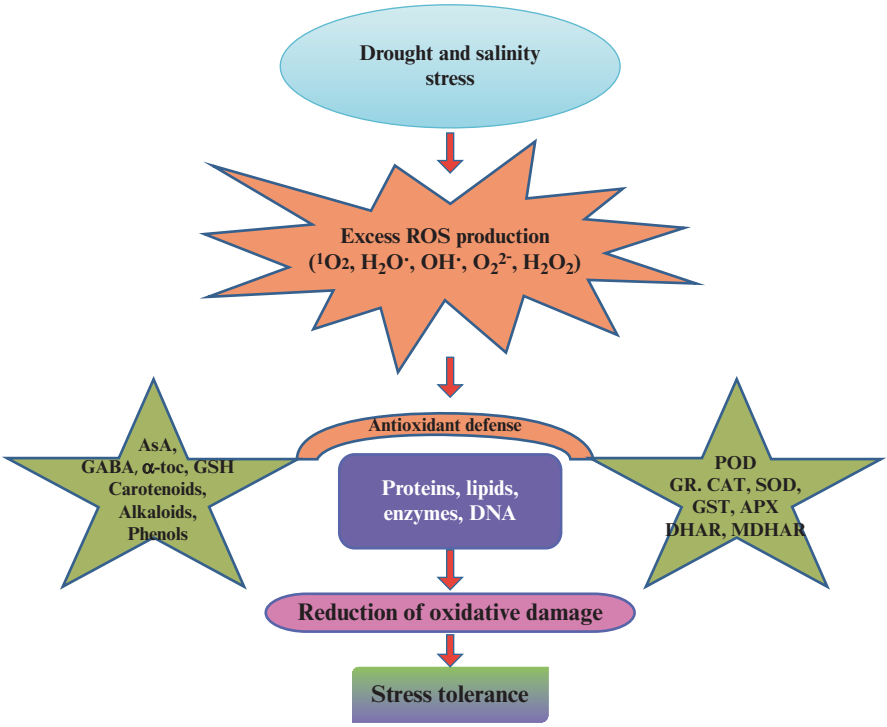

Fig. 5.2 Role of antioxidant enzymes in the ROS scavenging mechanism. Exposure to drought and salinity leads to generation of ROS, including singlet oxygen ($^1O_2$), perhydroxyl radical (H_2O), superoxide hydroxyl radicals (O_2^{2-}), hydroxyl radicals (OH), and hydrogen peroxide (H_2O_2). *ROS* reactive oxygen species, *SOD* superoxide dismutase, *CAT* catalase, *POD* peroxidase, *GR* glutathione reductase, *APX* ascorbate peroxidase, *GABA* γ-aminobutyric acid, *GSH* reduced glutathione, *MDHAR* monodehydroascorbate reductase, *DHAR* dehydroascorbate reductase, *GST* glutathione *S*-transferase

functioned at higher rates to suppress an increased ROS formation under salt stress. The significant increase in the activities of SOD, POD, APX, and GR in the NaCl-stressed leaves of *H. marinum* was highly correlated with the temporal regulation of the constitutive isoenzymes as well as the induction of new isoenzymes. Lower level of lipid peroxidation also revealed a higher free radical-scavenging capacity and protection mechanism of *H. marinum* against high salinity (300 mM NaCl) than *H. Vulgare*. Our previous reports (Ahmed et al. 2013b) indicated that CM72 had a higher malondialdehyde (MDA) content than XZ5 not only under D + S treatments but also under drought alone, suggesting less oxidative damage in Tibetan wild barley than cv. CM72. The essential role of antioxidative systems for maintaining a balance between the overproduction of ROS and their scavenging to keep them at appropriate levels for signaling and reinstatement of metabolic homeostasis is well established.

5.7.7 Compatible Solutes

Compatible solutes are low molecular weight and highly water-soluble compounds that are usually nontoxic even at high cytosolic concentrations. Plants accumulate compatible solutes, such as Pro and GB, sugars in response to drought and salinity to facilitate water uptake (Hare et al. 1998; Ashraf and Foolad 2007). In addition to osmotic adjustments, these osmolytes were suggested to be important for protecting cells against increased levels of ROS accumulation under stress conditions. Major contributors to osmotic adjustment were revealed to be K^+ in the early stages of stress and molecules including GB, Pro, and glucose, in the late stress (Nio et al. 2011).

Pro accumulates in the cytosol and the vacuole during stress (McNeil et al. 1999) and was shown to protect plant cells against damages caused by $^1O_2$ or HO (Matysik et al. 2002). By quenching $^1O_2$ and directly scavenging HO, Pro might be able to protect proteins, DNA, and membranes (Smirnoff and Cumbes 1989; Matysik et al. 2002). In the recent study, drought stress alone and D + S combined stress caused a marked increase in GB content in XZ5 and XZ16, more so than in CM72 (Ahmed et al. 2013b). Enhanced GB levels in Tibetan wild barley may exert protection on enzyme activity, including enzymes associated with sugar and amino acid metabolism (Chen et al. 2007), leading to greater increases in soluble sugars and Pro in Tibetan wild barley than control. Thus, it is proposed that the two Tibetan wild barley genotypes may acquire more protection than cv. CM72 under stressed environment due to the elevated levels of GB and the greater osmotic protection from higher levels of soluble sugars and Pro.

5.7.8 Plant Secondary Metabolism

Plant produces a large variety of secondary metabolites through several metabolic pathways in normal condition. But different stresses either biotic or abiotic trigger the plant secondary metabolism that results in enhanced production of plant

secondary products. Generally, precursors of secondary metabolic pathways are the products of the primary metabolism. To a large extent, secondary metabolites derive from three biosynthetic routes, namely the phenyl propanoid, isoprenoid, and alkaloid pathways. The major source of aromatic secondary metabolites in plants is the phenylpropanoid pathway (Irti and Faoro 2009).

Elevated phenol and flavonoid content were observed under single and combined stresses in the two Tibetan wild genotypes (Ahmed et al. 2014). In salt stressed *H. vulgare*, significantly higher concentration of flavonoids was observed (Ali and Abbas 2003). The content of protochatechuic acid, caffeic, and chlorogenic acids was increased following drought stress in *Matricaria chamomilla* (Kováčik et al. 2009). Ahmed et al. (2013c) also observed that the increase of phenolic compounds in the tissue prevented the formation of ROS in Tibetan wild and cultivated barley under combined drought and salinity stresses. In addition, the induced expression of genes related to secondary metabolism (*GST, PPO, SKDH, PAL, CAD,* and *chi2*) was demonstrated under all stress conditions in wild barley and accompanied an increase in the activities of the respective enzymes, with the greatest increase observed in XZ5. During rehydration and recovery, the activities of all enzymes increased except for phenylalanine ammonialyase (PAL) and cinnamyl alcohol dehydrogenase (CAD), which increased only in XZ5 (Ahmed et al. 2014).

5.7.9 Ultra-Morphology of Plants

Drought and salt stress leads to disintegration of fine structure of chloroplast, instability of the pigment protein complexes, destruction of chlorophylls, and changes in the quantity and composition of carotenoids (Dubey 1997). A wide array of variation has been observed in many studies regarding the effects of salinity stress on chloroplast ultrastructure like swelling of thylakoid membranes of chloroplast in the mesophyll cells of sweet potato leaves (Mitsuya et al. 2000) and also reduced numbers and depth of the grana stacks, and enlargement of starch grains in the chloroplasts of potato (Bruns and Hecht-Buchholz 1990). Hernández et al (1995) observed disorganized thylakoid structure of the chloroplasts, increased number and size of plastoglobuli, and decreased starch content in chloroplasts of plants exposed to drought and salinity stress. Whereas, chloroplasts aggregation, distortion of cell membranes with no signs of grana or thylakoid in chloroplasts were observed in tomato plants exposed to salt stress (Khavari-Nejad and Mostofi 1998). Elevation in the level of NaCl increased swelling of thylakoids and reduced chlorophyll fluorescence in barley seedlings (Zahra et al. 2014). Chloroplasts and mitochondria were affected in a variety-specific manner under all adverse treatments. The organelles of the drought-tolerant wheat cultivar Katya were better preserved than those in the sensitive variety Sadovo. Leaf ultrastructure can be considered as one of the important characteristics in the evaluation of the drought susceptibility of different wheat varieties (Grigorova et al. 2012). The effect of drought and salinity alone and in combination on endosperm starch and protein composition varied with genotypes and treatments. Under drought stress, the endosperm of CM72 grains had smaller

starch granules, especially B-type granules, which were located adjacent to crushed cell layer (CCL), while many A-type starch granules in this region were either pitted or showed surface erosion. The appearance of pitting can be associated with degradation of the proteinaceous layer, exposing the starch granule to severe stress. However, XZ5 and XZ16 showed more protein deposited on the surface of starch granules under drought stress (Ahmed et al. 2013c).

5.8 Identification of QTLs Controlling Drought and Salinity Tolerance in Barley

Quantitative trait locus (QTL) mapping is a powerful approach for locating genomic regions controlling complex traits (Gyenis et al. 2007). By linking phenotypic and genotypic data, QTL mapping enables the identification of the action, interaction, numbers and chromosomal locations of loci affecting particular traits (Miles and Wayne 2008). Large numbers of barley mapping populations have been developed to map genes and QTLs controlling agronomic and quality traits (Table 5.1) and have been reviewed by Fox et al. (2003). Several barley populations have been developed to map the QTLs for drought tolerance in both controlled environments and Mediterranean field trials. These included Tadmor x (ER/Apm) RIL population (Teulat et al. 1998), Derkado x B83-12 DH population (Forster et al. 2004), Apex x ISR101-23 (Pillen et al. 2003), and Barke x Hor11508 populations (Talame et al. 2004).

Kalladan et al. (2013) used advanced backcross quantitative trait locus (AB-QTL) analysis of a BC_3-doubled haploid population developed between the cultivated parent Brenda (*H. vulgare* ssp. *vulgare*) and the wild accession HS584 (*H. vulgare* ssp. *spontaneum*) to study the contribution of wild barley in improving various agronomic and seed quality traits under postanthesis drought. QTL analysis indicated that wild barley contributed favorably to most of the traits studied under both control and drought conditions. A total of seven hotspot QTL regions with colocalizing QTL for various traits harbored more than 80 % of the stable QTL detected in their study. For yield and 1000-grain weight and their respective drought-tolerance indices, most of the QTLs were derived from Brenda. On the other hand, for traits like seed length and seed nitrogen content, all the QTLs were contributed by HS584, the parent with higher trait value.

Many QTL studies carried out using wild barley as a donor parent for various traits indicated that it is a potential source for trait improvement (Nevo 1992; Volis et al. 2000; Pillen et al. 2004; Li et al. 2005, 2006; Rostoks et al. 2005; Schmalenbach et al. 2009; Schnaithmann and Pillen 2013). In addition, *H. vulgare* ssp. *spontaneum* was also found to possess positive alleles for abiotic stresses such as drought and salt (Talame et al. 2004; Suprunova et al. 2007; Ceccarelli et al. 2007; Lakew et al. 2011, 2013). Major hindrances to the utilization of wild species in crop improvement using conventional breeding are the quantitative nature of most of the agronomic traits and the linkage drag of undesirable genes present in wild species

Table 5.1 Enhancing drought and salinity tolerance in barley lines/varieties using marker-assisted selection

Stress	QTL used	QTL donor line/cultivar	Line/cultivar developed	Trait improved	References
Drought	AB-QTL	Apex/ISR101–23	BC2F2 population	For various agronomic and malting quality traits	Pillen et al. (2003)
Drought	AB-QTL	Brenda/HS584	BC3-doubled haploid population developed	Improved various agronomic and seed quality traits	Kalladan et al. (2013)
Drought	81 QTLs were used, out of which six (1H-3, 2H-1, 3H-2, 4H-3, 1H-5, 3H-1 and 3H-4) were for grain yield	*Hordeum spontaneum*	Backcross population	Improved grain yield, and reduced negative impact of drought on grain filling	Baum et al. (2003), Talame et al. (2004), Tuberosa and Salvi (2006)
Salinity	Identified QTLs were located on chromosomes 2H, 1H, 4H, 6H, and 5H	Steptoe x Morex and Harrington x TR306	Two doubled haploid (DH) populations	Those of QTLs controlling salt tolerance at germination and at the seedling stage	Mano and Takeda (1997)
Salinity	A spring barley collection of 192 genotypes were used to identify QTLs on chromosome 6H and 4H by a 1000 SNP marker set	From a wide geographical range	This study can be used for targeting candidate gene(s) for salt tolerance and uptake/transportation of both Na^+ and Cl^- which are important factors for salt-tolerance improvement of barley	Improved biomass production, chlorophyll content, plant height, tiller number, leaf senescence and shoot Na^+, shoot Cl^- and shoot, root Na^+/K^+ contents	Long et al. (2013)
Drought and salinity	MQTL were located on chromosomes 2H (drought) and 5H (salinity)	*Hordeum vulgare*	MWG2262BE37M33-160 and E36M50-81-E38M61-302, could be better for developing makers for salinity and drought stresses tolerance	In this study, 26 genes (some genes with two functions) associated with antioxidation (7), electron/ion transportation (11), Ca^{+2} ion binding (3), ATP binding/ATPase activity (7), or phosphorylation (7) were colocated with the five-type MQTL	Li et al. (2013)

QTL quantitative trait locus, *MQTL* meta-QTL, *AB-QTL* advanced backcross QTL, *SNP* single nucleotide polymorphism

(Wang and Chee 2010). One of the breeding strategies to overcome the problem of linkage drag associated with wild genotypes during breeding programs is AB-QTL analysis, which combines QTL detection with the introduction of favorable alleles into the targeted variety (Tanksley and Nelson 1996). In barley, AB-QTL analysis was first reported by Pillen et al. (2003) using a BC_2F_2 population developed between the cultivar Apex and the wild accession ISR101-23 for various agronomic and malting quality traits. Some of the other studies for improving drought tolerance in barley include Baum et al. (2003), Ceccarelli et al. (2004), Forster et al. (1997), Grando et al. (2001), and Ivandic et al. (2003).

Wild barley *H. spontaneum* has been recognized as an important source for drought tolerance. A QTL identified on chromosome 4H from *H. spontaneum* consistently increased grain yield across six test environments with an average yield increase of 7.7% (Pillen et al. 2003). Talame et al. (2004) identified two QTLs on chromosomes 2H and 5H with relative yield increase ranging from 12 to 22% under dry conditions. These QTLs could be used as target chromosome regions for the integration of wild barley genes for yield improvement under drought. Lu et al. (1999) suggested that drought tolerance in wild barley is related to their differing genetic abilities of osmotic adjustment under drought conditions. Thus, further genetic mapping and marker-assisted transfer of the osmotic-adjustment genes harbored in the wild progenitor could improve resistance of cultivated barley grown in water-limited environments.

Traditional QTL mapping or biparental QTL mapping based on a single segregating population derived from two homozygous parental genotypes has been the commonly used approach for genetic dissection of salt tolerance in barley and to identify candidate genes (Mano and Takeda 1997; Xue et al. 2009; Ellis et al. 2002; Witzel et al. 2009). This approach provides valuable information on genomic regions that control quantitative traits but it also has limitations due to poor sampling of the allelic variation present in the barley gene pool for each of the loci affecting salt tolerance, lack of segregation, and poor resolution of this type of mapping QTLs. Mano and Takeda (1997) identified QTLs controlling salt tolerance at germination and the seedling stage in barley by interval mapping analysis using marker information from two doubled haploid (DH) populations derived from the crosses, Steptoe × Morex, and Harrington × TR306. The results revealed that the QTLs for salt tolerance at germination in the DH lines of Steptoe x Morex were located on chromosomes 4H, 6H, and 5H, and in the DH lines of Harrington/TR306 on chromosomes 1H and 5H. In both DH populations, the most effective QTLs were found at different loci on chromosome 5H. Genetic linkage between salt tolerance at germination and ABA response was found from QTL mapping. The QTLs for the most effective ABA response at germination were located very close to those for salt tolerance on chromosome 5H in both crosses. The QTLs for salt tolerance at the seedling stage were located on chromosomes 2H, 1H, 6H, and 5H in the DH lines of Steptoe x Morex, and on chromosome 5H in the DH lines of Harrington x TR306. Their positions were different from those of QTLs controlling salt tolerance at germination, indicating that salt tolerance at germination and at the seedling stage was controlled by different loci.

Long et al. (2013) demonstrated that a spring barley collection of 192 genotypes from a wide geographical range was used to identify QTLs for salt-tolerance traits by means of an association mapping approach using a 1000 single nucleotide polymorphism (SNP) marker set. Linkage disequilibrium (LD) decay was found with marker distances spanning 2–8 cM depending on the methods used to account for population structure and genetic relatedness between genotypes. The association panel showed large variation for traits that were highly heritable under salt stress, including biomass production, chlorophyll content, plant height, tiller number, leaf senescence, shoot Na^+, shoot Cl^-, and shoot, root Na^+/K^+ contents. The significant correlations between these traits and salt tolerance (defined as the biomass produced under salt stress relative to the biomass produced under control conditions) indicate that these traits contribute to (components of) salt tolerance. Association mapping was performed using several methods to account for population structure and minimize false-positive associations. This resulted in the identification of a number of genomic regions that strongly influenced salt tolerance and ion homeostasis, with a major QTL controlling salt tolerance on chromosome 6H, and a strong QTL for ion contents on chromosome 4H (Long et al. 2013).

Recently, Li et al. (2013) confirmed that the distribution of meta-QTL (MQTL) was similar to that of the initial QTL. Many of these MQTL were located on chromosomes 2H (drought) and 5H (salinity). It inferred that chromosomes 2H and 5H were important for barley abiotic stress tolerance. As expected from trait correlations, 22.8 % of these MQTL displayed overlapping confidence intervals (CIs). These overlapping regions were mainly on chromosomes 1H, 2H, and 4H. The results indicated that the tolerance to diverse abiotic stresses were associated with each other in barley (Li et al. 2013).

5.9 Molecular Approaches for Improvement of Modern Barley

The high-throughput omics analysis, including transcriptomics, proteomics, and metabolomics, will improve comprehensive understanding of drought and salt stress-induced changes in gene-protein-metabolite (Urano et al. 2010; Sicher et al. 2012). Transcriptomics and proteomics analysis have been widely used in salt-tolerance studies (Du et al. 2008; Zhang et al. 2012). Currently, metabolomics are developed and applied in understanding multiple physiological processes in plants, in combination with other platforms such as transcript profiling and proteomics. Major approaches currently used in plant metabolomics are metabolic fingerprinting, metabolite profiling, and targeted analysis. Main analysis methods include gas chromatography-mass spectrometry (GC-MS), liquid chromatography-mass spectrometry (LC-MS), capillary electrophoresis-mass spectrometry (CE-MS), Fourier transformation cyclotron resonance-mass spectrometry (FT-ICR-MS), and nuclear magnetic resonance (NMR; Nicholson et al. 1999; Shulaev et al. 2008). In recent years, metabolomics analysis is being widely used to investigate abiotic stress tolerance of plants (Shulaev et al. 2008;

Oliver et al. 2011). In barley root, the metabolite profiling was analyzed in response to drought (Sicher et al. 2012), and combined stress of high temperature and drought (Rizhsky et al. 2004). Metabolome changes were also reported in cultivated barleys in response to salt stress (Widodo et al. 2009, Wu et al. 2013). In these context, several categories of genes which respond to the stress could be differentiated (Fig. 5.3): genes that encode protective but metabolically inactive polypeptides, such as dehydrins, chaperones (including proteases), genes for metabolic pathways leading to the synthesis of low-molecular osmolytes which increase stress tolerance, radical scavengers, or compounds with both functions, and regulatory proteins such as transcription factors, protein kinases, phospholipase C, or 14-3-3 proteins.

Most of the drought- and salt-tolerance genes belong to large gene families with high-sequence similarity distribute in a genome, which brings difficulty in identifying the specific locus for a specific function. More recently, genomic technologies have provided high-throughput integrated approaches (Bartels and Sunkar 2005) to investigate global gene expression responses not only to drought but also to other

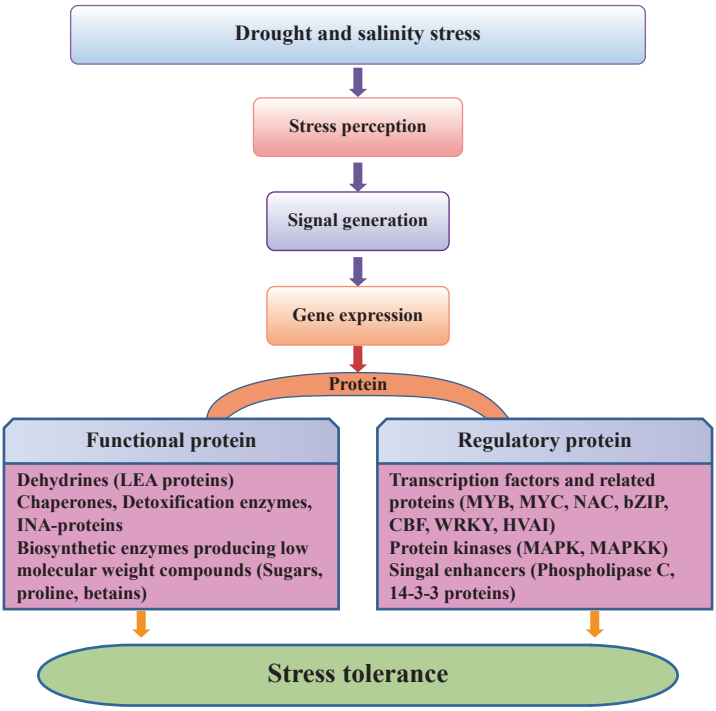

Fig. 5.3 Stress tolerance factors produced in adaptive responses of a barley plant to drought and salinity stress. *CBF* C-repeat binding factor, *MYB* myeloblastosis oncogenes, *LEA* late embryogenesis abundant, *INA* ice nucleation-active protein, *MYC* v-myc avian myelocytomatosis viral oncogene homolog, *bZIP* basic leucine zipper, *MAPK* mitogen-activated protein kinase, *MAPKK* mitogen-activated protein kinase kinase, *HVA1* ABA-inducible protein PHV A1, *WRKY* c-terminal wrky domain, *NAC* nascent polypeptide-associated complex protein

abiotic stresses (Chaves et al. 2003). Microarray profiling under drought stress has been carried out in different plant species such as *Arabidopsis* (Oono et al. 2003), rice (Rabbani et al. 2003), barley (Ozturk et al. 2002; Talame' et al. 2007), and wheat (Mohammadi et al. 2007). These studies identified differentially expressed transcripts of genes involved in photosynthesis, ABA synthesis and signaling, biosynthesis of osmoprotectants, protein stability and protection, reactive oxygen detoxification, water uptake, and a myriad of transcription factors including several members of the zinc finger, WRKY (c-terminal wrky domain), and bZIP (basic leucine zipper) families. Du et al. (2011) showed that two dehydrin genes might contribute to improved drought and salt tolerance of Tibetan and wild barley. Hv-WRKY38 is a barley gene coding for a WRKY protein, whose expression is involved in cold and drought stress response which was mapped close to the QTL region (Mare et al. 2004). Hv-WRKY38 was early and transiently expressed during exposure to low nonfreezing temperature, in ABA-independent manner. Furthermore, it showed a continuous induction during dehydration and freezing treatments. The aquaporin, dehydrin, C-repeat binding factor (CBF) genes, and Hv-WRKY38 may be putative candidate genes that underlie the QTL effect on salt tolerance. Differentially regulated proteins predominantly had functions not only in photosynthesis but also in detoxification, energy metabolism, and protein biosynthesis. The analysis indicated that de novo protein biosynthesis, protein quality control mediated by chaperones and proteases, and the use of alternative energy resources, i.e., glycolysis, play important roles in adaptation to drought and heat stress (Rollins et al. 2013).

Transcriptional factors (TFs) play important roles in the regulation of gene expression in response to abiotic stresses such as drought and salinity. TFs are powerful targets for genetic engineering of stress tolerance, because overexpression of a single TF can lead to the up-regulation or down-regulation of a wide array of stress response genes. Until now, transcription factors have been the most appealing targets for transgenic barley improvement, due to their role in multiple stress-related pathways. Dehydration-responsive element-binding protein 1 (DREB1)/CBF and DREB2 gene function in ABA-independent gene expression while ABA-responsive element (ABRE)-binding protein (AREB)/ABRE binding factor (ABF) functions in ABA-dependent gene expression. NAC (nascent polypeptide-associated complex protein) and MYB (myeloblastosis oncogenes)/MYC (v-myc avian myelocytomatosis viral oncogene homolog) are involved in abiotic stress-responsive gene expression (Uauy et al. 2006). In another study, a barley LEA protein, HVA1 (ABA-inducible protein PHV A1), was also overexpressed in wheat, and the overexpressors were observed to have better drought tolerance (Bahieldin et al. 2005). Transgenic wheat obtained with *Arabidopsis* DREB and HVA1 protein overexpression was also shown to produce higher yield in the field under drought conditions, but further studies are required to confirm their performance under different environments (Bahieldin et al. 2005). The transformation of oat and rice with the barley HVA1 gene also improved drought and salt tolerance (Xu et al. 1996; Oraby et al. 2005). It is not unreasonable to predict in the following decades: genetically modified (GM) wheat will be transferred to the fields as a common commercial crop. However, to pace this process,

new transgenics methodologies should be developed since the current methods are laborious and time-consuming. In a recent study, drought enhancement of bread wheat was established with the overexpression of barley HVA1, using a novel technique, which combines doubled haploid technology and *Agrobacterium*-mediated genetic transformation (Chauhan and Khurana 2011). Most of the transformed genes are from model plants such as *Arabidopsis* and rice or from wheat and barley cultivars. These approaches could be applied to wild relatives whose genes may have stronger effects. This hypothesis awaits experimental confirmation and field testing.

Plant miRNAs are approximately 20–24-nucleotide noncoding RNAs that specifically base pair to and induce the cleavage of target mRNAs or cause translational inhibition (Zhang et al. 2006b; Shukla et al. 2008). They have diverse roles in plant development, such as phase transition, leaf morphogenesis, floral organ identity, developmental timing, and other aspects of plant development (Lu and Huang 2008; Rubio-Somoza and Weigel 2011). To date, numerous miRNAs from diverse plant species have been identified and functionally characterized in plant development as well as stress response to biotic and abiotic environmental factors (Eldem et al. 2013). More than 40 miRNA families in plants have been associated with response to abiotic stress such as salt and drought (Sunkar 2010; Covarrubias and Reyes 2010). For instance, miR167, miR168, miR171, and miR396 were found to be drought-responsive miRNAs in *Arabidopsis* (Liu et al. 2008). In search of potential miRNAs involved in drought response in barley, some of the miRNAs, such as miR156, miR171, miR166, and miR408, were observed as differentially expressed upon dehydration (Kantar et al. 2011). miR166 is an example of many drought-responsive miRNAs that were previously characterized as crucial for cell development. It posttranscriptionally regulates class-III homeodomain-leucine zipper (*HD-Zip III*) transcription factors, which were demonstrated to be important for lateral root development, axillary meristem initiation, and leaf polarity (Hawker and Bowman 2004; Boualem et al. 2008). It is likely that differential regulation of miRNAs in different tissues is important for adaptation to stress in plants. For example, four miRNAs displayed tissue-specific regulation during dehydration in barley: miR166 was up-regulated in leaves, but down-regulated in roots; and miR156a, miR171, and miR408 were induced in leaves, but unaltered in roots (Kantar et al. 2011). Studying drought-responsive miRNAs and their target gene expression in individual cell types will provide greater insights into miRNA target networks that operate in a cell- or tissue-specific manner under drought stress. Zhou et al. (2013) reported that the overexpression of miR319 impacts plant development and enhances plant drought and salt tolerance. The miR319-mediated down-regulation of target genes in transgenic plants may have caused changes in various biological processes, including those associated with water retention capacity, leaf wax synthesis, and salt uptake beneficial to plants responding to salinity and water deficiency. The manipulation of miR319 target genes provides novel molecular strategies to genetically engineer crop species for enhanced resistance to environmental stress. An increasing understanding of the role of miRNAs in drought and salinity tolerance will enable the use of miRNA-mediated gene regulation to enhance plant drought and salinity tolerance.

Although tremendous efforts have been applied to breed drought- and salt-tolerant barley by conventional and molecular approaches, truly drought and salt-tolerant barley cultivars have not been produced that can go to farmer's field. The promising drought- and/or salt-tolerant genotypes are still in the laboratory and experimental fields. To overcome this bottleneck from the laboratory to the farmer's field, breeding programs should target specific environments and pyramid tolerance genes because drought and salt stresses are complex and variable in different environments and in different years.

5.10 Conclusions and Future Perspectives

Crop production under field conditions can be decreased by several abiotic stresses and the studies on multifactor interactions are of greater importance than analyses of only one stress. A combination of drought and salinity stress affects the plants to a larger degree and plant reaction cannot be directly extrapolated from the response of plants to individual effect of these two stresses. In the case of drought tolerance, plants potentiate to maintain the metabolic activities even at lower level of tissue water potential by accumulating intracellular osmoprotectants such as Pro, GB, amino acids, and soluble sugars. Besides, scavenging of ROS by enzymatic and nonenzymatic antioxidants, cell membrane stability, expression of aquaporin, and stress-related proteins such as LEA (late embryogenesis abundant) are also the vital mechanisms of drought and salinity stress tolerance.

Marker-trait associations are being identified by the development of a high density SNP assay platform that provides sufficient marker density for genome-wide scans and LD-led gene identification (Waugh et al. 2009). Projects are aiming to exploit the discriminatory LD observed in landrace and wild barley populations for fine mapping and gene identification (e.g., ExBarDiv: http://pgrc.ipk-gatersleben.de/barleynet/projects_exbardiv.php). Highly significant associations can be identified between genome-wide SNPs and drought and salt tolerances in wild progenitors, landraces, and varieties. These approaches offer the possibility of identifying novel allelic variation that may be of considerable value to future crop improvement (Waugh et al. 2009).

Advances are still needed to efficiently explore the extensive reservoir of drought and salt-tolerant alleles within wild germplasm deciphering: (1) the molecular networks those lost during domestication and modern breeding (Fu and Somers 2009); (2) the high-throughput screening of wild germplasm for drought/salt tolerance and their regulation of fitness components; (3) the molecular basis of chromosomal recombination; and (4) the potential regulatory relationship between coding and noncoding regions. This will increase the availability of sequence information and will encourage new breeding strategies by transferring single and multiple interacting networked loci/QTLs from wild relatives to commercial varieties via marker-assisted selection. The International *Triticeae* Mapping Initiative and the Barley Genome Sequencing Consortia are serving as platforms for international collaborative projects

that will ensure the use of extensive drought- and salt-tolerance gene pools for cereal crop improvement.

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Chapter 6
Combined Abiotic Stress in Legumes

Santiago Signorelli, Esteban Casaretto, Jorge Monza and Omar Borsani

6.1 Legume Family: Agronomic Relevance

A major source of protein in the human diet is of animal origin. The production of beef and mutton is based on natural pastures or supplementation based on grains (feedlot). Sown pastures can be monospecific or may be ultrasimple, simple or complex of different species of the same botanical family or a family of different botanical blends. Within the latter group, are mixtures of grasses and legumes.

From the point of view of human and animal consumption, legumes belonging to the subfamily Papilonideae are relevant. This includes seeds and forage legumes such as peanut, beans, chickpea, broad beans, lentils, soybean, among others. Some species of the genus *Medicago, Lotus* and *Adesmia* can be used as forage or green manure, thus enhancing the contents of nitrogen in the soils.

Forage legumes have been widely spread in the world due to the great agronomic importance that they possess. The species of this plant family are an invaluable component of pastures, mainly due to their ability to fix atmospheric nitrogen through symbiotic association with several bacteria collectively called *rhizobia*. Second, legumes have a high nutritional value, especially proteins and minerals (Ca^{+2} and Mg^{+2}), which makes them essential for the production of forage. Legume crops also play a critical role as main protein sources in vegetarian diets. Tolerance

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to environmental abiotic stress is one of the ways to improve the productivity of legumes and aid in harnessing their potential nutritional value. Identification of biochemical and physiological characters which contribute to improve the yield in legumes under limiting conditions is a main objective of plant breeders for agricultural and cattle-rearing regions. Thus, this chapter intends to provide an understanding of the mechanisms involved in the combined stress-tolerance responses in legumes.

6.2 Environmental Stresses Induce Varied Plant Responses

Plants are frequently subjected to stress—environmental condition that adversely affects the growth, development and productivity thereof. Biotic stress can be imposed by organisms such as viruses, bacteria and fungi, while abiotic stress can be due to an excess or deficit in some environmental factor. Among the environmental conditions that cause damage are excess water, water deficit, soil salinity, extreme temperatures, insufficient mineral nutrients in the soil and high- or low-light radiation (Bohnert and Sheveleva 1998; Bray et al. 2000).

Resistance or susceptibility to stress depends on the species, genotype and stage of development of the plant. Resistance mechanisms can be grouped into two categories—those that prevent exposure to stress and the other that results in tolerance. Certain morphological features such as sunken stomata and deep roots are examples of resistance mechanism that can prevent stress. However, other mechanisms of resistance are achieved by acclimation, i.e. the maintenance of internal homeostasis of the various organelles in response to changing environmental factors (Bray et al. 2000).

Plants acclimate to manage the different types of stress triggering a wide range of responses from the perception of stress at the cellular level, leading to the activation of a very large number of genes. Key components of the stress response are the stimulus itself, transducers, signal molecules, transcription regulators, responsive genes that trigger morphological, biochemical and physiological adaptation involved in this situation. In turn, the duration and severity with which stress is imposed determine how the plant will respond (Pastori and Foyer 2002; Bray et al. 2000).

Unlike resistance to biotic factors, resistance to water stress and other abiotic factors, despite being clearly genetic, is not a result of the action of a specific gene (Zhu et al. 1997). The ability of plants to withstand water stress is a multigenic trait and biochemical pathways responsible for products or processes that improve the overall strength can act additively, and also synergistically (Bohnert et al. 1995).

It is reported that several genes responsive to water stress not only perform their functions protecting cells by producing metabolically important proteins under water deficit but also in the regulation of genes involved in signal transduction in response to stress. Thus, these gene products are classified into two groups: The first group includes proteins that are involved in stress tolerance such as channel

proteins involved in the movement of water across membranes, enzymes necessary for the biosynthesis of osmolytes, proteases and macromolecules that can protect membranes, among others. The second group includes factors involved in the regulation of signal transduction and gene expression, such as protein kinases, transcription factors and 14-3-3 proteins, among others (Bray 1997; Shinozaki and Yamaguchi-Shinozaki 1997).

Higher temperatures primarily affect photosynthesis, in particular CO_2 assimilation because Rubisco activation is inhibited. Plants exposed to excessive temperatures have specific metabolic cellular response characterized by low protein synthesis, and induction of the synthesis of heat shock proteins (HSPs). In addition to altering the pattern of gene expression, the high temperature can damage cellular structures such as organelles and cytoskeleton (Bray et al. 2000; Tang et al. 2007).

Water stress and high temperatures interact strongly with each other and have opposite effects on photosynthesis. For example, in response to high temperature, plants open their stomata to cool their leaves by transpiration, but if there is also water deficit condition, plants would not be able to open the stomata and hence leaf temperature will increase (Rizhsky et al. 2002). While both types of stress have been extensively studied individually, few studies (Lu and Zhang 1999; Rizhsky et al. 2002; Rizhsky et al. 2004) focused on impacts of combined heat and water stress—a common situation prevailing under field conditions. It is possible that combination of these stress factors can alter the metabolism of the plant differently, compared to when a single stress is imposed (Xu and Zhou 2006).

6.2.1 Plants Response to Water Stress

Water deficit is one of the most widespread environmental factor stresses that occurs when the transpiration rate exceeds the absorption of water from the root system. Water deficit at the cellular level may result in an increase of solute concentration, changes in cell volume, disruption of water potential gradient, turgor loss, loss of membrane integrity and protein denaturation. The ability of the plant to respond to water deficit and survive depends on mechanisms that involve the integration of cellular responses throughout the plant (Bray et al. 2000).

Water deficit is a common plant environmental stress that dramatically limits growth and development. Water stress can trigger a significant decrease in crop productivity and quality, especially evident in grain and forage legumes. *Lotus japonicus* is a well-established model legume closely related to forage legumes such as *Lotus corniculatus, Lotus tenuis* and *Lotus uliginosus* (Choi et al. 2004; Díaz et al. 2005a). Alfalfa is a legume species with great plasticity that can succeed in semiarid, subhumid and humid regions and for that reason is called the "queen of forage legumes". However, it requires well-aerated and deep soils and is morphologically and physiologically adapted to withstand prolonged water deficiencies. In marked contrast to their drought-tolerant nature, these plants are very sensitive to a lack of oxygen that is common in flooding soils.

Legumes are typically subjected to a variety of different environmental stresses such as water stress. At the cellular level, this stress induces overproduction of reactive oxygen species (ROS; Fig. 6.1), such as hydrogen peroxide (H_2O_2), superoxide radical ($O_2^{\bullet-}$) and hydroxyl radical ($^{\bullet}OH$), which are responsible for oxidative damage associated with stress (Dat et al. 2000). Plants respond to stress using different enzymatic and non-enzymatic antioxidant systems. Oxidative stress responses may involve increased activity of superoxide dismutase (SOD), catalase (CAT) and ascorbate–glutathione cycle activities such as glutathione reductase (GR) or ascorbate peroxidase (APX), which can confer greater tolerance against a specific environmental stress (Sade et al. 2011). Increased levels of non-enzymatic soluble antioxidants including glutathione (GSH), ascorbic acid and tocopherols are also produced in response to water stress-induced oxidative stress (Feng et al. 2004). Plant antioxidant defence systems normally provide adequate protection against ROS damage under optimal growth conditions. The generation of higher levels of ROS may overcome the defence provided by these systems and result in oxidative stress (Mittler 2002; Noctor and Foyer 1998; Valderrama et al. 2006). Cellular damage caused by oxidative stress includes lipid peroxidation, which increases in various tissues during water stress and is also a common marker of oxidative stress (Sade et al. 2011).

In response to water deficit, plant cells also accumulate low-molecular-mass compounds termed compatible solutes, mainly proline, glycine betaine, sugars and polyols, in the cytoplasm to control the ionic balance in the vacuoles (Parida and Das 2005). Among these solutes, proline has been associated with different

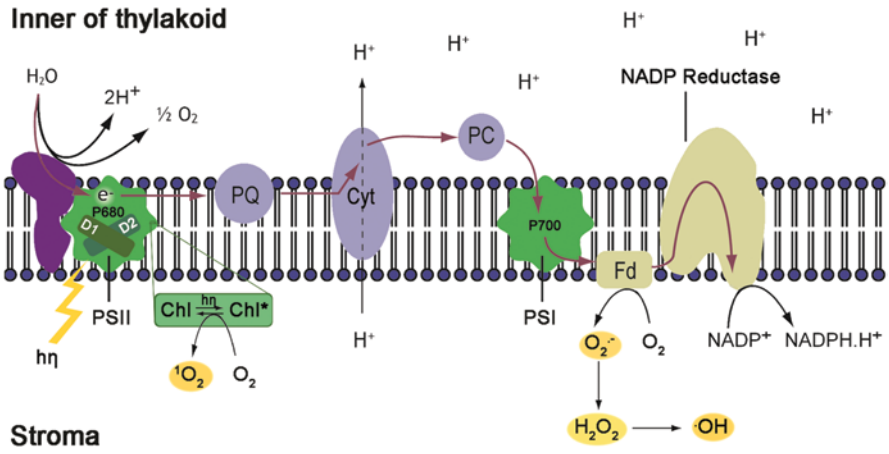

Fig. 6.1 ROS production in the chloroplast. *Chl* chlorophyll, *Chl** excited chlorophyll. *PSI* photosystem I, *PSII* photosystem II. *Cyt* cytochrome, *PQ* plastoquinone, *PC* plastocyanin. Superoxide ($O_2^{\bullet-}$) can be produced by electron transfer to oxygen. Hydrogen peroxide (H_2O_2) is produced from superoxide by spontaneous dismutation or SOD activity. Hydroxyl radicals ($^{\bullet}OH$) are produced from hydrogen peroxide by homolysis or Fenton reaction in the presence of Fe^{3+}. Singlet oxygen is generated from oxygen by energy transfer from excited chlorophylls

functions, such as being a free radical scavenger, a cell redox balancer, a cytosolic pH buffer and a stabilizer for subcellular structures, especially during osmotic and salt stresses (Szabados and Savouré 2010).

During drought establishment, plants exhibit a decrease in stomatal conductance with the consequent decrease in CO_2 assimilation. Stomatal closure has been considered as the main reason for the inhibition of photosynthesis under drought. However, it was demonstrated that limiting stomatal water losses is not so important to maintain photosynthetic activity. For example, it has been observed in leaves of various species, reductions in photosynthesis occur without apparent effects on stomatal conductance (Teskey et al. 1986; Hutmacher and Krieg 1983), suggesting that factors independent of stomatal behaviour impact photosynthesis in plants subjected to drought.

The use of split root system has helped in gaining knowledge about the impact of drought on the process of nodulation in legumes (Larrianzar et al. 2014). Nodule number is mainly regulated at the systemic level through a signal which is produced by nodule/root tissue, translocated to the shoot and transmitted back to the root system. This process involves shoot Leu-rich repeat receptor-like kinases. In contrast, local and systemic mechanisms regulate nitrogenase activity in nodules (Esfahani et al. 2014). Under drought and heavy metal stress, the regulation is mostly local, whereas the application of exogenous nitrogen seems to exert a regulation of nitrogen fixation both at the local and systemic levels (Marino et al. 2007).

6.2.2 Response of Plants to Heat Stress

High temperature at early sowing resulted in poor crop establishment due to failure of seed germination, emergence and reduced vigour (Khalaffalla 1985; Weaich et al. 1996). In such situations, avoidance mechanisms, such as transpiration, leaf rolling, hairiness or wax layers, may play a role in dissipating the heat load. However, in general, transpiration is the most important heat-dissipating system through latent heat loss (Kramer 1983).

Plants exposed to high temperatures, at least 5 °C above their optimal growing conditions, exhibit cellular and metabolic responses required for the plants to survive under this condition (Guy 1999). These effects include changes in the organization of organelles, cytoskeletal reorganization and membrane functions, accompanied by a decrease in the synthesis of some proteins and overexpression of HSPs, the production of phytohormones such as abscisic acid (ABA) and antioxidants and other protective molecules (Bita and Gerats 2013; Maestri et al. 2002; Bray et al. 2000). Under heat stress, about 5% of plant transcripts (~1500 genes) are up regulated, twofold or more (Rizhsky et al. 2004; Larkindale and Vierling 2008; Finka et al. 2011). A significant fraction of these transcripts encode heat-induced chaperones. For example, 88 out of 1780 in *Arabidopsis thaliana,* and 117 out of 1509 in wheat, are associated with HSP-based protection mechanism (Liu et al. 2008; Ginzberg et al. 2009; Bokszczanin and Fragkostefanakis 2013).

There are many transcripts-encoding proteins involved in calcium signalling; protein phosphorylation; phytohormone signalling; sugar and lipid signalling and metabolism; RNA metabolism; translation, primary and secondary metabolisms; transcription regulation and responses to different biotic and abiotic stresses (Mittler et al. 2012; Huve et al. 2011). Changes in ambient temperature are sensed by plant sensors positioned in various cellular compartments. The increased fluidity of the membrane leads to activation of lipid-based signalling cascades and to an increased Ca^{2+} influx. Signalling by these routes leads to the production of osmolytes and antioxidants as a response to heat stress. This stress also brings about changes in respiration and photosynthesis and thus leads to a shortened life cycle and diminished plant productivity (Barnabás et al. 2008).

The early effects of heat stress comprise of structural alterations in chloroplast–protein complexes and reduced activity of enzymes (Ahmad et al. 2010). The photochemical modifications in the carbon flux of the chloroplast stroma and those of the thylakoid membrane system are considered the primary sites of heat injury (Wise et al. 2004), as photosynthesis and the enzymes of the Calvin–Benson cycle, including ribulose 1,5-bisphosphate carboxylase (Rubisco) and Rubisco activase are very sensitive to low increases of temperature, and it is suggested to be one of the primary determinants of heat-dependent reduction in photosynthesis (Maestri et al. 2002; Morales et al. 2003). Heat inactivation of Rubisco is reversible (Salvucci and Crafts-Brandner 2004; Kim and Portis 2005). However, moderate heat stress has been shown to alter the thylakoid permeability and electron transport (Schrader et al. 2007; Zhang and Sharkey 2009), and this inhibition of electron transport is associated with enhanced membrane permeability, disorganization of photosystem II (PSII) and antenna tertiary structure, and disruption of the water splitting and oxygen evolving system (Huve et al. 2011). Other specific responses of heat stress on photosynthetic membranes include the swelling of grana stacks and an aberrant stacking. Such structural changes are accompanied by ion leakage from leaf cells exposed to heat and changes in energy allocation to the photosystems (Wahid and Shabbir 2005; Allakhverdiev et al. 2008). The maintenance of cellular membrane function under heat stress is thus essential for sustained photosynthetic and respiratory performance (Chen et al. 2010). The detrimental effects of heat on chlorophyll and the photosynthetic apparatus are also associated with the production of ROS (Guo et al. 2007). By increasing chlorophyllase activity and decreasing the amount of photosynthetic pigments, heat stress ultimately reduces the plant photosynthetic and respiratory activity (Sharkey and Zhang 2010).

Homeostasis, in general, including biosynthesis and compartmentalization of metabolites, is disturbed in high-temperature-challenged plant tissues (Maestri et al. 2002). Among the primary metabolites, accumulating in response to heat stress are proline, glycine betaine or soluble sugars (Wahid 2007).

Heat stress results in the misfolding of newly synthesized proteins and the denaturation of existing proteins. Protein thermostability is provided in part by chaperones (Ellis 1990). In this sense, the exacerbation of combined heat and other stress could be due to the loss of function of some enzymes that are overexpressed in response to other stress.

6.3 Effect of Water Stress–Heat Stress Combination on Different Plant Processes

L. corniculatus and *Trifolium pratense* are legumes used in agriculture as a forage source. These species are both perennial herbaceous plants used in temperate grassland and can be nodulated by *rhizobia*. Nevertheless, lotus is better suited to soils with water restriction and has a superior tolerance to water stress (Peterson et al. 1992). In the field, mainly during summer, these plants are commonly exposed to environmental stresses such as water stress and high temperatures, which in fact are considered to be the most important environmental factors limiting plant growth and development (Berry and Bjorkman 1980; Yordanov et al. 1986; Sinsawat et al. 2004).

6.3.1 Proline Accumulation

The accumulation of proline is known to be a good indicator of water stress in *L. corniculatus* (Díaz et al. 2005b). However, the responses to combination of stresses are not a mere additive effect of the single stresses. For example, some plants that tend to accumulate proline in water stress conditions replace it with sucrose as the major osmoprotectant when subjected to a combination of water stress and heat stress (Rizhsky et al. 2004). In *L. corniculatus* water stress and heat individually produce proline accumulation, but concomitant imposition of both stresses produced a higher accumulation of proline. In contrast, *Trifolium Pratense*-accumulated proline in water stress conditions but not under heat stress and the imposition of the combined stress produced only a slight increase in proline concentration compared to unstressed plants (Signorelli et al. 2013b). Thereby, for *L. corniculatus*, proline accumulation is a parameter that can be used as a stress marker to assess water stress and heat stress conditions, as well as the combination of both. However, proline accumulation in legumes cannot always be considered a good indicator of stress condition when two or more stresses are present. It is also known that proline accumulation under heat stress decreases the thermotolerance of the plant, probably because of an enhancement in the production of ROS via the Pro/P5C cycle (Lv et al. 2011). In *T. pratense,* it was suggested that blocking proline accumulation might be a strategy to avoid self-toxicity during heat stress (Signorelli et al. 2013b). This hypothesis correlated with the lipid peroxidation estimated by thiobarbituric reactive substances (TBARS), as *T. pratense* did not show an increase in lipid peroxidation under heat conditions. Moreover, *T. pratense* has a lower lipid peroxidation content than *L. corniculatus* when water stress and heat stress are combined—a treatment in which *L. corniculatus* accumulates the highest levels of proline.

On the other hand, it has been demonstrated that proline can act as an osmolyte under severe dehydration (Verslues and Sharp 1999). The non-accumulation of proline and the greater leaf area of *T. pratense* are important disadvantages of

this species compared to *L. corniculatus* when water loss must be prevented. In a comparative analysis of *L. corniculatus* and *T. pratense* subjected to water stress and heat, it was observed that *T. pratense* did not survive 5 days of combined stress, while lotus was still alive (Signorelli et al. 2013b). In concordance, higher dry-matter yield was observed in *L. corniculatus* compared to *T. pratense* under field conditions subjected to summer water stress (Peterson et al. 1992).

6.3.2 Oxidative Stress

Most stresses induce ROS and alter the antioxidant–enzymatic response (Mahalingam and Fedoroff 2003). However, little is known about how two or more stresses affect the ROS production and the antioxidant response. Alterations induced by water and heat stress on antioxidant response and oxidative damage in the model legume *L. japonicus* (Sainz et al. 2010), in the forage legumes *L. corniculatus* and *T. pretense* has been reported (Signorelli et al. 2013b).

SOD is the main enzymatic system responsible for cell detoxification and is well documented in several plant species to increase in response to water deficit and heat stress (Alscher et al. 2002). In *L. corniculatus*, the activity of Mn-SOD and Fe-SOD increased as a consequence of water stress and combined stress (Fig. 6.2), but it did not change under heat stress (Fig. 6.2). In the related model specie *L. japonicus,* Cu/Zn-SOD immunodetection and the isoenzyme-specific activity assays confirmed that high-temperature treatment provoked a reduction in the Cu/Zn-SOD protein content and activity. This is consistent with a failure to convert $O_2^{\bullet-}$ to H_2O_2 in the combined heat–drought condition. Additionally, in spite of the decreased Cu/ZnSOD in the high-temperature treatment, the accumulation of $O_2^{\bullet-}$ remains low,

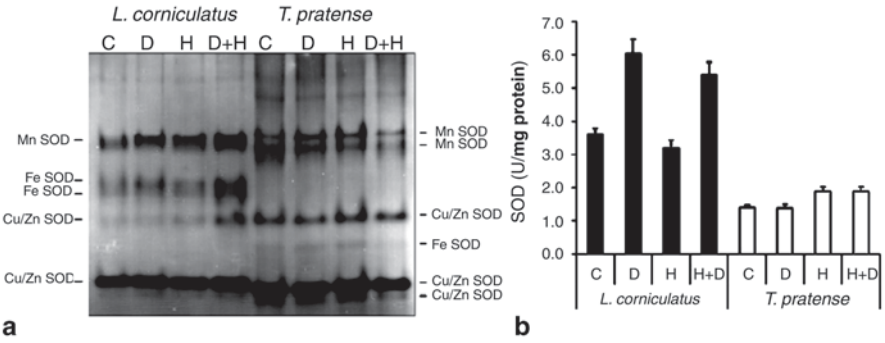

Fig 6.2 SOD activity under drought and combined heat and drought stress. **a** SOD isoforms profile. *C* control; *D* drought; *H* heat at 42 °C; D + H drought + heat at 42 °C. 40 and 200 mg of protein were loaded in *L. corniculatus* and *T. pratense,* respectively. The gel is the most representative of three replicates of native gels. **b** Total in vitro SOD activity. *C* control; *D* drought; *H* heat at 42 °C; D + H drought + heat at 42 °C. One unit of SOD was defined as the amount of enzyme that inhibits the rate of cytochrome c reduction by 50 %. *Bars* indicate the relative standard deviation. (Figure modified from Signorelli et al. 2013b)

and this is likely because high temperature does not induce accumulation of this ROS (Sainz et al. 2010).

In *T. pratense,* however, no changes were observed in the activities of any SOD isoforms. The results of the quantitative enzyme activity assay demonstrated that total SOD activity is 2.6-fold greater in *L. corniculatus* than in *T. pratense,* and it is affected by the stress treatments. Heat did not modify the SOD activity in *L. corniculatus,* but the combination with water stress led to same level activity observed under water stress (Fig. 6.2). *T. pratense* showed a slight increase in the SOD activity by heat stress and combined stress (Fig. 6.2). In this case, for both legumes the response of SOD activity in the combined stress was the addition of responses in the individuals' stresses. It could be concluded that if one of the stresses that produce the induction of SOD activity is present, the induction of SOD activity will be warranted in the combined stress. In *L. japonicus,* heat stress led to a decrease on Cu/Zn-SOD contents, which also was observed under a combination of heat and water deficit (Sainz et al. 2010).

In *L. corniculatus,* CAT activity only increases during the combination of water stress and heat. However, in *T. pratense*, CAT enzyme activity increased with reference to control in response to water deficit, heat stress and combined stress, although no differences were observed among these stresses. In *T. pratense,* it was observed that any stress was able to induce CAT activity and the combination of both stresses did not lead to an additive effect on the enzyme activity. For *L. corniculatus,* it seems that any individual stress is not sufficient to induce CAT activity; however, the combination of stresses led to the induction of CAT, suggesting that more than one signal is required to induce this enzyme. In *L. japonicus,* the combination of heat and water deficit led to an increase in CAT activity, that was much higher than the activity observed when the stressors were imposed individually (Sainz et al. 2010).

Interestingly, the APX activity in *L. corniculatus* was inhibited by water stress condition, while in *T. pratense,* this activity was inhibited only in the combined stress treatment. This enzyme is inactivated by nitration (Begara-Morales et al. 2014), which is reported to occur under several abiotic stresses (Corpas et al. 2013). For example, for *L. japonicus,* a closely related species, it was observed that water deficit induces a nitro-oxidative stress that was also reducing APX activity (Signorelli et al. 2013c). We speculate that the different stressful situations are also inducing nitro-oxidative stress in these plants, and this could explain the decay in enzyme activity.

Both *L. corniculatus* and *T. pratense* leaves showed $O_2^{\bullet-}$ accumulation only in the water deficit–heat stress combination, as was previously observed in the model legume *L. japonicus* (Sainz et al. 2010). The higher SOD activity in water stress conditions with respect to controls, would allow this species to deal with the $O_2^{\bullet-}$ induced mainly by water stress. However, the increase of Mn-SOD and Fe-SOD isoform activity by water stress was lost under high-temperature conditions, resulting in an increase of $O_2^{\bullet-}$ in the combined treatment. In *L. japonicus,* similar results were obtained with Cu/Zn-SOD, showing that deleterious effects of heat stress on SOD activity might be a general response for this legume genus (Sainz et al. 2010).

The differences detected between both species are mainly explained by changes in the Cu/Zn- SOD isoforms. In *T. pratense,* H_2O_2 accumulation showed the same pattern; however, in *L. corniculatus,* the highest accumulation of ROS was observed under water deficit. These results clearly demonstrate that combination of stress situations cannot be always considered the additive responses of individual stresses.

L. corniculatus showed an increase in TBARS content as a consequence of water deficit, heat stress and a combination of these. But *T. pratense* did not produce any increase in TBARS content under heat stress. As proline antioxidant protection function under stress conditions is now in discussion (Signorelli et al. 2013a), the absence of proline accumulation in T. pratense may be an advantage under heat stress by avoiding the Pro/P5C cycle which, as previosuly mentioned, could result in higher ROS production via the Pro/P5CS cycle (Lv et al. 2011). However, proline accumulation might be critical under combined stress because the osmolyte function seems to be important when water stress is established.

6.3.3 Photosynthesis

Water stress and heat combination affects the rate of photosynthesis due to an increase in photoinhibition, a process that can be enhanced when more types of abiotic stress coexist (Takahashi and Murata 2008). Under stress conditions, the possibility of overexcitation of PSII increases. This can cause a decline in the photosynthetic rate as the process of photoinhibition increases due to the necessity to dissipate, through nonradiative processes, the excess of absorbed energy (Takahashi and Murata 2008; Baker 2008). Because the capacity of photoprotection is limited, certain conditions can lead to damage and loss of active PSII reaction centres. Under severely high temperatures, in combination with water stress, the photosynthetic apparatus is the primary site of damage. On the contrary, photosystem I is more resistant to heat than PSII (Sayed et al. 1989; Hu et al. 2004; Havaux 1993). Once photoinhibition is established, the PSII reaction centre is simultaneously repaired via removal, synthesis and replacement of degraded D1 protein (Ohad et al. 1984; Kyle and Ohad 1986), a protein of reaction centre of PSII (Fig. 6.1). The observed photoinhibitory damage is the net result of a balance between photodamage and the repair process (Samuelsson et al. 1985; Lidholm et al. 1987; Shyam and Sane 1989). Several studies have reported a good correlation between changes in chlorophyll fluorescence parameters in response to environmental stresses, such as heat, chilling, freezing and salinity (Bonnecarrére et al. 2011; Smillie and Hetherington 1983; Yamada et al. 1996; Hakam et al. 2000). Others authors have linked the decrease in the maximum quantum yield of PSII (F_V/F_M) to the physical dissociation of the PSII reaction centres that lead to photoinhibition, and this assay was used to identify tolerant wheat cultivars (Abdullah et al. 2011).

In *L. corniculatus,* no changes of the maximum quantum efficiency, evaluated as F_V/F_M, were observed in any treatment until the 5th day, when the combined

treatment showed a significant decrease in the F_V/F_M parameter. In contrast, in *T. pratense,* this fluorescence parameter slightly decreased from the 1st day in the heat and combined treatment, but no changes were observed under water stress conditions.

L. corniculatus showed a slight decrease in the amount of D1 protein after water stress treatment. However, there was no decrease in the protein content when the control and heat conditions were compared. The D1-complex profile of *T. pratense* was also analysed, and the western blot showed a very different result when compared with the *L. corniculatus* profile. The total D1 protein content in *T. pratense* did not change in any treatment, but a difference was found in the ratio between the free protein and the complex form. In the treatments where heat was involved, an increase in free D1 protein together with a decrease in the D1-complex form was evident, but it should be considered that this result might be a consequence of the high hydrophobicity of these complexes, which makes their isolation difficult. Regarding D2, in *L. corniculatus,* the results were similar to those observed with D1; namely, when water stress was present in the treatments, a reduction in the amount of D2 protein was observed. Surprisingly, in the combined treatment, the D2 protein was not detected. In contrast, in *T. pratense*, no significant changes were observed in D2 protein levels.

The chlorophyll fluorescence parameter that was evaluated showed that *L. corniculatus* had a significant decrease in the maximum quantum efficiency at the end of the combined treatment. The low F_V to F_M ratio indicated photoinhibition, a process that can act as determinant of plant performance during a stress condition (Abdullah et al. 2011). In *T. pratense,* only a small decrease in the F_V/F_M values was observed from the 1st day and in the treatments involving heat stress.

Analysis of PSII proteins in the two legumes with contrasting water stress responses shows an effect that is stress- and species-specific. The D1 and D2 subunit content is decreased in *L. corniculatus* in both treatments involving water stress, showing certain adaptability in response to water stress. Interestingly, the decrease in the D2 levels was pronounced in the combined treatment, and this is well correlated with the decrease in the maximum quantum efficiency, suggesting the presence of a disassembling process. The D2 subunit is of particular interest because it represents the initial point for the assembly of the PSII as a whole (de Vitry et al. 1989; Komenda et al. 2004; Minai et al. 2006). The expression of the gene that encodes the D2 subunit of the PSII reaction centre is regulated post-transcriptionally by an RNA-binding protein (Schwarz et al. 2007). Modifications induced by the stress in this post-transcriptional regulation could be a possible explanation for the absence of D2 in *L. corniculatus* subjected to the combined stress treatment.

In *T. pratense,* the total content of D1 and D2 did not change, but we observed an increase in the free form of D1 in treatments involving heat. One possible explanation is that the turnover of D1 is taking place in the heat treatments, and this is evident based on the increase of free D1 together with a reduction of the D1–D2 complex, as well as a decrease in the F_V/F_M values.

6.4 Waterlogging and Salinity: A Combined Stress in Legumes

Salt stress is certainly one of the most serious environmental factors limiting the productivity of crop plants (Ashraf and O'Leary1999). Salinity reduces the ability of plants to take up water, causing rapid reductions in growth rate, along with an array of metabolic changes identical to those caused by water stress (Munns 2002).

High salt concentration in the external solution of plant cells produces several deleterious consequences. First, salt stress causes an ionic imbalance (Niu et al. 1995). The homeostasis of not only Na^+ and Cl^- but also K^+ and Ca^{+2} ions is disturbed (Rodriguez-Navarro 2000; Hasegawa et al. 2000; Serrano et al. 1999). As a result, plant survival and growth will depend on adaptations that re-establish ionic homeostasis, thereby reducing the duration of cellular exposure to ionic imbalance. Second, high concentrations of salt impose a hyperosmotic shock by decreasing water and causing loss of cell turgor. This negative effect in the plant cell is thought to be similar to the effects caused by drought. Third, reduction of chloroplast stromal volume and generation of ROS, in salt-induced water stress, are also thought to play important roles in inhibiting photosynthesis (Price and Hendry 1991). On the molecular level, these responses are manifested as changes in the pattern of gene expression (Maggio et al. 2002).

The process of salinization results from the interaction between climate, geomorphology, hydrology, land use and surface water properties and dynamics of the salts. Regions with salinity are frequently associated with geographical localization with inundation events; thus it is not infrequent that salt and flood stress occurs simultaneously.

Salinity and waterlogging interact adversely to reduce production of crops and pastures, as very few species used in agriculture can tolerate the combination of both stresses (Barrett-Lennard 2003). Moreover, annual pasture legumes are particularly sensitive to combined salinity and waterlogging (Bennett et al. 2009).

One of the most important consequences of energy limitation under anoxia is altered redox state of the cell. Under low oxygen pressure conditions, the intermediate electron carriers in electron transport chain become reduced, affecting redox-active metabolic reactions. Therefore, for maintaining redox homeostasis cells need to regulate NADH to NAD ratio under flooding (Chirkova et al. 1992). Saturated electron transport components, the highly reduced intracellular environment and low-energy supply are the factors favourable for ROS generation. The consequences of ROS formation depend on the intensity of the stress as well as on the physicochemical conditions in the cell (i.e. antioxidant status, redox state and pH). As was mentioned for other stresses, ROS accumulation may cause damage to different cell structures and biomolecules. H_2O_2 production during O_2 deprivation was observed in the plant cells (Blokhina et al. 2001), and its degradation was found to play an important role in waterlogging tolerance in non-legume plants (Lin et al. 2004).

A trait that is essential for root survival during water logging or flooding is the development of aerenchyma (Armstrong 1979). Aerenchymas are cortical airspaces

that provide a low-resistance internal pathway for the movement of O_2 from the shoots to the roots, where it is consumed in respiration and may also reoxidize the rhizosphere (Armstrong 1970; Armstrong 1971, 1979). In legumes, aerenchyma may also be important for supplying O_2 and N_2 to root nodules (Walker et al. 1983; James et al. 1992; Zook et al. 1986; Pugh et al. 1995). Tolerance of *Melilotus siculus* to waterlogging is associated with the production of a highly porous phellem, a type of secondary aerenchyma, on taproots and upper lateral roots (Verboven et al. 2011).

Studies with plant species sensitive or tolerant to flooding–salt stress combination have shown that the rate of transport of Na^+ and Cl^- to the shoot is critical to define the response. The ions transport rate increases significantly under combined stress in comparison with salinity alone (Barrett-Lennard 2003). For more tolerant species, there is only small or even no increase in shoot Na^+ and Cl^- in response to combined salinity and waterlogging (Colmer and Flowers 2008), presumably due to better root aeration. Moreover, in perennial legumes such as *Trifolium repens L*. (Rogers and West 1993) and *Liolaemus tenuis* (Teakle et al. 2007), high root porosity was associated with better shoot ion regulation under combined salinity and waterlogging. Comparisons of annual pasture legumes in growth, ion regulation and root porosity demonstrate that *M*. *siculus* has exceptional tolerance to combinations of salinity and waterlogging (Teakle et al. 2012). Enhanced root aeration would avoid energy deficits that could impair ion transport processes in roots, which determines delivery of Na^+ and Cl^- to shoots via the xylem (Barrett-Lennard 2003; Teakle et al. 2007; Colmer and Flowers 2008). Thus, traits of importance for tolerance to combined salinity and waterlogging are likely to include high root porosity, leading to decreased shoot Na^+ and Cl^- concentrations.

6.5 Metabolic Changes in Responses to Stress Combination

It is well known that the effect of a combination of different stresses on plants can be quite different from those generated when plants are subjected to individual types of stress (Rizhsky et al. 2002). Table 6.1 represents a summary of how the combination of different stresses affects some parameters in legumes.

With reference to antioxidant responses, different patterns are observed when more than one stress is imposed. However, it seems that in most cases the addition of other stress did not alter the response. It implies that the signal molecules that induce the expression of antioxidant enzymes probably are the same in different stresses and so the imposition of both stresses is redundant. In other cases, the effect of simultaneous stresses produces deleterious effects. For example, for APX and CAT, one stress produces the induction of the activity (or at least a normal level of activity), but the imposition of two stresses could produce a more nitrosative condition in the cell leading to the nitration of the enzyme, which is known to decrease the activity of these enzymes.

Table 6.1 Effects of stress combination on main parameters studied in legumes

		Response			
	Evaluated parameter	Negative correlation	Unchanged	Additive response	Synergistic effect
Antioxidant enzymes	APX				
	SOD				
	CAT				
	GR				
	POX				
Oxidative stress	H_2O_2				
	TBARS				
	Electrolyte leakage				
	$O_2^{\circ-}$				
Photosynthetic activity	F_V/F_M				
	CP47				
	D1				
	D2				
Metabolites	Proline				
	Ascorbic acid				

Darker shading indicates that the particular response is supported by more evidence. Data obtained from following legume species under various combined stresses: *L. corniculatus, T. pratense* subjected to combined drought and heat (Signorelli et al. 2013b), *L. japonicus* subjected to combined drought and heat (Sainz et al. 2010), *Vigna unguiculata* subjected to combined CO_2, UV-B radiation and temperature stress (Singh et al. 2010), *Vigna radiata* (Siddiqui 2013) and *Phaseolus vulgaris* subjected to combined zinc and high irradiance stress (Michael and Krishnaswamy 2011).

APX ascorbate peroxidase, *SOD* superoxide dismutase, *CAT* catalase, *GR* glutathione reductase, H_2O_2 hydrogen peroxide, *TBARS* thiobarbituric reactive substances, $O_2^{\bullet-}$ superoxide radical, F_v/F_M photosystem II, *POX* peroxidase

Among the oxidative stress markers, synergistic effect was the most commonly observed response. Most stresses are accompanied by an increment of ROS production, and the source of ROS is different for different stresses (Mahalingam and Fedoroff 2003; Wrzaczek et al. 2013). Thus, when more than one stress is present, it induces ROS from different organelles, and hence the total ROS tends to be higher in combined stress scenarios. Less commonly, a negative correlation or an unchanged response is observed. In one case of negative correlation observed for H_2O_2, it was suggested that the reduction in SOD activity in combined stress as opposed to in single stress was responsible for the lower H_2O_2 in the former. In the other case, induction of CAT activity only in the combination of stress was suggested to be the cause of lower H_2O_2 levels.

Photosynthetic activity does not show a defined pattern, maybe due to lack of information. Even with the limited data, it can be seen that in all the cases examined, D1 was unchanged by the imposition of combined stresses. D2 protein had a synergistic effect in combined stress. It is important to point out that in drought and heat stress were considered in these studies, and some of these responses were observed in *T. pratense* and in two related species such as *L. japonicus* and *L. corniculatus*. Other species should be evaluated to see the conservation in the response of D2, which is suggested to disassemble to induce inhibition of photosystem activity, and protect cells from oxidative damage caused by its own activity.

6.6 Forage Legumes Field Productivity and Combined Environmental Stress

Legumes have a high level of productive diversification and flexible utilization. The same species can be usefully exploited for different purposes such as soil protection from erosion; green manure crop; mulching; cover crop in vineyards, orchards and firebreak lines; high quality honey production; landscape enhancement and medicinal use. Consequently, forage legumes were adapted to a wide range of soil types, climatic conditions and management systems (Sánchez-Díaz 2001).

Legumes, as many other crops, have been bred to maximize productivity (forage or grain). But this productivity is always affected by adverse environmental factors. Perennial forage legumes are a good model to analyse the responses of adaptability of plants under field conditions. This is because during the whole plant growth and development cycle, plants are subjected to various types of abiotic stresses, both singly and in combinations.

Low temperatures and periods of water saturation in soils are common during the winters in many regions and in the other side periods of low water regime combined with high temperatures are common during summers. To these we must add other combinations of stresses such as periods of high radiation or toxic ions (Na^+ or heavy metals) produced by changes in the physicochemical conditions of the soils.

Further, abiotic stress can affect the legume plants at different developmental stages. So legumes growing under field conditions must have adaptation process triggered by stress in seedling, vegetative or reproductive stages. For example, for seedling emergence, the optimal conditions in the field are established at the end of winter (Fig. 6.3).

Legumes are adapted to different environmental conditions by setting the developmental stages, such as reseedling capacity that is an important characteristic for the perpetuation of *L. corniculatus*. Yield of *L. corniculatus* during 3 years with seed set and without seed set, reveal the importance in reseedling (Fig. 6.4, Ayala and Carámbula 2009).

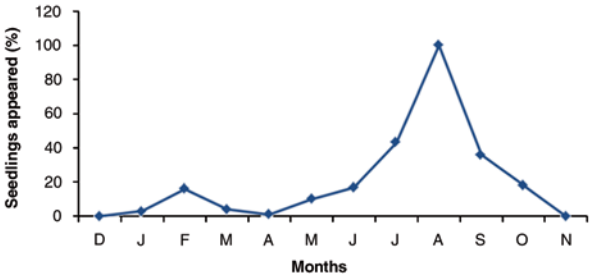

Fig. 6.3 Seedlings emergence of *L. tenuis* during a typical of temperate zones from south hemisphere (Ayala and Carámbula 2009)

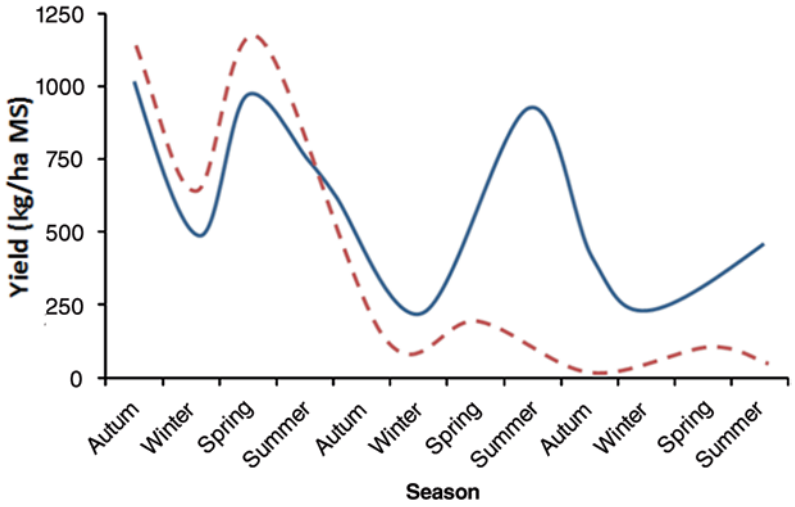

Fig. 6.4 Seasonal dry matter production of *L. corniculatus* under two-seed set management. With seed set (*solid line*) and without seed set (*dashed line*). (Ayala and Carámbula 2009)

This suggests that the tolerance in reproductive stages should be accompanied by physiological responses to deal with water restriction and high temperatures. Another key physiological mechanism in the survival of legumes is their ability to mobilize carbohydrates to storage tissues that can be located on the crown, root or rhizome (Castillo et al. 2012). However, it is critical that the photosynthetic activity remains active during the stress period to achieve significant accumulation of sugars allowing regrowth of the shoot after stress.

6.7 Breeding Approaches for Improving Tolerance to Combined Abiotic Stresses

Selection for one abiotic stress tolerance in the field is very challenging due to interactions among the different stresses. Thus, the only strategy to identify the traits to be applied in field for breeding tolerant genotypes is by performing experiments

under controlled environment conditions. Regardless of the screening method, a key objective for plant breeders is to develop an effective set of stress combination markers that can be used to improve legume crop species. Controlled environmental conditions allow the dissection of each one of different stress effect and the identification of principal targets affecting plant tolerance. Breeding for stress tolerance requires efficient screening procedures, identification of key traits in diverse donor or tolerant lines and understanding their inheritance and molecular genetics. Statistical package applied to plant breeding will facilitate the identification of markers in a multi-trait multi-environment way (Malosetti et al. 2004).

Several quantitative trait locus (QTL) studies relating to various abiotic stress tolerances have already been reported showing it is possible to improve and accelerate the breeding process in plant species without sequenced genomes (Chandra et al. 2004). In order to transfer these traits, classical breeding requires the establishment of rapid and cost-effective screening procedures and implementing these using breeding approaches such as association mapping or genomic selection procedures.

For the complete sequencing of the different important legumes, genome opens the possibility of fine mapping of the QTLs. In this perspective, gene identification for combined stress tolerance in legumes using genetic map information and genome data is an achievable goal (Heffner et al. 2009; Hirayama and Shinozaki 2010).

Phenotypic and physiological characterization along with RNA sequencing analysis of plants subjected to drought, heat, salt, flooding stress or their combination would confirm that the simultaneous imposition of different types of stress presents unique but varied aspects that includes alteration of respiration rate, decreased photosynthesis, stomatal closure, high leaf temperature and redox homeostasis. Thus, deep phenotyping methodologies, genome-based selection and massive RNA sequencing technologies emerge as a promising avenue for the development of multiple abiotic stress-tolerant crops.

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Chapter 7
Interactive Effects Between Ozone and Drought: Sorrow or Joy?

Sacha Bohler, Ann Cuypers and Jaco Vangronsveld

7.1 Introduction

The industrial revolutions of the eighteenth and nineteenth centuries marked the beginning of industry and technology, as we know it today (Ashton 1997; Hull 1999). In the eighteenth century, the first commercially available steam engine was one of many breakthroughs that improved transport and industrial processes. Unfortunately, it was also the first step towards the extensive use of fossil fuels, initially in the form of coal. The nineteenth century brought forward the invention of the combustion engine using fuel derived from petrol. The increasing use of fossil fuels also marked the dawn of anthropogenic pollution, which has increased ever since and reached its preliminary peak in the twenty-first century.

In the 1970s, acid rain was the major concern of environmentalists (van Breemen et al. 1984; Shortle and Bondietti 1992), and damaged vast areas of vegetation. Later, in the 1980s, depletion of the ozone layer had everybody worried (Solomon et al. 1986). Today, climate change is on the mind of the general population, including policy makers and researchers. Even though still largely rejected by climate change opponents, the reports of the Intergovernmental Panel on Climate Change (IPCC) presents ample proof that the earth's average temperature is increasing, polar ice caps are melting, ocean levels are rising and extreme weather conditions are becoming more and more frequent (Solomon et al. 2007; Stocker et al. 2014). This is for a

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larger part due to anthropogenic atmospheric pollution, primarily brought on by the combustion of fossil fuels. Molecules like carbon dioxide (CO_2), methane (CH_4), nitric and sulphur oxides (NO_x, SO_x) are directly emitted into the atmosphere, while other molecules like ozone (O_3) are formed from reactions between pollutants and atmospheric constituents. CO_2 and ozone are two of the main greenhouse gases that cause the retention of heat in the atmosphere and lead to the observed increase in temperatures.

7.2 Ozone

Ozone is a secondary pollutant, i.e. it is not directly emitted, but produced as a consequence of primary pollutants. Nitric dioxide (NO_2), mostly emitted by car and industrial exhausts, reacts with solar UV radiation and fragments to form nitric monoxide (NO) and an activated oxygen atom ($O^{\bullet -}$). The reaction between $O^{\bullet -}$ and atmospheric oxygen (O_2) leads to the formation of ozone (Andreae and Crutzen 1997; Renaut et al. 2009; Fig. 7.1). Molecules like volatile organic compounds (VOC) also intervene in the complex reactions. Background ozone concentrations in the troposphere (the layer of air that expands from the earth's surface up to an altitude of about 10 km) have increased by 500 % during the past century (Marenco 1994). Even though, in recent years, steps have been taken to reduce the emission of ozone-forming pollutants, results are inconclusive (Jonson et al. 2006).

An ozone-enriched atmosphere induces a situation of stress in plants. Ozone is easily absorbed through stomata and instantly fragments in contact with the plant

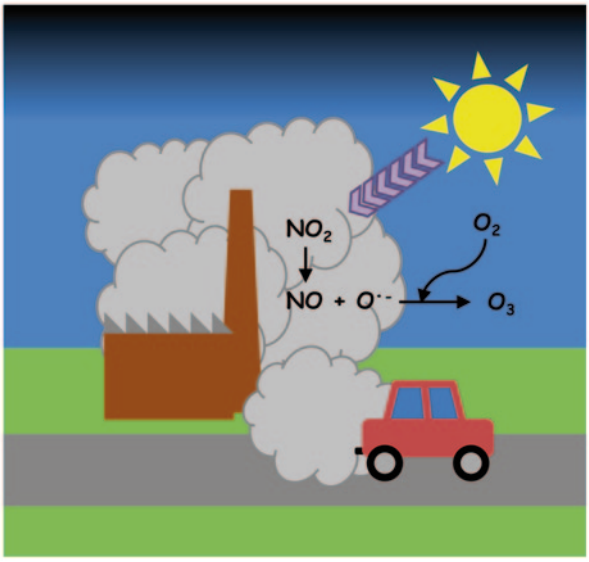

Fig. 7.1 Simplified scheme of the formation of the secondary pollutant ozone in the troposphere. NO_2 nitric oxide; O_3 ozone; $O^{\bullet -}$ activated oxygen atom; O_2 atmospheric dioxygen

tissue. The highly energetic ozone molecule reacts with cell wall components and causes the formation of reactive oxygen species (ROS). These highly reactive molecules in turn diffuse into the cells where they can damage proteins, genes, lipids and other biomolecules. The high oxidative potential of ROS interferes with cell signalling and regulation, including induction of programmed cell death (Kangasjärvi et al. 2005), and may eventually lead to the death of exposed plants. Leaf chlorosis, formation of necrotic patches and an increase in the number of senescing leaves are the most visible symptoms of ozone stress (Bohler et al. 2007, 2013).

7.3 Drought

Drought is the prolonged absence of rain that leads to a transient water deficit in the soil and concomitantly a stress situation for plants. Not only does drought depend on precipitation but also on the speed of water evaporation from the soil (Sherwood and Fu 2014). The occurrence of drought is variable among the different regions of the earth and dependent strongly on climatic regions. Droughts are common in arid regions; but in recent years, occurrences have also become more frequent in moderate climate, posing a threat for crops and forests (Kreuzwieser and Gessler 2010; Ciais et al. 2005). Recent considerations show that background dryness is as important for hydrological changes as acute occurrences of drought, and needs to be given more importance in the evaluation of the effect of climate change on hydrological changes (Sherwood and Fu 2014).

In plants, drought induces a decrease in the internal water potential. The first response is usually a reduction in stomatal conductance to reduce evaporation and save water (Warren et al. 2007). Further effects involve an accumulation of osmotically active solutes, to increase internal osmotic potential and improve water retention and absorption (Evers et al. 2010). Visible symptoms of drought include stunted leaves and an increase in leaf senescence (Bohler et al. 2013; Munn-Bosch and Alegre 2004).

7.4 Co-Occurrence of Ozone and Drought

Due to the meteorological conditions favouring both ozone formation and drought (i.e. a succession of warm days free of cloud cover), both are very likely to occur simultaneously. This can have drastic consequences for vegetation, if the effects of both stresses are synergistic. However, the effects of ozone and drought can also be antagonistic, in which case a simultaneous occurrence might be beneficial to plants. It has been postulated that a stomatal closure induced by drought may reduce the flux of ozone into the plant and thus be protective. In this chapter, a closer look is taken on the current state of understanding of the physiological, biochemical and molecular effects that ozone and drought in combination have on plants.

7.5 Stomatal Conductance: Protection Against Ozone by Drought

One of the common characteristics between ozone and drought exposure is the significant involvement of stomata. While ozone enters plants through the stomata, water vapour escapes through them; therefore, stomatal closure would theoretically protect plants against both stresses (Fig. 7.2). While observations have shown that stomatal conductance is an excellent marker for the severity of drought (Medrano et al. 2002), the situation is less evident for ozone. Reports have concluded that stomatal behaviour is not consistent, but dependant on many factors (Wittig et al. 2007). This has been mainly attributed to stomatal sluggishness in multiple publications (Hoshika et al. 2012, 2014; Paoletti and Grulke 2010; Dumont et al. 2013). It has been shown that this delayed response time of stomata varies among species (Hoshika et al. 2012; Paoletti and Grulke 2010), severity of stress (Hoshika et al. 2012) and on seasonal changes (Hoshika et al. 2014). It has furthermore been concluded that the sluggish behaviour of stomata under ozone exposure can lead to perturbations in the response to water deficit (Hoshika et al. 2014).

Stomatal closure during drought has been proposed as a protective measure against ozone exposure if both stresses are present simultaneously. However, this phenomenon has not been consistently observed. It has been shown that the interactive effect between ozone and drought is dependent on many factors, e.g. species (Wagg et al. 2012; Ribas et al. 2005; Biswas and Jiang 2011; Pell et al. 1993), sequence of appearance (Bohler et al. 2013; Le Thiec et al. 1994), severity (Le Thiec et al. 1994), time of day (Le Thiec et al. 1994), developmental stage (Alonso et al. 2001; Skärby et al. 1998) or season (Pell et al. 1993). Biswas and Jiang (2011) showed, for instance, that, under conditions of combined ozone and drought stress, the ozone-sensitive modern winter wheat cultivar (*Triticum aestivum* L. cv. Xiaoyan 22) improved its tolerance against ozone, while the ozone-tolerant primitive wheat (*Turgidum* ssp. *durum*) lost ozone tolerance. Le Thiec et al. (1994) and Bohler et al. (2013) hypothesized that the order of occurrence could play an important role in the combined effect. An early drought could lead to a decrease in stomatal conductance

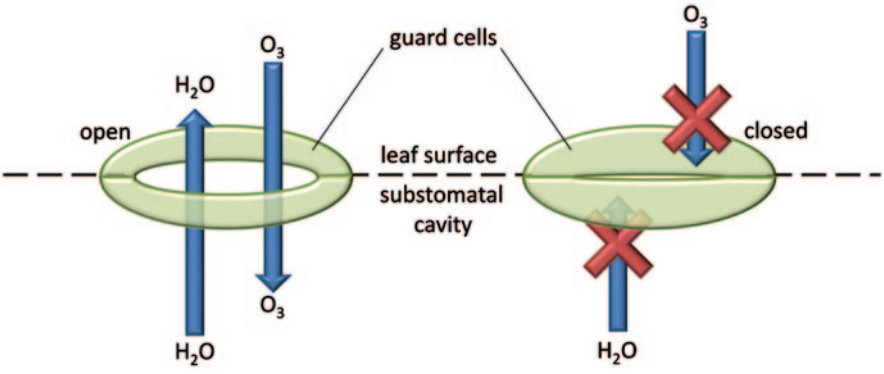

Fig. 7.2 Stomata forming a physical barrier against ozone absorption and water vapour loss

and a subsequent protection against a later ozone exposure, while the appearance of drought during pre-existing ozone stress would suffer under the appearing sluggishness of stomata, initially caused by ozone.

The sluggishness of stomata under ozone stress is most likely due to a perturbation of the abscisic acid (ABA)-induced stomatal regulation by ethylene (Fig. 7.3). Wilkinson et al. (Wilkinson and Davies 2009) showed that ozone-treated *Leontodon hispidus* present a reduced sensitivity to exogenously applied ABA and that stomata display a decreased response to a gradual drought. They furthermore measured an increase in ethylene production in ozone-exposed *L. hispidus*, while observing no change in ABA concentrations. Most importantly, it was determined that the application of 1-Methylcyclopropene (1-MCP), which prevents ethylene from binding to its receptors, restored the sensitivity of stomata to externally applied ABA and to soil drying (Wilkinson and Davies 2009). This shows that ozone-induced emission of ethylene is responsible for the sluggish behaviour of stomata, leading to increased effects of drought, rather than protective effects against ozone. Ethylene-mediated inhibition of ABA-induced stomatal closure was also shown by Tanaka et al., independently of ozone exposure (Tanaka et al. 2005).

7.6 Biomass Changes and Visible Symptoms

Estimations predict that ozone may cause up to 30% loss in biomass of crop plants, and up to 10% in forest species (Fuhrer 2009; Broadmeadow 1998). Drought may lead to yield loss as well, as was shown by a 30% decrease in plant productivity after the 2003 summer drought in Europe (Ciais et al. 2005). Decreases in biomass are indeed to be expected as a consequence of both ozone and drought exposure, since both phenomena may lead to a decrease in net photosynthetic rate (A) and thus in the net CO_2 fixation (Wittig et al. 2007; Biswas and Jiang 2011; Flexas et al. 2002). A decrease in biomass production can include reduced seed weight and number (Biswas and Jiang 2011; Flexas et al. 2002). Coinciding appearance of ozone and drought has been shown to have a cumulative effect on the decrease of seed biomass (Biswas and Jiang 2011).

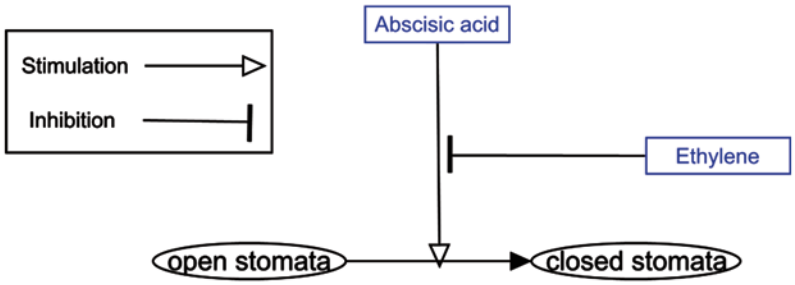

Fig. 7.3 Simplified representation of the interactive effects of ethylene and abscisic acid on stomatal closure, drawn in PathVisio (van Iersel et al. 2008)

Visible symptoms of ozone and drought are quite characteristic and can allow differentiation between both types of stress. Whereas ozone leads to the formation of necrotic patches and irregular chlorotic discoloration of leaves, drought rather induces a homogenous and gradual discoloration of leaves that in addition appear stunted and droopy (Bohler et al. 2013). It has furthermore been shown that, in poplar saplings, the combined effect of ozone and drought leads to an additive display of both symptoms (Bohler et al. 2013). In contrast, a field survey by Showman (1991) determined that in 1988 (a year with particularly high ozone levels in combination with drought), less ozone-related injuries were observed as compared to 1989 (a year with lower ozone concentrations and less drought). Besides, Matyssek et al. (2010) discussed that in 2003 (an exceptionally dry summer), the impact of ozone on beech trees at a test site in Kranzberg forest (Germany) was most likely reduced by drought, and the detrimental effects on radial and whole-stem volume increment were most likely due to the water deficit. These are further indications that protection manifested by drought may be very dependent on specific environmental conditions and that even if drought has a protective effect against ozone, the aftermath of drought itself may be equally or more detrimental than ozone.

7.7 Carbon Metabolism

Physiological measurements of ozone-exposed plants have shown that net photosynthetic rate, maximum rate of RuBisCO-mediated carboxylation and carboxylation efficiency can be decreased (Biswas and Jiang 2011). This can partly be attributed to a decrease in stomatal conductance, but there are indubitably further reasons for these effects. It has been clearly shown on multiple occasions, and for many species, that the enzyme RuBisCO is affected by ozone stress. Studies have shown reduced enzyme activity and abundance of RuBisCO subunits, but also of RuBisCO activase (Bohler et al. 2007, 2010, 2013; Pelloux et al. 2001; Brendley and Pell 1998). It is likely that the enzyme itself is damaged during ozone exposure, as illustrated by the increase in abundance of a degradation fragment of RuBisCO in poplar (Bohler et al. 2013). In addition, a number of enzymes of the Calvin cycle have been shown to be decreasing in abundance in response to ozone (Bohler et al. 2007, 2010, 2013). The fact that these are mostly redox-regulated enzymes (RuBisCO activase, fructose-1,6-bisphosphatase, sedoheptulose-1,7-bisphosphatase and ribulose-5-phosphokinase) is understandable in the context of oxidative stress, since the Calvin cycle is activated during the day by the reduction of key enzymes. In an oxidative environment, these enzymes can become inhibited, which might also be the cause for lower abundances.

During periods of water deficiency, various observations have been made for RuBisCO. Parry et al. suggested that the reduction in activity of RuBisCO in droughted tobacco was due to increases in the inhibition of the enzyme (Parry et al. 2002), while Pelloux et al. (2001) determined that the abundance of both RuBisCO and RuBisCO activase transcripts and proteins in Aleppo pine hardly changed during drought. Nevertheless, Sergeant et al. detected increases in the abundance of the

previously discussed fragment of RuBisCO in droughted oak (Sergeant et al. 2011). Similar results were found in the combined stress experiment from Bohler et al., although it was shown that the increase in fragment abundance was less strong for drought than for ozone (Bohler et al. 2013).

Not many publications discuss the primary carbon metabolism of plants exposed to both ozone and drought. Pelloux et al. detected that RuBisCO and RuBisCO activase abundance were not changed during double stress (Pelloux et al. 2001). Bohler et al. (2013) reported that in poplar, a very similar set of proteins were differentially abundant in combined stress compared to ozone alone, but that the differences were less severe. This clearly shows the existence of an interactive effect between both stresses, but not necessarily a protective effect, since visual and morphological symptoms were reported to be either similar or cumulative.

On the level of the chloroplast electron transport chain, many subunits of photosystems and ATPase have been reported to be significantly lower in abundance after ozone stress (Bohler et al. 2007, 2011) while drought appears to have an effect only on ATPase (Flexas et al. 2002; Tezara et al. 1999). Furthermore, ozone appears to induce an early increase in ferredoxin–$NADP^+$–oxidoreductase, indicating a need for reducing power that is consistent with the appearance of oxidative stress (Bohler et al. 2013). Nevertheless, this is only transient, since after longer exposure to ozone levels of ferredoxin–$NADP^+$–oxidoreductase decrease, possibly due to an overwhelmed system. The consistent reduction in photosystem subunits is most likely caused by the accumulation of ATP and NADPH in the chloroplast, in consequence of the decrease in Calvin cycle activity. These observations have not been made in drought; and according to the results of Bohler et al. (2013), the combined stress causes a very similar response, albeit to a lesser extent.

7.8 Antioxidant Metabolism

One of the main differences between ozone and drought is the induction of oxidative stress, which is predominant during ozone exposure but less characteristic of drought. Whereas ozone itself fragments into ROS and leads to a strong accumulation (Langebartels et al. 2002; Pellinen 1999), drought response mostly uses ROS as internally produced signalling molecules (Yao et al. 2013), although severe drought may lead to photo-oxidative stress as well (Foyer and Noctor 2000). Consequently, accumulation of ROS is likely to be considerably higher during ozone stress, and more closely located to chloroplasts in drought. Experiments show an increase in activity and/or abundance of antioxidant enzymes like peroxidases, catalases and superoxide dismutases in plants exposed to ozone (Alonso et al. 2001) and of glutathione reductase and superoxide dismutase during drought (Alonso et al. 2001; Huseynova et al. 2014). Alonso et al. (2001) detected decreases in antioxidant enzyme activities in the combined stress compared to ozone or drought separately, deducing that the cumulative effects of both stressors may overwhelm defence systems. Similar observations were made by Wellburn et al. (1996). Among antioxidant molecules, ascorbate is particularly important during ozone response. The

apoplast is the first location of ozone attack, and consequently apoplastic ascorbate and ascorbate peroxidase are the primary defence against ozone (Sanmartin et al. 2003; Luwe et al. 1993). In drought, cytosolic and chloroplastic ascorbate-dependent detoxification is of more importance, but appears to be dependent on species (Mittler and Zilinskas 1994; Zhang and Kirkham 1996). Nevertheless, Kronfuß et al. showed that in Norway spruce, total needle ascorbate was increased significantly by ozone, while apoplastic ascorbate was increased significantly by drought and a combined exposure led to a significant increase in both. Combined stress may therefore increase the reduction potential and improve protection against oxidative stress (Kronfuß et al. 1998). Reduction potential is considerably dependent on plant species, and it has been proposed that resistance to ozone is associated with both ozone flux and reduction potential (Dizengremel et al. 2008). Similarly, the interactive effect of ozone and drought may differ, depending on how much antioxidant molecules and enzymes are induced by either of the stresses.

7.9 Conclusions

Since tropospheric ozone accumulation and soil drying are caused by similar meteorological conditions, both situations are likely to emerge in parallel in nature. Even though the combination of two stress conditions very often causes cumulative effects, it was proposed that the stomatal closure induced by drought may be able to protect plants against the influx, and hence the detrimental effects of ozone. However, as is commonly the case, observations do not consistently corroborate these expectations. It appears that the response caused by the combination of both stresses is determined by many environmental and phenotypical factors.

One of the main relevant factors appears to be the sequence of events. The primary appearance of ozone is likely to cause disturbances in the reactivity of stomata. A subsequent drought will cause delayed and limited stomatal closure, allowing continuous entry of ozone into the plant. Drought preceding ozone, on the other hand, will cause stomatal closure early in the sequence and cause a natural barrier against ozone absorption. The individual and combinatorial consequences of ozone and drought exposure can be affected by a number of additional factors such as species, ozone flux and antioxidant capacity, sensitivity to ozone and drought, time of day and vegetative season.

Only few studies have investigated the effects of combined ozone and drought exposure on plant metabolism (Bohler et al. 2013; Pelloux et al. 2001). Neither of them (Bohler et al. 2013; Pelloux et al. 2001) identified any major synergistic or antagonistic effects. In addition, the use of high throughput molecular approaches is quite rare for this topic. This is regrettable, since high throughput techniques like transcriptomics, proteomics and metabolomics present some considerable advantages versus targeted experiments. Where specific studies rely on prior knowledge and a clearly stated hypothesis, high throughput techniques approach a subject without any prior bias. This approach may easily lead to new discoveries that were previously unpredicted and therefore unconfirmed. In addition to the wealth

of information that can be obtained, bioinformatic methods exist that can analyse, represent and combine high-throughput measurements to an extent where the interpretation becomes highly intuitive.

In nature, plants are often exposed to multiple constraints, but often research is carried out on a single stressor. This is unavoidable for understanding the response of plants to any particular stress, but the results apply neither to natural conditions nor to expectations. Therefore, the study of combinations of constraints that naturally co-occur is of major importance, as is the use of new technologies, to unravel the response of plants against environmental stresses, so that crops and forests can be protected and maintained for future generations.

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Chapter 8
Effect of High Temperature and Water Stress on Groundnuts Under Field Conditions

Vijaya Gopal Kakani, Timothy R. Wheeler, Peter Q. Craufurd and Rao C. N. Rachaputi

8.1 Introduction

In the semiarid environments of the world, which contribute to 90% of global groundnut production, high temperature and water stress often occur together (Nix 1975; Kramer 1980). The effects of drought under field situations are well established in groundnut (e.g. Williams et al. 1986; Nageswara Rao et al. 1988; Chapman et al. 1993a). Reports of the effects of increased temperature, both air and soil, in groundnut fields are available in the literature (e.g. Williams et al. 1975b; Sivakumar et al. 1993). However, high-temperature studies on groundnut growth and development are confined to controlled environment conditions (e.g. Wheeler et al. 1997; Prasad et al. 1999a, 1999b, 2000; Craufurd et al. 2003).

High-temperature studies conducted on groundnut by Prasad et al. (1998, 1999a, 1999b, 2000) use a high-temperature treatment for a period of 12 h, with temperature changing as a square wave. Such uniform temperature fluctuations do not occur in natural environments. Temperatures under field conditions follow a more sinusoidal pattern, reaching the peak during the afternoons (Fig. 8.1). To confirm the findings of studies of high-temperature effects on groundnuts conducted in a controlled environment, field studies in natural, hot environments are essential. Studies evaluating the effects of both drought and high temperature in groundnut

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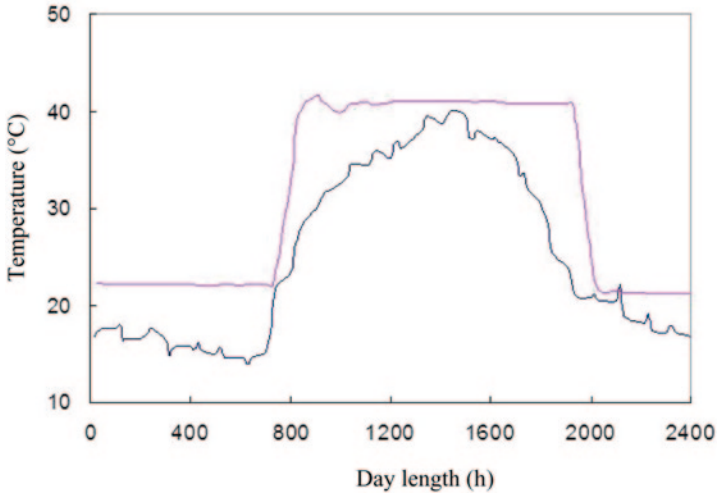

Fig. 8.1 Diurnal temperature cycle under natural (−) hot environment at ICRISAT, India, and controlled (−) high temperature treatment (40/22 °C—day/night with 12 h photo-thermoperiod)

have not been conducted so far under field conditions. Such studies under controlled environment did not result in any definite conclusions (Craufurd et al. 1999).

This study evaluated the effects of high-temperature stress on groundnut yield, and its interaction with drought, under field conditions. The objectives of this study are: (1) to investigate the effects of water stress and high temperature on growth, development and yield of groundnut grown in the semiarid tropics; (2) to test the possible interaction between water stress and high temperature observed under controlled environment, on yield and yield components under field conditions.

8.2 Materials and Methods

8.2.1 Location, Weather and Soil

An experiment to study the interaction between high temperature and water stress was conducted at two sowing dates, at the International Crops Research Institute for Semiarid Tropics (ICRISAT), Patancheru, Hyderabad, India. The ICRISAT is located in semiarid tropics at an altitude of 545 m above sea level (ASL), 17°32’ N latitude, 78°16’ E longitude.

A mini weather station (Fig. 8.2a) was set up to record daily values of temperature and incident solar radiation. Air and soil temperatures were measured using copper–constantan thermocouples. Air temperatures were measured at canopy level and soil temperatures at a 10-cm depth (i.e. in the podding zone; Fig. 8.2b). Solar radiation

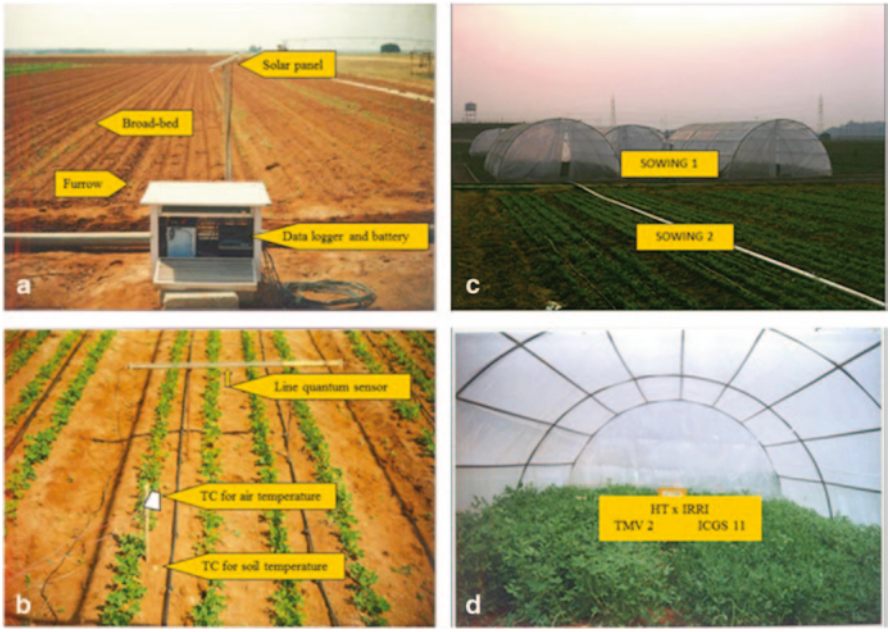

Fig. 8.2 Pictures showing **a** broad bed and furrow system with mini—weather station; **b** Line quantum sensor and thermocouples (TC) for measuring air (inside the cup) and soil temperature (10 cm below soil surface), **c** Layout of heat tunnels in the field, and **d** Inside of the high temperature × irrigation treatment heat tunnel

received above the crop canopy was measured in each treatment using line quantum sensors (LI-191SB, LI-COR Ltd). Measurements were logged at 10-s intervals and averaged every 15 min throughout the crop-growth period. Daily weather was also collected from a meteorological observatory located within 500 m of the experimental site.

The soil at the experimental site was a reddish-brown alfisol, a member of isohyperthermic family of Udic Rhodustalf. Soil pH was 6.5. Depth of soil in the site was 120 cm. The soil moisture was 20 % w/w at field capacity and 8 % w/w at permanent wilting point. These soils are well drained with moderate permeability.

8.2.2 Field Preparation and Experimental Design

The field site was ploughed to a depth of 30 cm with mouldboard and disc ploughs 15 days before sowing. The ploughed field was then laid into broad beds (1.2-m wide) and furrows (0.3-m wide), in an East–West direction (Fig. 8.2a). The beds were then levelled and compacted. Four furrows at 30-cm spacing and 5-cm depth were then opened on the bed surface along the length of the bed. The whole area was then divided into two halves, one for each sowing. Each sowing composed ten beds of

60 m length. The main irrigation treatments had a bulk bed in between to restrict water seepage between treatments.

The experiment was planned at two sowing dates to ensure that the crop was exposed to high temperature during the sensitive period of flowering. Sowing 1 was on 21st January and sowing 2 was on 26th February. The experimental design was split–split plot with two irrigation regimes as main plots—irrigated (IR) and fully irrigated—replacing 100% of crop evapotranspiration (ET_C) and water-stress (WS) irrigation with only 40% of ET_C from flowering to harvest otherwise fully irrigated; temperature treatments as subplots—ambient temperature (T1), high-temperature sowing 1 (T2), ambient temperature sowing 2 (T3) and high-temperature sowing 2 (T4); and genotypes TMV2 and ICGS11 as sub–subplot.

8.2.3 Cultivars and Sowing

TMV 2: This cultivar was released in 1940. TMV 2 is a Spanish botanical type, a selection from 'Gudhiatham Bunch' and a local variety. It is widely adapted, well suited for rainy and summer season cultivation in southern India. This cultivar is moderately tolerant to water stress and high temperature.

ICGS 11/ICGV 87213: This cultivar was released in 1986. This cultivar is a Spanish botanical type, selection from natural hybrid population of Robut 33-1. It has above-average tolerance to end-of-season drought. It is also photoperiod insensitive, adapted to post-rainy season cultivation in India and performs well in West Africa.

Prior to sowing, seeds were treated with fungicide mixture, Thiram + Captan (3:1). Seeds of cultivars TMV 2 and ICGS 11 were sown manually, 5 cm deep and 10 cm apart in furrows made at 30 cm spacing on broad beds. An iron chain with tags at 10 cm spacing was used to ensure that each plot received the required number of plants. Soon after emergence, gaps were filled for ungerminated seeds. Appropriate weed, pest and disease control measures were taken to maintain a healthy crop stand.

8.2.4 Stress Treatments

8.2.4.1 Irrigation

Immediately after sowing, all plots were irrigated using an overhead sprinkler system. A second sprinkler irrigation was given after 7 days to help seedlings emerge. A drip irrigation system was then installed to provide adequate irrigation to the growing seedlings. The drip irrigation system is shown in Fig. 8.2b and c. The drip emitters were calibrated so that each supplied 10 L h^{-1} of water to crop plants. This ensured that all plants in the plot were supplied with equal amount of water. Plots were irrigated at 3-day intervals. Fully irrigated plots were replaced with water equal to that lost through evaporation. Water stress (WS) plots were irrigated with

40 % of that given to fully irrigated plots, from anthesis to harvest. The amount of water supplied to an irrigated plot (L) was calculated using

$$L = \text{Plot area} \times ET_C \tag{8.1}$$

$$ET_C = \text{Evaporation} \times K_c, \tag{8.2}$$

where K_c is the crop coefficient with a value of 0.8 for groundnut (Doorenbos and Pruitt 1992) crop during the reproductive development period. The evaporation data were obtained from the weather station at ICRISAT, which is given as:

$$\text{Evaporation} = \text{Open} - \text{pan evaporation} \times \text{Kpan} \tag{8.3}$$

Open-pan evaporation was obtained from United States Department of Agriculture (USDA) class A type pan and Kpan with a value of 0.7 as the pan coefficient. Water use efficiency (WUE) was calculated as the ratio of above-ground biomass dry weight (including pod weight) to the amount of water supplied.

8.2.4.2 Temperature Treatments

Plants were exposed to high temperatures by covering them with plastic tunnels supported by an iron frame, referred to from now on as ‘heat tunnel’. Plants in the high-temperature treatment were covered with heat tunnel from flowering to 20 days after flowering (DAF), the most sensitive period for temperature stress (Prasad et al. 1999a). Temperature inside the heat tunnel was controlled so as not to exceed 42–43 °C by opening and closing the flaps of the heat tunnel. This also ensured that humidity did not build up in the heat tunnel. The polythene sheet (400 μ thick) used allowed 80 % transmittance of light for plants in the heat tunnel, and the surface was cleaned regularly for any settled dust to maintain transmittance levels.

8.2.5 Crop Development

The time of the key reproductive stages (R1, R2, R3 and R8; Boote 1982) were recorded in each plot. Observations were made daily on ten plants per plot. The crop was considered to have reached a particular reproductive stage when 50 % or more of the plants were at that stage of development.

8.2.6 Growth Analysis

Sampling of plants was done once in the vegetative stage, before flowering, and at weekly intervals after imposition of water and temperature stress treatments. An area of 0.6 m^2 (0.5 × 1.2 m) from each plot was sampled at each harvest. A

subsample of five plants was tagged at flowering in each of the harvest areas. Daily flower production was recorded on these plants from flower appearance for a period of 30 days. These plants were also used to determine leaf area and partitioning of dry matter to leaves, stems, and pods. Observations were also made on plant height, node and leaf number, peg and pod number on plants of the subsample. To determine dry weights, plant components of the subsample and the remaining part of the large sample was oven dried at 80 °C for 3–4 days and weighed. Total dry matter and pod yields were recorded at harvest maturity in all replications of the experiment using an area of 4 × 1.2 m.

8.2.7 Statistical Analysis

All the data were analyzed using an analysis of variance procedure (ANOVA) for split–split plot design in Genstat 5 (Genstat 5 Committee, 1997). All percentage values were angular transformed before analysis to ensure homogeneity of variances. Pod dry weight values were multiplied by a factor of 1.65 to account for energy spent to synthesize oil in the seed (Duncan et al. 1978). Statistical significance was tested by applying F-test at <0.05, <0.01 and <0.001 level of probability, represented by *, ** and ***, respectively.

8.3 Results

8.3.1 Weather (Temperature and Relative Humidity)

A range of temperatures was imposed during flowering by using two sowing dates combined with heat tunnels. As photoperiod did not vary much at the experimental site (mean 12 h ± 45 min), and the genotypes used were insensitive to photoperiod, results are described in terms of differences in mean temperature between treatments, rather than by sowing dates. Daily maximum and minimum temperatures recorded during the crop period in all the four treatments are presented in Fig. 8.3. Temperatures to which different development phases were exposed to in each of the temperature treatments are given in Table 8.1.

A combination of sowing dates and heat tunnels gave mean temperatures from sowing to maturity of 26.3° (T1), 27.3° (T2), 29.0°(T3) and 29.7 °C (T4). The heat tunnels were capable of raising day temperature by >10 °C compared to ambient (Fig. 8.3). During the 20-day high-temperature treatment at flowering, mean temperatures were 33.8° (T1), 41.6° (T2), 38.7° (T3) and 43.5 °C (T4). Increase in soil temperature was also observed with increase in air temperature (Table 8.1). Temperature of the soil was highest in the T4 treatment where air temperature was highest.

Average daily relative humidity (RH) in the ambient treatments T1 (sowing 1) and T3 (sowing 2) was 48.4 % (SE ± 0.95) and 44.3 (SE ± 0.98), respectively (Fig. 8.3).

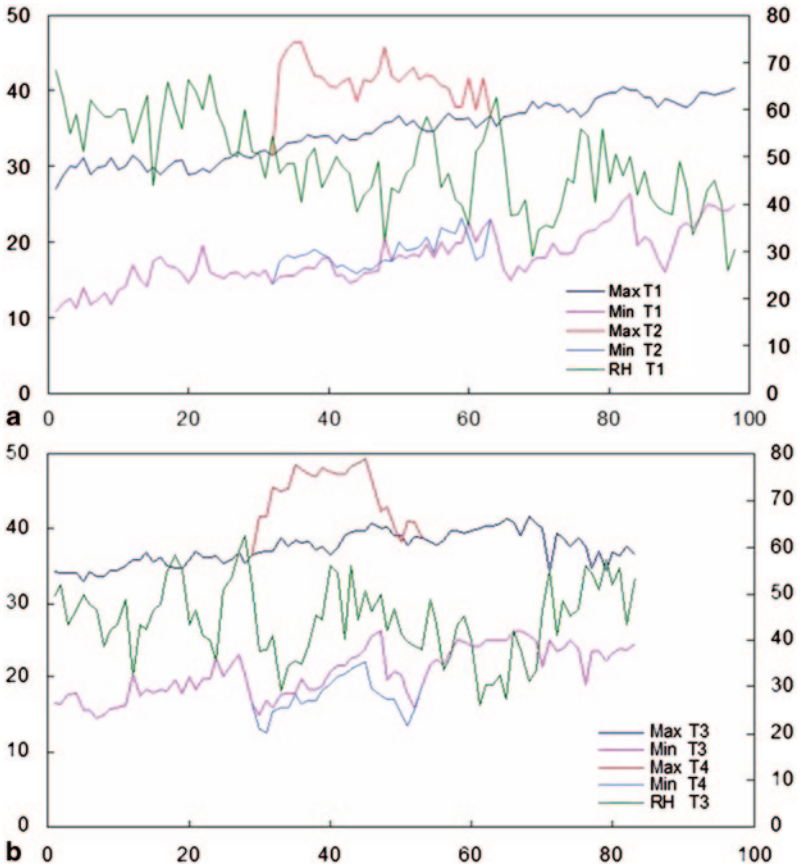

Fig. 8.3 Daily maximum and minimum air temperatures recorded under ambient- and high-temperature conditions and relative humidity in **a** early and **b** late sown groundnut crop

The calculated vapour pressure deficit (VPD) values were 1.82 and 2.26 kPa in T1 and T3, respectively. It was not possible to record RH in the T2 heat tunnel due to lack of instruments and therefore VPD could not be estimated in T2. The RH level in T4 during the 20-day period of high temperature averaged to 57% (SE ± 1.12), slightly above that of the ambient T3 treatment. VPD was therefore slightly lower in T4, 2.06 kPa than in T3 (2.26 kPa).

Due to lower ambient temperature in T1 (sowing 1), heat tunnels for T2 were kept closed during the greater part of the day to achieve the target temperature of >40 °C. This led to a buildup of humidity in the heat tunnel near to saturation, which must have reduced the VPD. A better control of humidity was achieved in the T4 heat tunnel treatment (sowing 2), keeping the heat tunnel open to reduce the maximum temperature which at times was >48 °C. These very high temperatures were achieved because ambient temperatures were much higher at the second sowing (>38 °C).

Table 8.1 Average maximum (Max), minimum (Min) and mean air temperatures (°C), soil temperatures (°C) and relative humidity (%) recorded during different developmental stages of groundnut in the four temperature treatments to which the crop was exposed in the field

Developmental Stage	Treatments											
	T1			T2			T3			T4		
	Max	Min	Mean	Max	Min	Mean	Max	Min	Mean	Max	Min	Mean
	Max	Min	Mean	Max	Min	Mean	Max	Min	Mean	Max	Min	Mean
Air temperature (°C)												
Sowing–R1	30.2	14.8	22.5	30.7	15.2	23.0	35.2	18.1	26.7	35.3	18.1	26.7
R1–R3[a]	33.9	16.5	25.2	41.6	17.4	29.5	38.7	20.3	29.5	43.5	18.05	30.8
R3–R8	37.8	20.4	29.1	38.6	20.3	29.5	38.6	23.7	31.2	38.6	23.7	31.2
Soil temperature (°C)												
Sowing–R8	25.8	25.3	25.5	29.4	26.2	27.8	25.9	25.2	25.5	32.3	25.2	30.8
Relative humidity (%)												
Sowing to R1	87.5	30.0	58.7	87.5	30.0	58.7	71.3	22.8	47.0	71.3	22.8	47.0
R1–R3[a]	73.3	22.2	47.7	NA	NA	NA	64.4	22.3	43.3	70.3	44.2	57.1
R3–R8	64.3	23.4	43.8	64.3	23.4	43.8	58.9	26.2	42.5	58.9	26.2	42.5

Developmental stages: R1 = Beginning flower; R3 = Beginning pod; R8 = Harvest maturity
NA not available
[a] High temperature period

8.3.2 Analysis of Variance

The ANOVA table (Table 8.2) for 2 × 3 × 2 (WS × Temp × Geno) split–split plot analysis with three replications at final harvest shows the main effects and interactions between the treatments. No significant interaction could be recorded at final harvest for temperature and water stress treatments. However, a significant interaction for water stress and temperature was recorded for only peg and pod number in the harvest made immediately after imposing high-temperature treatments (i.e. at 54 DAS). Otherwise, only main effects of temperature and water stress, and their interaction with genotypes, could be observed in the various harvests made for growth analysis in the study. Hence, results recorded only at final harvest are presented.

8.3.3 Water Use and Water Use Efficiency

The cumulative amount of water supplied to IR (100% of ET_C) and water stress (40% of ET_C) treatments is presented in Table 8.3. No monitoring was possible of evaporation in the high-temperature treatments T2 (sowing1) and T4 (sowing 2). Hence, similar amounts were supplied to ambient- (T1 and T3) and high-tempera-

Table 8.2 Analysis of variance with mean square and treatment significance for growth and development parameters recorded at final harvest

Source	Df	VWT	BM	PWT	FLN	PGN	PDN	HI	WUE
Replicate	2	6269	10997	986	173	85	5	0.0007	0.03
WS	1	30514*	94008*	17403	108*	356	156*	0.0114	0.05
Residual	2	891	3803	1259	3	114	5	0.0024	0.008
Temp	2	3768	20915*	16570**	180	375*	3	0.0640***	0.305*
WS × Temp	2	5276	670	2553	103	214	29	0.0139	0.044
Residual	8	2176	4516	1179	69	86	18	0.0032	0.019
Geno	1	681	21776**	14754***	2342***	3589***	668***	0.0648***	0.070*
WS × Geno	1	657	5487	2344**	289*	23	84*	0.0022*	0.007
Temp × Geno	2	564	1572	3448***	181*	1171***	175**	0.0225***	0.002
WS × Temp × Geno	2	1088	2953	457	17	222	5	0.0001	0.0097
Residual	12	833	1554	218	38	93	17	0.0004	0.0094

All weights are g m^{-2} and all numbers are per m^2

df degrees of freedom, *BM* biomass, *FLN* flower number, *PGN* peg number, *PDN* pod number, *PWT* pod weight, *VWT* vegetative weight, *WUE* water use efficiency

*; **; *** indicate significance at 0.05, 0.01 and 0.001 levels of probability, respectively

Table 8.3 Cumulative amounts of irrigation (mm) supplied to irrigated (IR—100% of ET_C) and water stress (WS—40% of ET_C) plots during different stages of development

Development stage	TI and T2 IR WS		T3 and T4 IR WS	
Sowing–R1	121	121	204	204
R1–R3	98	43	201	89
R3–R8	355	183	234	91

Table 8.4 Effect of temperature treatments on WUE (g L^{-1}) and VPD (kPa) and normalized WUE (WUE × VPD (g kPa L^{-1})

Temperature treatments	WUE	VPD	WUE normalized for VPD
T1	0.88	1.82	1.6
T2	1.21	NA	NA
T3	0.58	2.26	1.3
T4	0.64	2.06	1.3
SED	0.055***		

*** indicates significance at 0.001 level of probability

ture (T2 and T4) treatments irrespective of irrigation treatment. Amount of irrigation given was higher in T3 and T4 treatments due to greater ET demand associated with the increase in ambient temperature in the second sowing.

Sowing date and temperature treatments significantly affected WUE (Table 8.4). At ambient temperature, WUE was higher in T1 (sowing 1) than T3 (sowing 2), and this was associated in part with a lower VPD. The highest WUE, 1.21 g m^{-2} L^{-1}, was recorded in T2, and both high-temperature treatments, T2 and T4, increased WUE compared to their respective ambient controls.

WUE was strongly affected by VPD, which was lower at sowing 1 (T1) than sowing 2 (T3). The normalized values of WUE for T1 and T3 were 1.6 and 1.3 g kPa L^{-1}, respectively. The higher WUE at sowing 1 was probably due to cooler mean temperatures (Table 8.1). The higher WUE in T4 compared to T3 is accounted for by the lower VPD in T4, which in turn is due to the high RH in the heat tunnel. Although RH was not measured in T2, RH was very high in the heat tunnel, and the high WUE in T2 is undoubtedly due to a higher RH. Accordingly, T2 has been excluded from further analysis.

No interaction between these factors could be recorded for WUE. Water stress treatments did not influence WUE. Main effects of temperature and cultivar were significantly affected by WUE. Genotype ICGS 11 recorded significantly ($p < 0.01$) higher WUE of 0.74 g m^{-2} L^{-1} compared to 0.65 g m^{-2} L^{-1} in TMV 2.

8.3.4 Effects of Temperature × Water Stress Interactions

Table 8.5 shows the interaction effects for temperature and water stress treatments. The effects of temperature and water stress interaction were apparent only in the

Table 8.5 Effects of temperature (mean of 20-day high temperature) and water stress treatments on peg and pod number (plant^{-1}) recorded in the harvest made immediately after the withdrawal of high-temperature treatments

Water stress treatments	Temperature treatments	
	T3 (29 °C)	T4 (31 °C)
Peg number		
Irri	15.81	7.96
WS	6.60	8.09
SED	2.35*	
Pod number		
Irri	3.72	1.74
WS	1.03	2.84
SED	0.76*	

Irri irrigated, *WS* water stress
* indicates significance at 0.05 level of probability

harvests made immediately after ending the 20-day high-temperature treatment. This interaction disappeared as the crop reached maturity. The interaction was significant (<0.05) between T3 and T4 for both peg and pod number at 54 DAS. High temperature imposed in the irrigated treatment (IR) decreased the peg (50%) or pod (54%) number. Water stress (WS) treatmentals reduced peg (68%) and pod (72%) number under ambient temperature conditions. However, a combination of high temperature (T4) and water stress (WS) increased peg, and in particular pod, number relative to WS or T4. In general, water stress effects were more severe than high-temperature effects.

8.3.5 Effects of Water Stress and Its Interaction with Genotypes

Water stress treatments significantly ($p<0.05$) altered the specific leaf area (SLA) of the plants. There was no water stress x sowing interaction for SLA values, but within a sowing there were differences between water stress treatments for SLA. The SLA values recorded in irrigated conditions was lower (180.3 cm^{-2} g) in sowing 2 compared to the values in sowing 1 (192.2 cm^{-2} g). Treatment WS (40% ET_C) increased the SLA in sowing 1 (201.2 cm^{-2} g), while it decreased the SLA in sowing 2 (163.8 cm^{-2} g).

Seasonal time course of biomass and pod weight in T1 is shown in Fig. 8.4. There was no immediate effect on biomass or pod weights of the 20-day high-temperature period. However, water stress treatment decreased biomass and pod weight throughout the stress period.

The main effects of water stress were recorded only for biomass due to significant ($p<0.05$) reduction in vegetative and pod weight. Vegetative (283.9 g m^{-2}) and pod weight (120.2 g m^{-2}) in irrigated treatments (100% ET_C) were reduced by 20 and 37%, respectively, due to water stress treatment (40% ET_C).

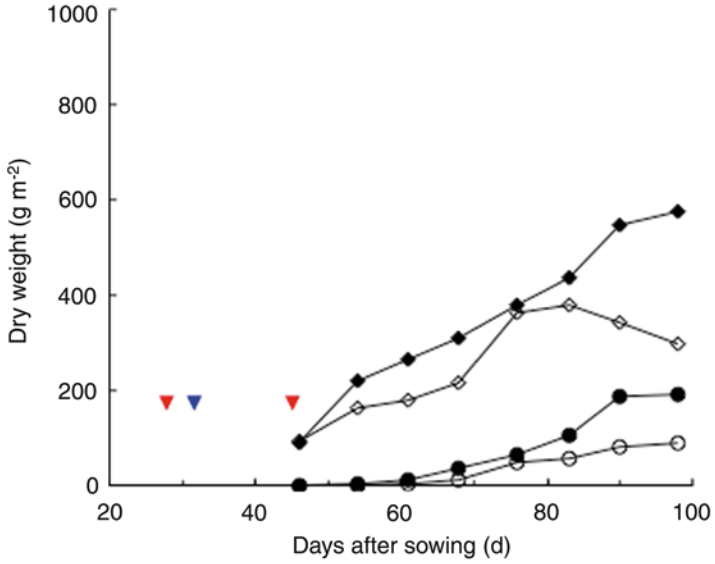

Fig. 8.4 Seasonal time course of biomass (*diamond*) and pod weight (*circle*) recorded in water stress treatments, Irri (100% ET_C—closed) and WS (40% ET_C—open) in T1 treatment; *red inverted triangle* indicates start and end of high-temperature treatment, while *blue inverted triangle* indicates start of water stress (WS—40% ET_C) treatment

Cultivars differed in their response to water stress treatments (Table 8.6). The interactions persisted until the final harvest. Cultivar ICGS 11 recorded significantly ($p<0.05$) higher values for flower number (40%), pod number (50%), pod yield (37%) and harvest index (HI; 31%), than TMV 2 under irrigated conditions (100% ET_C). When the genotypes were supplied with 40% ET_C, the differences for tolerance to water stress were clear between the genotypes. Flower number, biomass, pod yield and HI decreased by 14, 31, 42 and 14% in ICGS 11 and by 0, 23, 28 and 4% in TMV 2, respectively, compared to those obtained in the irrigated treatment. There was no effect of water stress treatments or its interaction with genotypes on peg and pod number and pod set.

8.3.6 Effects of Temperature and Its Interaction with Genotypes

Main effects of temperature were significant for biomass (Fig. 8.4). High temperature decreased biomass in T3 and T4 by 21 and 12%, respectively, compared to T1. The smaller decrease in biomass in T4 compared to T3 can be attributed to lower VPD in T4. Similar trend was also recorded for vegetative weight (data not presented).

The interaction of temperature treatments with water stress disappeared with advance in crop age, but temperature interactions with cultivar persisted until the

Table 8.6 Interaction between genotype and water stress treatments for flower number (plant^{-1}) at 30 DAA, pod number (plant^{-1}), pod yield (g m^{-2}) and harvest index as observed at final harvest

Water stress	Cultivar	
	TMV 2	ICGS 11
Flower number		
Irri	32	53
WS	34	44
SED	2.1*	
Pod number		
Irri	12	24
WS	11	17
SED	1.5*	
Pod yield		
Irri	91.9	148.5
WS	66.1	88.4
SED	6.4**	
Harvest index		
Irri	0.23	0.33
WS	0.21	0.28
SED	0.017*	

IR irrigated, *WS* water stress, *SLA* specific leaf area.
*, ** indicate significance at 0.05 and 0.01 levels of probability, respectively

final harvest. A temperature x cultivar interaction was recorded for flower number, pod number, pod yield and HI (Table 8.7).

Of the two cultivars, ICGS 11 was more tolerant to high temperature. In both cultivars, a decrease in pod yield and HI was recorded under high-temperature treatments, but the decrease was significantly less in ICGS 11 compared to the decrease in TMV 2. Cultivar ICGS 11 maintained a high pod yield and high HI under high-temperature treatments (T3 and T4). On the other hand, a severe decrease in pod yield and HI were recorded in TMV 2. The higher pod yield and HI in ICGS 11 can be attributed to greater flower fruit-set (i.e. ratio of pod to flower number) and pod number. In contrast, in TMV 2, reduction in flower number and fruit set was recorded, and so pod number was decreased on exposure to high temperature.

8.4 Discussion

Studies to identify temperature × water stress interactions (Craufurd et al. 1999) or to screen genotypes for heat tolerance have been conducted mainly in controlled environments (Prasad et al. 1999a, 1999b, 2000; Wheeler et al. 1997). Under these conditions, the temperature increase follows a square-wave pattern (Fig. 8.1).

Table 8.7 Interaction between genotype and temperature treatments for flower number (plant^{-1}) at 30 DAA, pod number (plant^{-1}), pod yield (g m^{-2}) and harvest index as observed at final harvest

Cultivar	Mean temperature treatments (°C)			SED
	T1(27)	T3 (29)	T4 (30)	
Flower number				
TMV 2	35	35	28	4.2*
ICGS 11	42	55	50	
Pod number				
TMV 2	16	10	10	2.4**
ICGS 11	15	24	22	
Pod yield				
TMV 2	140.0	51.0	42.8	15.26***
ICGS 11	142.2	103.8	109.4	
Harvest index				
TMV 2	0.36	0.18	0.14	0.024***
ICGS 11	0.34	0.29	0.28	

TMV 2 Spanish botanical type, a selection from 'Gudhiatham Bunch' and a local variety
*, **, *** indicate significance at 0.05, 0.01 and 0.001 levels of probability, respectively
SED Standard Error of Difference of Means

Hence, an interaction between temperature and water stress occurs on plant growth during the entire 12 h of photo-thermo period, providing a longer period for the interaction to influence the growth and development of the crop plant under study. However, under field conditions, increase in day temperature follows a more or less sinusoidal pattern (Fig. 8.1), and high air temperature effects on plant in field occur for a short duration of only 3–4 h. Furthermore, the temperature of plant canopy can be higher than that in controlled environment under similar air temperatures due to associated radiative heating in semiarid tropic (SAT) regions (Guilioni et al. 2000). Hence, the interaction between the stress events that occur under controlled environment might be different from those occurring in the field. If true, this would have important implications for using controlled environment facilities for screening for water and temperature stress.

Temperature increase across the treatments, T1 to T4, (Table 8.1) was achieved by using plastic heat tunnels in the field. Humidity was controlled in these heat tunnels by opening the heat tunnel doors for brief periods during the day; nonetheless, an increase of humidity in these heat tunnels did occur, particularly at sowing 1 (i.e. T2). The normalized WUE values observed in this study were less (1.6–1.3 g kPa L^{-1}) than recorded by other researchers (e.g. 3.5 g kPa L^{-1} by Ong et al. 1987; 1.9 g kPa L^{-1} by Mathews et al. 1988) due to the higher temperatures to which the crop was exposed. This experiment was conducted during the hot summer season of India and warmer temperatures would have caused an increase in evaporation with less water available for transpiration by the plants. Water stress inhibits leaf expansion and stem elongation through a reduction of relative turgidity (Slatyer 1955; Allen et al. 1976; Vivekanandan and Gunasena 1976), thus altering both leaf

and stem morphology as observed in this study thus causing a reduction in growth, resulting in lower WUE.

Increase in WUE of the plants in T2 and T4 indicates that less water was lost from the soil through evaporation due to higher humidity in the heat tunnels. Lee et al. (1972) recorded that increase in humidity from 50 to 95 % increased the flower number, peg number and vegetative weight. Similar observations were made in this study, notably in T2 where the RH was near saturation compared with 48 % under ambient conditions. The use of heat tunnels resulted in clear temperature differences across treatments. These heat tunnels can thus be used in the field to screen groundnut genotypes for high temperature tolerance, as humidity control can be achieved with experience in using the heat tunnels (T2 vs. T4).

The effects of temperature and water stress on various components of groundnut as recorded at final harvest are shown in the flow diagram (Fig. 8.5). The field study also confirms the observations made under controlled environment studies (Kakani 2001) that the interaction for temperature and moisture stress is transient and disappears with release of a stress treatment. The interaction between temperature and water stress treatments was recorded in the harvests made immediately after the withdrawal of high-temperature treatment (T4).

The interaction between water and temperature stress was significant only for peg and pod number. This interaction is due to the sensitivity of the reproductive processes such as pollen germination and fertilization to high temperature. In a controlled environment with a maximum temperature of 37 °C for 10 days, a decrease in pod number of 43 % was recorded at 50 DAS (Kakani 2001). On the other hand, in field, a temperature of 43.5 °C was imposed for 20 days that caused a reduction of only 46 % in pod numbers. This lesser decrease in pod number can be attributed to the greater tolerance to high temperature of the genotypes used in the field (ICGS 11 and TMV 2) study compared to those in a controlled environment (ICGV 86015 and ICG 796). Observations made on membrane thermostability and cardinal temperatures for pollen germination and tube growth (Kakani et al. 2002) also show that genotypes tested in field were more tolerant than those tested in a controlled environment.

The reasons for the existence or disappearance of the interaction can be attributed to the moisture level at that particular stage of crop growth. In the controlled environment study, the interaction with high temperature occurred when the moisture content in water stress treatment was 60 % available soil moisture (ASM). Similar to controlled environment pots, WS plots in field were at 100 % ASM until the initiation of water stress at 30 DAS. Time was required to bring down the moisture level to 40 %, which can be seen from the trends in biomass and pod yields (Fig. 8.4). Estimates of soil water content by simple water balance as shown below in WS × T2 treatment; assuming water loss of ET_C from soil, indicate that the water content of soil at the end of the high-temperature treatment was about 62 % ASM.

It can also be seen that biomass or pod yields in the water stress treatments are lower than irrigated treatments only after 50 DAS and remain less until the final harvest. This suggests that the interaction of water stress with high temperature would also have occurred at a moisture level of 60 % ASM, as observed from the

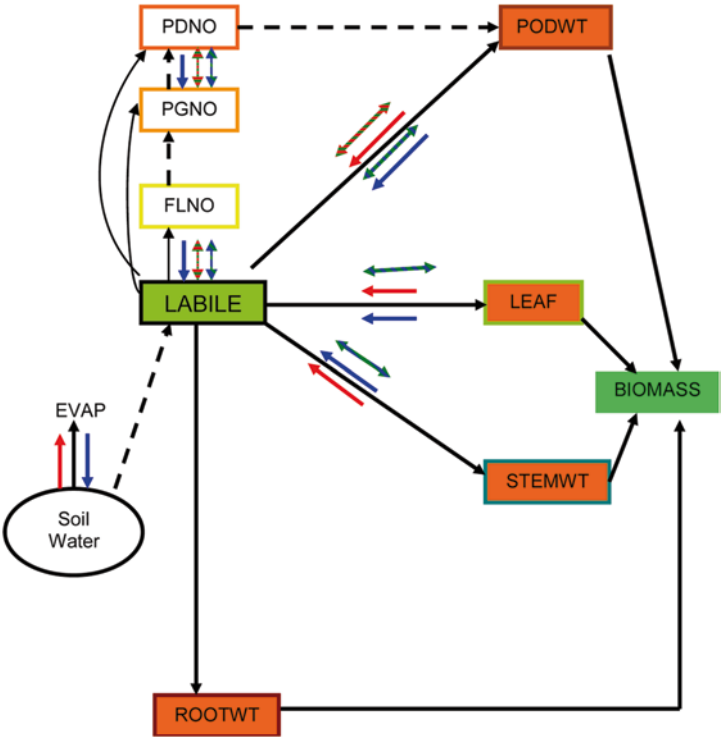

Fig. 8.5 Summary of the combined high temperature and water stress effects on growth and development of groundnut in SAT. (*Thick arrows* = main routes for assimilate translocation; *Thin black arrows* = routes for minor use of assimilates; *broken arrow* = information flow; *red arrow* = temperature effects; *blue arrow* = water stress effects; *red and green arrow* = interaction of temperature and genotype; *blue and green arrow* = interaction of water stress and genotype; *WT* weight; *Labile* = current and stored assimalte pool). Direction of *red/blue arrows* opposite to assimilate route indicates negative effects. Pod number (*PDNO*); peg number (*PGNO*); flower number (*FLNO*); pod weight (*PODWT*); root weight (*ROOTWT*); stem weight (*STEMWT*)

pot studies. Model PNUTGRO was run to simulate the ASM in the irrigated and WS treatments and presented in Fig. 8.6.

The simulations concur with soil water calculations and both confirm that the soil moisture was around 60 % ASM at the end of high-temperature treatment. The ASM averaged to 70 % from sowing to harvest in irrigated plots. In the case of WS plots, ASM averaged to 40 % during the stress period, even though the plants experienced a severe stress of around 25 % towards harvest.

Controlled environment and field studies also suggest that when soil moisture is around or less than 40 % ASM, critical for groundnut (Wright and Nageswara Rao 1994), water stress dominated the stress effects. Water is a reactant or substrate for many reactions in plant (Kramer and Boyer 1995), and the rate at which these reac-

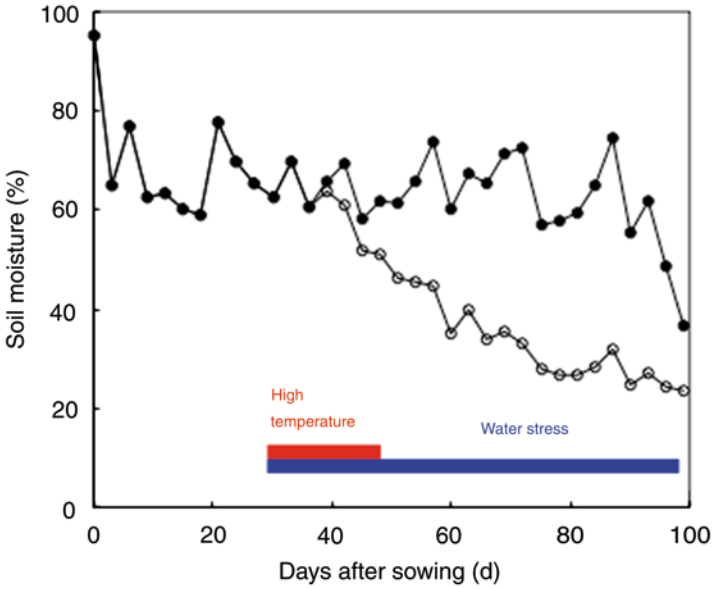

Fig. 8.6 Simulated values of percentage soil moisture in irrigated (● received 100% ET_C) and water stress (○ received 40% of ET_C from flowering) treatments in sowing 1 from sowing to harvest

tions occur is affected by temperature (Johnson and Thornley 1985). Thus, when water stress goes below 40% ASM, available substrate is limited, and hence the role of the temperature is reduced on the reaction rates in the plant. The reduction in dry weight of groundnut plants on exposure to water stress was due to severe decrease in the amount of water available for transpiration (Fig. 8.7), as evaporation was constant irrespective of the water stress treatment.

The increase in SLA value in 40% ET_C treatment of sowing 1 could be attributed to a decrease in biomass causing a decrease in leaf weight but not in leaf area. On the other hand, decrease in SLA of 40% ET_C treatment in sowing 2 could be attributed to decrease in both leaf weight and leaf size. To account for a decrease in SLA, the transpiration efficiency (*TE*) and transpiration (*T*) values were derived from the equations of Wright et al. (1996). The values of *TE* (Table 8.8) are similar in response irrespective of water stress treatment and sowing date.

The decrease in SLA in 40% ET_C of sowing 2 can be attributed to the severe reduction in transpiration, which could cause a decrease in leaf size along with a decrease in leaf weight. The decrease in leaf weight and biomass due to reduced transpiration can be attributed to reduced CO_2 assimilation (Hsiao 1973). Similar decrease in dry matter due to reduced photosynthesis under water stress conditions were reported in groundnuts by Hubick et al. (1986).

There were differences in the values for *T* obtained (T_{sla}) from SLA in the above table using the equations (8.4–8.6) and *T* values (T_{sim}) obtained from the simulations made using the PNUTGRO model. The total ET_C during the crop growth from 30 DAS, when 40% ET_C treatment was initiated, T_{calc} and T_{sim} are depicted in Fig. 8.8.

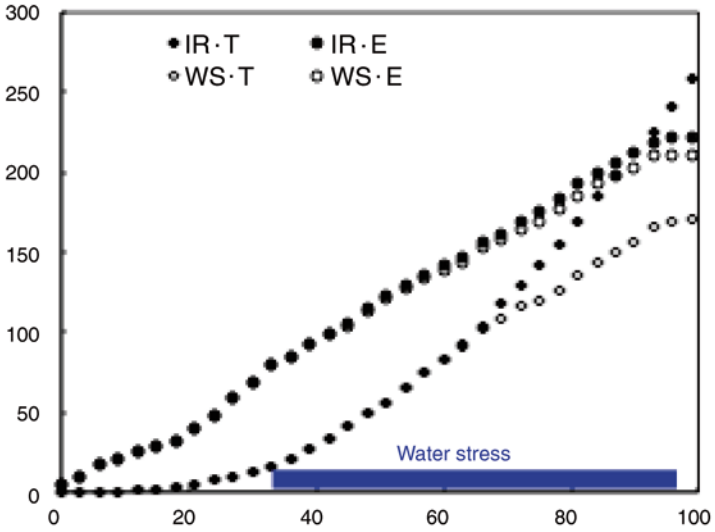

Fig. 8.7 Simulated values of cumulative soil evaporation (E_S) and transpiration (T) values in irrigated—*IR* (supplied with 100 % ET_C) and water stress (supplied with 40 % of ET_C from flowering) treatments in sowing 1 from sowing to harvest

Table 8.8 Observed specific leaf area (SLA, $cm^2\ g^{-1}$) and vapour pressure deficit (VPD, kPa) in water stress treatments, carbon isotope discrimination (Δ), transpiration efficiency (TE, $g^{-1}\ kg^{-1}$), transpiration during stress periods (T, L) derived from SLA values using the equations described by Wright et al. (1996)

Water stress treatment	SLA	Δ=0.03(SLA)+14	*K*=14.4–0.53(Δ)	VPD	TE=*k*/ VPD	T (mm)
Sowing 1						
IR	192	19.76	3.92	1.82	2.16	208
WS	201	20.04	3.78	1.82	2.08	116
SED	1.14*					
Sowing 2						
IR	180	19.41	4.11	2.26	1.82	165
WS	164	18.91	4.37	2.26	1.94	99
SED	1.28*					

IR irrigated, *WS* water stress, *SLA* specific leaf area
* indicates significance at 0.05 level of probability

$$k = -0.53\ \Delta + 14.4\,(\mathrm{Pa}) \tag{8.4}$$

$$\Delta = 0.03\ \mathrm{SLA} + 14.0 \tag{8.5}$$

$$T = \text{above-ground biomass}\left(\text{leaf} + \text{stem}\right) / TE\left(\mathrm{L}\right) \tag{8.6}$$

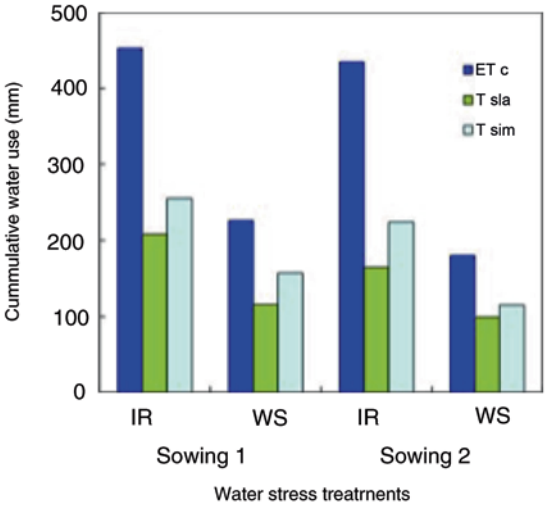

Fig. 8.8 Amount of water received by the crop (ET_C) and cumulative transpiration values derived using SLA (T_{sla}) and simulated by PNUTGRo model (T_{sim}) in irrigated (100% ET_C) and water stress (40% of ET_C) treatments in sowing 1 and sowing 2 from flowering to harvest

The T_{sim} values obtained from the PNUTGRO model are greater than T_{sla} values. The greater T_{sim} values can be attributed to the greater amounts of biomass predicted by crop model under these conditions. The *TE* values are lower compared to those obtained by Wright et al. (1996). This could be due to the high VPD of 1.82–2.26 under field conditions. These values are comparable to those obtained by Hubick et al. (1986) when groundnut studies were conducted in glasshouse at a VPD of 2.2. Similar to those reported here were obtained by Azam ali et al. (1989) at VPD of 2.1 kPa in drying soil; Mathews et al. (1988) at 1.9 kPa in dry season with occasional irrigation.

The results from this field study clearly show that both temperature and water stress decrease pod yields in groundnut, but the cultivars used in this study differed in their responses to temperature and water stress. Temperature moderately reduced total biomass or vegetative weight (leaf+stem). In contrast, a severe decrease in pod yield was recorded due to high temperature. However, under water stress conditions, a greater decrease in biomass and vegetative yield occurred along with a decrease in pod yield. This provides evidence to suggest that crop plants react differently to environmental stresses and adopt different strategies to overcome the stress events occurring at a particular location.

Pod yield decrease under water stress conditions can be attributed to a decreased source (vegetative weight), and in one cultivar to a slight decrease in partitioning. Such decrease in vegetative weight has been recorded in many experiments (Wright et al. 1991; Sarma and Sivakumar 1989, 1990). There exists evidence in literature for this decrease in pod yield under water stress conditions (Nageswara Rao et al. 1988; Ravindra et al. 1990; Williams et al. 1986). Thus, under water stress conditions, pod yield is source limited. Decrease in partitioning was also recorded in earlier studies by Greenberg et al. (1992).

Genotypes used in this study differed in their response to temperature treatments. Genotypes did not differ in their vegetative weight, indicating that source was not limiting. Thus, processes like photosynthesis or respiration, responsible for source, are not much altered. In contrast, pod yield was reduced in both the genotypes.

A greater reduction in pod yield of >70% occurred in TMV 2, while it was only around 23% in ICGS 11. This indicates that ICGS 11 is more tolerant to high temperature than TMV 2. The greater tolerance of ICGS 11 to high temperature can be attributed to maintenance of a significantly higher partitioning under increasing temperature conditions. This higher partitioning is due to greater sink strength in ICGS 11 than in TMV 2. Such genotypic differences for reduction in pod yield when exposed to high temperature were reported in several independent studies (Talwar et al. 1999; Prasad et al. 1999a, 2000; Wheeler et al. 1997). In a screening study conducted in 1991 in Sahelian region of Africa, Ntare et al. (2001) demonstrated that groundnut genotypes significantly differ in their pod yields in hot environments due to the effects on partitioning.

Under water stress conditions, a greater reduction in vegetative weight and pod yield occurred in ICGS 11 than in TMV 2. Although the reductions were greater in ICGS 11 under water stress, this genotype had higher vegetative and pod yield under irrigated conditions. This is due to greater accumulation of assimilates and higher partitioning of these assimilates to pod yield (Table 8.6). Under water stress, only a slight decrease in flower number occurred in ICGS 11, which did not significantly influence the peg and pod number. No such decrease in flower number occurred in TMV 2. In addition, the genotype ICGS 11 had a higher WUE when compared to TMV 2. This allowed the genotype to accumulate greater biomass even under water stress conditions. Hence, genotype ICGS 11 was tolerant to both high temperature and water stress conditions over TMV 2.

8.5 Conclusions

It can be inferred from this study that genotypes that are tolerant to water stress are also tolerant to high temperature under field conditions. Mechanisms that a genotype adopts to overcome stresses differ. However, genotypes with the ability to establish greater biomass and with a significantly greater partitioning of biomass to pod yield would be suitable for sustaining higher yields in SAT areas with high temperature and water stress. Genotypes with greater WUE are also more useful for the SAT. Thus, screening of groundnut genotypes for both temperature and water stress tolerance in field conditions are essential before recommending them for SAT and before using them for further breeding of new genotypes to these stresses. Controlled environments can be used for screening genotypes to high temperature for specific processes and experiments under field conditions need to be adopted to identify the various mechanisms for tolerance involved.

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Chapter 9
The Response of Plants to Simultaneous Biotic and Abiotic Stress

Nicky J Atkinson, Ritushree Jain and Peter E Urwin

9.1 Introduction

Plants have developed specific mechanisms that allow them to detect precise environmental changes and respond to complex stress conditions, minimising damage whilst conserving valuable resources for growth and reproduction. Plants activate a specific and unique stress response when subjected to a combination of multiple stresses (Atkinson et al. 2013; Suzuki et al. 2014), and consequently the imposition of single stresses individually may be suboptimal for developing and testing stress-tolerant plants (Mittler and Blumwald 2010). This is particularly true for signalling pathways that can act antagonistically such as the combinations of biotic and abiotic stresses (Anderson et al. 2004; Asselbergh et al. 2008a). There is an urgent need to understand the nature of multiple stress responses in plants and to create avenues for developing plants that are resistant to multiple stresses yet maintain high yields. In this chapter, we consider the effects of biotic and abiotic stresses acting simultaneously on plants, with an emphasis on elucidating the molecular mechanisms involved.

Evidence in the literature from field, laboratory and molecular studies suggests that plants respond to a specific combination of stresses in a manner distinctly different from the additive response to the individual stresses (Atkinson et al. 2013; Prasch and Sonnewald 2013; Rasmussen et al. 2013; Rizhsky et al. 2004; Suzuki et al. 2014; Iyer et al. 2013). Plants must produce an appropriate response to specific multiple stress conditions, as often the individual stresses may elicit opposing reactions. For example, heat stress often causes plants to open their stomata in order to cool the leaves, but under drought conditions this would be disadvantageous as

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more water would be lost (Rizhsky et al. 2004). Further, increased transpiration caused by heat stress could enhance the uptake of salt or heavy metals, heightening the damage from these factors (Mittler and Blumwald 2010). The cost of plant defence is likely to be reduced if specific genes have more general roles in different stress responses, thus explaining the overlap between stress response pathways (Asselbergh et al. 2008a; Bergelson and Purrington 1996; Herms and Mattson 1992). This hypothesis is supported by studies showing that certain molecular signalling pathways (AbuQamar et al. 2009; Dubos et al. 2010; Mengiste et al. 2003; Narusaka et al. 2004; Vannini et al. 2006; Zhang et al. 2006).

Plants exposed to a pest or pathogen often show increased susceptibility to an abiotic stress such as water deficit (Audebert et al. 2000; Cockfield and Potter 1986; English-Loeb et al. 1997; English-Loeb 1990; Khan and Khan 1996; Smit and Vamerali 1998). Conversely, the long-term abiotic stress can weaken defences and cause enhanced susceptibility to pathogen attack (Amtmann et al. 2008; Goel et al. 2008; Mittler and Blumwald 2010). The number of reports in the literature that have focussed on the interaction between biotic and abiotic stresses is growing, but is still limited: this chapter reviews that literature, with additional in-depth analysis of rice, an increasingly important crop plant in the study of stress tolerance.

9.2 The Challenge of Simultaneous Biotic and Abiotic Stresses in Agriculture

Crops in field environments experience a wide range of environmental perturbations during development that could limit their productivity. When plants are grown under suboptimal environmental conditions, a yield gap is observed and thus the actual average yield obtained is much lower than the maximum yield potential of the particular crop (Lobell et al. 2009). The yield gaps for three major cereal crops—wheat, rice and maize—are 40, 75 and 30 % respectively, in major growing areas of the world (Fischer et al. 2009). The major factors responsible for the yield gap in crop species can be classed as: (i) abiotic factors, such as temperature extremes, insufficient water or minerals or (ii) biotic factors, such as bacterial, viral, fungal or insect attack (Gaspar et al. 2002). These environmental stresses are responsible for large-scale crop loss each year and with the predicted climate change, such losses are expected to increase. Nearly 50 % of crop yield losses each year are comprised of abiotic stresses (Wang et al. 2003). The predicted climate change, characterised by an increase in temperature, an increase in concentration of greenhouse gases, an intensified hydrological cycle and an increase in troposhperic ozone levels, will have a multifaceted effect on crop growth and productivity. The results from free-air carbon dioxide (CO_2) experiments (FACE) have established that an increase in CO_2 levels in the atmosphere will lead to photosynthetic carbon gain, increased nitrogen-use efficiency and decreased water use in the leaves, but the yield gain in crop species will be much smaller than anticipated (Leakey et al. 2009). Also, the change in hydrological cycle will cause frequent extreme events of floods and storms in coast-

al areas accompanied by drought and reduced soil moisture in the drier regions, resulting in reduced productivity (Schmidhuber and Tubiello 2007). The anticipated rise in temperature will lead to a shorter life cycle and increased biomass in plants. Temperature changes outside the typical range during the major growth stages of crop plants will highly affect the productivity (Moriondo et al. 2011). Currently, pests and pathogens account for 15 % of the annual crop loss across the globe (Maxmen 2013). The increase in temperature and precipitation will alter the geographic distribution and host range of various pests and pathogens (Newton et al. 2011). The predicted changes will leave crop plants vulnerable to a large number of biotic and abiotic environmental stresses, acting upon them simultaneously.

Traditional molecular studies designed to explore plant stress responses have been driven by systems that artificially impose one particular stress or exogenous application of hormones on model plant species grown in laboratory conditions. The results of such studies have enhanced our understanding of the signalling cascades and hormonal pathways that mediate plant responses towards various stresses and have been used in achieving tolerance to biotic and abiotic stresses. However, the plants engineered for tolerance to a single biotic or abiotic stress in the laboratory have repeatedly failed to attain similar results in the fields (Atkinson and Urwin 2012; Mittler 2006). This is because the crops in the field encounter more than one type of stress at any given point in time, and with the prophesied climate change model the incidences of simultaneous biotic and abiotic stresses on plants are bound to increase.

The effect of climate change on plant–pest interactions has been widely reviewed in recent years (Chakraborty 2005; Garrett et al. 2006; Gregory et al. 2009; Luck et al. 2011; Newton et al. 2011; Scherm 2004). The response of plants to a combination of biotic and abiotic stresses is tailored to the exact nature of the stresses and there can be additive, negative or interactive effects of each of the individual responses (Atkinson and Urwin 2012). Evidence suggests that increased CO_2 levels in the atmosphere will lead to suppression of plant defence responses by the manipulation of the hormonal signalling pathways. Soybean plants show the down-regulation of jasmonic acid (JA) and ethylene (ET) pathways resulting in the reduction of cysteine protease inhibitors under increased CO_2 levels that in turn reduce the plants' defence against coleopteran pathogens (Zavala et al. 2008). At the same time, the increased CO_2 levels also result in the increased global expression of salicylic acid (SA) in soybean plants (Casteel et al. 2012). The increased CO_2 levels are likely to provide legumes with a photosynthetic advantage and protection against drought-induced loss in N_2 (Rogers et al. 2009). In tomato plants, elevated CO_2 levels have resulted in decreased resistance to the root-knot nematode (RKN) *Meloidogyne incognita* (Sun et al. 2010). Apart from elevated levels of CO_2, temperature plays an important role in plant–pathogen interactions (Fu et al. 2009; Zhu et al. 2010). Temperature-dependent resistance is seen towards blast disease in rice, broomrape in sunflower and clover, downy mildew in musk melon and stripe rust in wheat (Balass et al. 1993; Eizenberg et al. 2004; Eizenberg et al. 2009; Fu et al. 2009; Webb et al. 2010). An increase in temperature will also lead to more rapid development, increased reproductive potential and more generations of pests and pathogens in a season. These changes in pest life cycle and productivity could cause unprecedented damage to the crops in one season (Scherm 2004).

Drought can aid pest and pathogen outbreaks in fields, at the same time pathogens can severely influence plant water relations and lead to low water potential in plant cells (Mattson and Haack 1987). The bacterium *Xylella fastidiosa* causes pathogen-induced drought in grape by severe reduction of water potential (Choi et al. 2013). In the case of foliar pathogens, stomatal closure is the first physiological barrier in the defence response. Stomatal closure is also a drought avoidance strategy, thus drought-induced stomatal closure reduces pathogen entry into the plant tissue. Similarly, pathogen-induced stomatal closure helps the plant in efficient use of water (Sawinski et al. 2013). Drought enhances the symptoms of fungal charcoal rot disease in common bean (Mayek-Perez et al. 2002), and leads to reduction in plant water status and in turn increasing concentration of metabolites in the plant tissue. Increased concentration of defence compounds in drought-stressed tomato plants results in reduced susceptibility towards the herbivore *Spodoptera exigua* (English-Loeb et al. 1997). However, the change in herbivore's feeding behaviour also depends on the nature of the pest and its specificity towards the plant species (Gutbrodt et al. 2011). Drought stress can influence the interaction between two pathogens acting on the same plant and vice versa. Root-feeding herbivores can also enhance resistance against foliar herbivores by abscisic acid (ABA)-mediated hydraulic changes (Erb et al. 2011). The plant response towards simultaneous infestation by a foliar herbivore (aphids), their parasitoids and a root herbivore is also altered by drought stress (Tariq et al. 2013).

Drought-induced changes in roots can interact or counteract root-specific pathogens. In water-dependent agricultural ecosystems, drought can increase the incidence of soil-borne disease, especially plant-parasitic nematodes (PPNs). Drought and PPN infection are the two biotic and abiotic stresses that are often encountered simultaneously by rice plants in the fields. Drought can increase susceptibility of rice to root-knot nematode infection in all ecosystems, especially in aerobic rice cultivation. Cyst nematodes (CNs) can contribute to the drought-related losses in rice by causing reduced stomatal conductance and reduced leaf water potential (Audebert et al. 2000). A study on simultaneous drought and CN infection on *Arabidopsis* has revealed that under simultaneous biotic and abiotic stress, the plant responses are dominated by abiotic stress-responsive changes (Atkinson et al. 2013).

An integrated approach should be used to test resistance traits under a range of stress treatments (Mittler and Blumwald 2010). It is crucial to impose the stresses simultaneously and treat each set of environmental conditions as an entirely new stress to truly characterise the response of plants to multiple stresses (Mittler 2006).

9.3 Transcriptomic Studies of Simultaneous Biotic and Abiotic Stresses

Traditionally, plant molecular responses to multiple stresses have been predicted by comparing the results from two or more individual transcriptomic studies conducted independently by exposing plants to a singular stress. The results obtained by these comparisons identify the genes that might be involved in general stress responses of a plant, but fail to highlight the genes that might play an important role when plants

are simultaneously exposed to a combination of biotic and abiotic stresses. Evidence suggests that the response towards a pair of simultaneous biotic and abiotic stress is not always additive of the responses seen towards these stresses individually. Plants treat each set of simultaneous stresses as a different environmental condition and tailor their response specifically to it (Atkinson and Urwin 2012). This may involve differential regulation of a new set of genes that were not induced or repressed by any of the stresses individually and vice versa (Mittler 2006). A systematic study performed in *Arabidopsis* exploring transcriptomic response to simultaneous application of flagellin and change in temperature determines that nearly 49.3 % of the changes seen as a response to combinatorial stress could not have been predicted by just studying the response to each of these stresses singly. The number of differentially expressed genes increases with severity and complexity of the combination of stresses (Rasmussen et al. 2013). When *Arabidopsis* plants are subjected to virus infection in combination with drought and/or heat, the transcriptomic responses are much more severe in the triple stress, followed by simultaneous virus and heat and then simultaneous virus and drought stress treatment (Prasch and Sonnewald 2013). By comparing the response of *Arabidopsis* plants under single, double and triple stress, down-regulation of primary carbon metabolism was seen as plant's general response to stress. The abiotic stresses can significantly influence R-gene-mediated defence in plants by significantly reducing the expression of defence-related genes and in turn making plants highly susceptible to pathogen attack (Prasch and Sonnewald 2013). The study identified 11 genes that were differentially regulated in all stress combinations and 23 genes that were specifically regulated when plants were subjected to simultaneous heat, drought and virus infestation. When virus-infected plants were subjected to drought or heat stress, 175 and 309 genes were differentially regulated, respectively. In some cases, the transcriptomic response to combinatorial stress can be dominated by one of the stresses. Transcriptomic investigations of the combined effect of a biotic stress, *Aspergillus parasiticus*, and an abiotic stress, drought, in peanut, showed that the response to the combinatorial stress was more similar to the drought response alone with a very small proportion of multiple stress-specific responses (Luo et al. 2005). Similar results were seen in *Arabidopsis* plants simultaneously exposed to dehydration and infection with the CN *Heterodera schachtii*. Ninety-seven percent of the genes differentially expressed in leaves and roots under multiple stress treatment were also differentially expressed in drought-only treatment. Only 50 genes were expressed specifically in response to simultaneous drought and nematode infection (Atkinson et al. 2013).

9.3.1 Case Study: Rice Transcriptomic Responses to Simultaneous Biotic and Abiotic Stresses

A comprehensive investigation of systemic and local transcriptomic responses of rice towards drought and nematode stress, in isolation as well as in combination, was conducted using Affymetrix Rice GeneChip® arrays that provide maximum coverage of the rice genome, representing 57,381 transcripts from both japonica- and indica-type cultivars (Jain et al. unpublished). The replicate arrays for drought

and simultaneous drought and nematode stresses cluster in one group, whereas the control and nematode stress arrays form the other group. The experimental model was designed to mimic realistic stress conditions encountered by rice plants in the fields.

The transcriptome response to the application of simultaneous stresses was dominated by changes also observed in response to drought stress alone (95%), with some additional unique transcript changes (5%). Nearly 10% (4480) of the genes on the chip had a twofold expression change at a significant level ($p \leq 0.05$) in the roots, and a similar level was observed for drought stress. The transcriptomic changes were tissue specific with only 5% overlap between the roots and the leaves. A total of 297 genes showed multiple stress-specific regulation. Of these, 75% were up-regulated genes whilst 25% were repressed. The changes unique to simultaneous stress included novel members of gene families such as lipid-transfer protein genes (LTPLs) and cytochrome P450s, known to be involved in crosstalk between abiotic and biotic stresses. One of the genes highly induced specifically under multiple stresses was LTPL 11, a previously uncharacterised member of this stress-responsive protein family was known to be involved in pathogenesis as well as abiotic stress response in rice (Atkinson et al. 2013; Vignols et al. 1997). In *Arabidopsis*, LTPLs impart SA-mediated response and signal transduction during fungal and bacterial pathogen attack (Maldonado et al. 2002; Molina and García-Olmedo 1997). Four cytochrome P450 genes were differentially regulated in response to simultaneous stress, two in leaves and two in roots (Jain et al. unpublished). Cytochrome P450s in *Arabidopsis* mediate crosstalk between the abiotic and biotic stress-responsive hormone pathways. They are involved in catabolism of ABA, the major abiotic stress-responsive hormone, deactivation of gibberellic acid and negative regulation of jasmonate pathway (Koo et al. 2011). The up-regulation of the α-amylase responsible for the degradation of sucrose and the down-regulation of starch synthase in multiple stressed plants indicate that multiple stresses significantly modulate carbohydrate metabolism. Drought stress affects α-amylase in leaves and thus modulates sugar metabolism (Jacobsen et al. 1986). Sucrose is required for plant growth, and it also acts as a signalling molecule by modulating a proton–sucrose symporter (Gupta and Kaur 2005).

The simultaneous stress response in rice is characterised by a unique set of genes that is not differentially regulated when any of the two stresses act individually on the plant, emphasising that the response to a combination of stresses is not additive but is interactive of the responses seen under the influence of any of the stresses singly.

9.4 Hormone Signalling and Master Regulators in Stress Interaction

Due to the complex interacting nature of plant stress responses, research aimed at developing stress-tolerant crops is increasingly focusing on the points of crosstalk between pathways, or master regulators (Denancé et al. 2013; Miller et al. 2010).

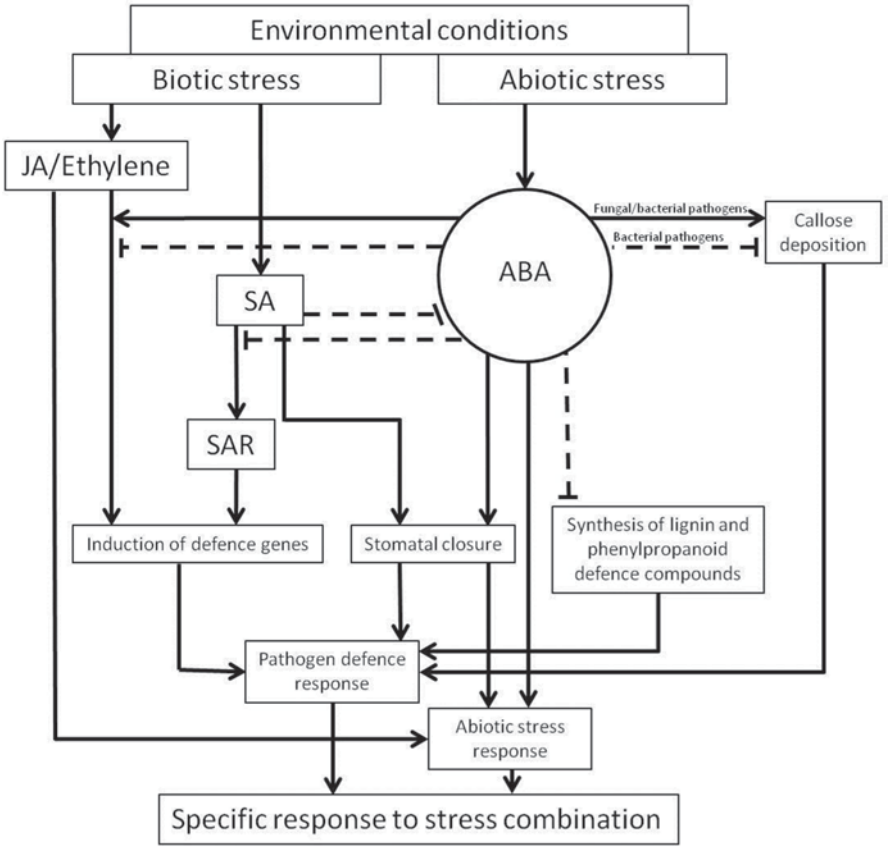

Fig. 9.1 The multifaceted role of abscisic acid (*ABA*) in plant biotic and abiotic stress responses. This figure summarises the main interactions of *ABA* with components of the pathogen defence pathway. ABA has both a positive and negative effect on various hormones and events involved in the response to biotic stress, as well as orchestrating the abiotic stress response. Positive regulation is shown by *solid arrows*, whilst negative regulation or inhibition is shown by *dashed bars*. *JA* jasmonic acid, *SA* salicylic acid, *SAR* systemic acquired resistance

Plant hormones are at the hub of this interaction, in particular ABA (Atkinson and Urwin 2012; Ton et al. 2009). ABA is central in the fine-tuning of stress responses and is now considered a global regulator that can control the switch in priority between the response to biotic or abiotic stress, allowing plants to respond to the most severe threat (Fig. 9.1; Anderson et al. 2004; Asselbergh et al. 2008a; Mauch-Mani and Mauch 2005; Ton et al. 2009). This dominant role of ABA may arise from its involvement in both the biotic and abiotic stress-regulatory networks.

Traditionally, ABA has been connected primarily with the response to abiotic stress, whilst defence against pathogens and other biotic stresses is determined by the mutual antagonism between SA, JA and ethylene signalling. New evidence suggests that ABA acts both synergistically and antagonistically with these defence pathways, with crosstalk at different levels (Asselbergh et al. 2008a; Atkinson and

Urwin 2012; Fujita et al. 2006; Yasuda et al. 2008). Its influence depends on the timescale of infection and the nature of the pathogen (Ton et al. 2009). In the early stages of defence against microbial invasion, ABA acts through the SA signalling pathway as a key strategy to induce stomatal closure and thus reduce infection (Melotto et al. 2006). After penetration, ABA is necessary for β-amino-butyric acid (BABA)-induced callose deposition as a defence against fungal pathogens (Ton and Mauch-Mani 2004), whilst during bacterial infection ABA can block callose production or indeed has a positive effect, a balance that depends on the external environmental factors such as light and glucose levels (De Torres-Zabala et al. 2007; Luna et al. 2011). Induced protection against the bacteria *Ralstonia solanacearum* in *Arabidopsis* is unexpectedly independent of SA, JA and ethylene and is instead dependent on ABA signalling and synthesis (Feng et al. 2012).

In the later stages of a pathogen infection, the hormones SA, JA and ethylene are induced by pathogen-associated molecular patterns (PAMPs) to regulate a broad spectrum of defensive compounds, processes that are generally inhibited by ABA (Asselbergh et al. 2008b; Ton et al. 2009). Treatment with ABA actually increases susceptibility to fungal and bacterial pathogens, a phenomenon demonstrated in *Arabidopsis*, tomato and potato (Asselbergh et al. 2008b; Audenaert et al. 2002; Henfling et al. 1980; Mohr and Cahill 2003) and in rice, where ABA treatment has been shown to cause a reduction in plant defence against the blast fungus Magnaporthe *grisea* (Koga et al. 2004). Furthermore, disruption of the ABA signalling pathway can improve defence against pathogens (Anderson et al. 2004; Asselbergh et al. 2007; Audenaert et al. 2002; Mohr and Cahill 2003). For example, *Arabidopsis* mutants with impaired ABA biosynthesis or signalling are more resistant to the necrotrophic fungi *Plectosphaerella cucumerina* (Sánchez-Vallet et al. 2012). On the analysis of transcription patterns in these mutants compared to wild-type plants, it was found that defence genes regulated by SA, JA and ethylene were specifically down-regulated by the ABA pathway. ABA treatment can repress the SA-mediated systemic acquired resistance (SAR) pathway in *Arabidopsis* and tobacco, and inhibits the accumulation of important defence compounds such as lignins and phenylpropanoids (Kusajima et al. 2010; Mohr and Cahill 2007; Yasuda et al. 2008). In contrast, SA is known to obstruct abiotic stress signalling, leading to drought susceptibility in maize when applied exogenously (Németh et al. 2002). In rice, resistance to the rice blast fungus *M. grisea* is mediated by the balance between ABA and SA (Jiang et al. 2010). ABA also antagonises JA and ethylene defence signalling through the repression of defence genes such as *PDF1.2* (Anderson et al. 2004), although JA production can contribute positively to tolerance against certain abiotic stresses such as chilling, salt, drought and osmotic stress (Santino et al. 2013).

This close association of ABA with defence signalling pathways may allow a subtle shift in environmental conditions to cause a dramatic difference in stress response, as any increase in ABA due to abiotic stress could repress the SA, JA and ethylene defence responses. As abiotic stress conditions such as drought tend to be a much greater threat to survival than biotic stresses, this would then allow plants to prioritise the response to the more urgent stress.

The fine-tuning in the regulation of stress responses by ABA may be partially controlled by the diversity amongst downstream signalling elements (Lee and Luan 2012). There are 14 members of the PYR/PYL/RCAR ABA receptor family, which in turn activate 6–9 members of the A-type PP2C phosphatases and at least 3 members of the SnRK2 kinases, known to carry out downstream protein phosphorylation and dephosphorylation events (Lee and Luan 2012; Ma et al. 2009; Wasilewska et al. 2008). Between them, these provide more than 200 signalling combinations that may activate similar or different downstream targets. These molecular components of the ABA signalling pathway may additionally provide opportunities for genetic engineering of stress tolerance in crop plants.

Points of crossover between hormone signalling pathways include several influential TFs, such as MYC2. This is activated by ABA (Abe et al. 2003), is a positive regulator of JA-responsive defence genes (Anderson et al. 2004; Pieterse et al. 2009), and in addition represses the SA pathway (Laurie-Berry et al. 2006). Members of the MYB and NAC TF family are also crucial controlling factors in multiple stress responses, and have been fully reviewed recently (Atkinson and Urwin 2012).

Large multi-protein mediator complexes may function to integrate downstream stress response signals from multiple sources (Balderas-Hernández et al. 2013). These are central components of transcription complexes in eukaryotes, which interact with ribonucleic acid (RNA) PolII and promote the assembly of TFs on promoter sequences (Bourbon 2008). In *Arabidopsis*, mediator is made up of at least 27 subunits, one of which is Med25, encoded by the *phytochrome and flowering time 1* (*PTF1*) gene. It regulates a multitude of signalling pathways by interacting with TFs central to the ABA and JA/ethylene cascades, such as MYC2 and ABA insensitive 5 (ABI5) which transcriptionally activates ABA-responsive genes (Balderas-Hernández et al. 2013).

Heat shock factors (HSFs) have also been identified as potential master regulators of the response to multiple stresses (Atkinson and Urwin 2012). These are TFs that act as molecular sensors of cellular stress-responsive reactive oxygen species (ROS) and induce the expression of heat shock proteins (Miller and Mittler 2006). As different stresses elicit different combinations of HSFs, they may contribute to the fine-tuning of stress response outcomes (Rizhsky et al. 2004; von Koskull-Döring et al. 2007; Yoshida et al. 2011). Recently, HSFA1b has attracted attention as a target for engineering stress tolerance in crops. Post-transcriptionally regulated during stress conditions, HSFA1b itself regulates 509 genes. When over-expressed in *Arabidopsis* it confers dehydration tolerance, resistance to bacterial pathogens and oomycetes, and improved seed yield under water-limited conditions. (Bechtold et al. 2013). In oilseed rape, its over-expression led to improved productivity characterised by an increased harvest index and seed yield. This is of particular interest given that many stress-tolerant *Arabidopsis* mutants over-expressing the ABA or SA signalling pathways show a diminished fecundity (Bechtold et al. 2013; van Hulten et al. 2006). Clearly to attain impact in the development of broad-spectrum stress-tolerant crop plants, improved disease and abiotic stress responses must go hand in hand with the maintenance of growth and yield characteristics.

9.5 Interaction of Volatile Compounds in Simultaneous Biotic and Abiotic Stresses

Plants interact with each other by emitting a unique blend of volatile organic compounds (VOCs). The intensity and chemical composition of VOCs emitted by a plant can define the physiological state of a plant and is an indication of the nature of the stress acting upon them. The ratio of various compounds in the volatile blend can hint to herbivorous insects or parasitic plants about the location of their potential host (Runyon et al. 2006; Tumlinson 2014). Some of the VOCs are specific to certain plant species. For example isothoicynates, volatile catabolites of the glucosinolates, are characteristic of the brassicaceous plants. Specialist brassica pests like the cabbage aphid *Brevicoryne brassicae* and the cabbage seed weevil *Ceutorhynchus assimilis* use isothiocyanates for host location (Bruce et al. 2005). However, as plants in nature may suffer from more than one stress at a time, it can be hypothesised that the multiple stresses will have a VOC signature different to any of the stresses acting individually on the plants (Blande et al. 2014). Abiotic stresses like heat, water stress, high-intensity light, ozone and salt stress lead to increased emission of volatile compounds including isoprene, monoterpenes and sesquiterpenes (Holopainen and Gershenzon 2010; Loreto and Schnitzler 2010). The emission under a biotic stress is dominated by terpenes and green leaf volatiles (GLVs), C_6 aldehydes, alcohols and esters of lipoxygenase cleavage of fatty acids (Holopainen and Gershenzon 2010). Two different stresses, two biotic or two abiotic stresses, are capable of initiating emissions of similar types of compounds that might suggest an underlying common signature for the biotic and abiotic stresses. In lima beans, exposure to ozone and spider mite infestation triggered the emission of (*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT) and (*E, E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene (TMTT; Vuorinen et al. 2004).

Similar to the molecular and physiological effects, simultaneous application of a biotic and an abiotic stress can have additive or opposing effects on the VOCs emission. Additive effects can result in an increase in emitted VOCs and also can increase susceptibility towards other stresses. Simultaneous exposure to ozone and infection with spider mites in lima beans gave a 31% increase in the emission of VOCs compared to plants exposed to single stress and also made plants more susceptible to secondary herbivore attack by predatory mites. In behavioural assays, the predatory mites preferred plants under dual stress over the plants that were just exposed to high levels of ozone. This preference was a result of increased ratio of (*E*)-β-ocimene in the emission blend of dual stressed plants (Vuorinen et al. 2004). An additive effect on emitted VOCs was also observed in the deciduous tree *Alnus glutinosa* during drought stress and simultaneous infection with the larvae of green alder sawflies. Concurrent application of the two stresses increased the emission of GLVs, monoterpenes and the markers of herbivory, (*E*)-β-ocimene and methyl jasmonate (Copolovici et al. 2014). The mild drought stress before larval attack in this case showed a priming effect and made plants less susceptible to herbivory, in contrast to the effect seen in lima beans under simultaneous ozone exposure and

spider mite attack. Perhaps the ozone dose used was insufficient to initiate a priming effect similar to drought stress. *Brassica napus* (oilseed rape) plants subjected to herbivory under elevated levels of ozone or CO_2 show contrasting interactions between the biotic and the two abiotic stresses. Terpenoid emission was increased in plants under elevated CO_2 and subjected to herbivory, but reduced in the elevated ozone and herbivory group. However, under both stress combinations plants became susceptible to herbivory as determined by olfactory tube assays (Himanen et al. 2009).

A detailed study to elucidate the effect of simultaneous biotic and abiotic stresses in maize plants was conducted using inoculation of caterpillar regurgitant in combination with changes in soil humidity, air humidity, temperature, light and mineral dosage. The amount and the composition of the VOCs emitted by the maize plants did not change with the abiotic conditions, but on simultaneous induction of biotic stress there was an increase in the VOCs emission under all stresses except the change in soil humidity. The composition of the emission blend also changed with simultaneous application of biotic and abiotic stresses. Table 9.1 gives a detailed overview of changes in VOCs under pairs of simultaneous biotic and abiotic stresses in different species. In most cases, simultaneous stresses change the composition and increase the amount of VOCs emitted by a plant, depending on the nature of the stresses applied. The VOCs emitted by stressed plants play a vital role in plant–pathogen interaction. A better understanding of VOCs emission under multiple stresses may be valuable for managing insect pests of crop species.

9.6 Points of Convergence Between Biotic and Abiotic Stress Signalling Pathways

Biotic and abiotic stress signal transduction is characterised by a complex arrangement of interacting factors. Certain gene products are now known to be central to both biotic and abiotic stress signalling, and may therefore control the specificity of the response to multiple stresses (Fujita et al. 2006; Mauch-Mani and Mauch 2005). Transcriptomic and genetic analyses have highlighted a number of putative candidates that might act as points of convergence, including TFs, map kinases, HSFs, ROS and small RNAs, and these discoveries have been fully reviewed recently (Atkinson and Urwin 2012).

9.6.1 Rice as a Case Study

As one of the most important crop plants worldwide and a model monocotyledon, rice is increasingly becoming a focus for applied plant stress research in the field and laboratory. Discoveries of key stress response genes in rice will provide direct opportunities for translational work to improve stress tolerance in cereal crops. Key

Table 9.1 Impact of simultaneous biotic and abiotic stresses on the emission of volatile compounds

Plant species	Biotic stress	Abiotic stress	Total VOCs in dual stress	(*Z*)-3-hexen-1-yl acetate	β-myrcene	(*E*, *E*)-α-farnesene	(*E*)-β-farnesene	linalool	DMNT	Indole	α-bergamontene	TMTT	Geranyl acetate	(*E*)-nerolidol	β-caryophyllene	(*E*)-β-ocimene	Methyl salicylate	1-penten-3-ol	(*Z*)-Hexen-1-ol	(*E*)-2-Hexenal	(*E*)-3-Hexenal	LOX products	α-Thujene	α-Pinene	Sabinene	Limonene	β-Elements	B-sesquiphellandrene
Zea mays	*SL*	Increased soil humidity (20 to 60–80%)	→	←	n.s	n.s	n.s	←	↔	↔	n.s	n.s	↔	n.s	↔	–	–	–	–	–	–	–	–	–	–	–	–	–
Zea mays	*SL*	Increased air humidity (20 to 45–65%)	←	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	s.n	–	–	–	–	–	–	–	–	–	–	–	–	–
Zea mays	*SL*	Increased temperature (17–37 °C)	←	←	n.s	n.s	n.s	n.s	n.s	n.s	n.s	←	n.s	←	←	–	–	–	–	–	–	–	–	–	–	–	–	–
Zea mays	*SL*	High light intensity (0 to 15,000–20,000 lm/m^2)	←	→	n.s	→	n.s	←	n.s	←	n.s	←	←	←	←	–	–	–	–	–	–	–	–	–	–	–	–	–
Zea mays	*SL*	Reduced fertilisation	→	n.s	n.s	→	n.s	↔	→	→	n.s	→	→	→	→	–	–	–	–	–	–	–	–	–	–	–	–	–
Zea mays	*SF*	Increased copper (0–80 µM)	←	←	–	–	↑	n.s	n.s	n.s	←	n.s	–	n.s	n.s	–	–	–	–	←	n.s	–	–	–	–	–	↑	–
Alnus glutinosa	*MP*	Drought	↑	n.s	–	–	–	–	↑	–	–	–	–	–	–	–	←	–	–	–	–	↑	–	–	–	–	↑	–

Table 9.1 (continued)

Plant species	Biotic stress	Abiotic stress	Total VOCs in dual stress	(*Z*)-3-hexen-1-yl acetate	β-myrcene	(*E*, *E*)-α-farnesene	(*E*)-β-farnesene	linalool	DMNT	Indole	α-bergamontene	TMTT	Geranyl acetate	(*E*)-nerolidol	β-caryophyllene	(*E*)-β-ocimene	Methyl salicylate	1-penten-3-ol	(*Z*)-Hexen-1-ol	(*E*)-2-Hexenal	(*E*)-3-Hexenal	LOX products	α-Thujene	α-Pinene	Sabinene	Limonene	β-Elements	β-sesquiphellandrene
Brassica napus	*PX*	Elevated O_3 (100 nL/L)	n.s	–	n.s	–	–	–	n.s	–	–	–	–	–	–	–	–	–	–	–	–	–	n.s	n.s	n.s	n.s	n.s	–
Brassica napus	*PX*	Elevated CO_2 (750 μL/L)	↑	–	↑	–	–	–	↑	–	–	–	–	–	–	–	–	–	–	–	–	–	↑	↑	↑	↑	↑	–
Phaseolus lunatus	*TU*	Elevated O_3 (150–200 nL/L)	↑	n.s	–	–	–	–	↑	–	–	n.s	–	–	–	↑	–	–	–	–	–	–	–	–	–	–	–	–

Plant pathogens: *SL Spodoptera littorali*, *SF Spodoptera frugiperda*, *MP Monsoma pulveratum*, *PX Plutella xylostella*, *TU Tetranychus urticae*

n.s. not significant, – not determined in particular study, ↕ no regular pattern but fluctuates significantly with the stresses, ↑ significant increase, ↓ significant decrease, *DMNT* (*E*)-4,8-dimethyl-1,3,7-nonatriene, *TMTT* (*E*, *E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene

components of the pathways in rice responding to multiple environmental stresses have already been elucidated. Members of the WRKY family of TFs are responsive to both biotic and abiotic stresses and play a vital role in fine-tuning plants' response to simultaneous stress. In rice, *WRKY13* antagonistically regulates the response to drought and bacterial disease by selectively binding to the *cis*-acting elements and specific sequences in the promoters of *SNAC1* and *WRKY45–1*. It can also auto-regulate its own expression by binding to its promoter (Xiao et al. 2013). *WRKY45* imparts resistance against the fungal and bacterial pathogens in rice by differential mechanisms (Shimono et al. 2012). The *WRKY45-1* allele negatively regulates ABA signalling and also increases plant susceptibility to bacterial pathogens, whilst the *WRKY45-2* allele positively regulates ABA signalling and increases resistance to bacterial pathogens (Tao et al. 2011). Both alleles positively regulate resistance to fungal blast disease (Tao et al. 2009). *WRKY76* transcription repressor plays opposite role in response to rice blast disease and cold stress; over-expression of the *WRKY76* results in increased susceptibility towards blast infection but increases tolerance to cold stress (Yokotani et al. 2013a). *WRKY82* enhances defence against biotic pathogens and tolerance against abiotic stress via the JA/ET pathways (Peng et al. 2011).

Several disease-resistant cultivars have different natural expression levels of *OsMYB4* leading to varying degrees of resistance to sheath blight and leaf blight diseases in rice (Singh et al. 2013). Ectopic expression of the rice *OsMYB4* TF enhances abiotic and biotic stress tolerance in many plants including *Arabidopsis*, tomato and apple (Pasquali et al. 2008; Vannini et al. 2006, 2007). The JA-induced *MYB* gene, *JAmyb,* is induced by high salinity, osmotic stress and ROS and its over-expression results in induction of JA-induced TFs that play an important role in biotic stress response (Yokotani et al. 2013b).

The *OsNAC6* gene acts as a transcription inducer for biotic and abiotic stress responses in rice. Constitutive over-expression of *OsNAC6* results in increased tolerance to dehydration and salt stress along with greater resistance to blast disease, but with growth and yield penalty (Nakashima et al. 2007). *OsNAC5* also enhances abiotic stress tolerance in rice and is responsive to JA, but does not cause any negative effect on plant growth (Takasaki et al. 2010). A plant-specific TF family, ethylene-responsive factor TFs, bind to the GCC sequence specifically found in the *PR* genes. These TFs are mainly involved in abiotic stress responses in plants. Four ethylene-responsive genes, *BIERF1-4,* are up-regulated by salt, drought, wounding and fungal pathogens (Cao et al. 2006).

In addition to TFs, various protein kinases (PKs) also act as the convergence points in biotic and abiotic stress pathways in rice. Out of the 17 known rice MAPK genes, five are induced by both biotic and abiotic stresses (Rohila and Yang 2007). *OsMAPK5* is the most studied rice MAPK; it confers ABA-mediated tolerance to abiotic stress and resistance to brown spot, whilst negatively regulating the response to rice blast fungus (Sharma et al. 2013). Members of the rice CDPK family are also involved in crosstalk between biotic and abiotic stresses. OsCDPK12 regulates genes involved in ROS scavenging in stressed plant cells

resulting in reduced accumulation of H_2O_2. The over-expression of *OsCDPK12* leads to positive regulation of salt tolerance and negative regulation of blast resistance (Asano et al. 2012). *OsCDPK13* is involved in the gibberellic acid-mediated response in rice leaf sheath and cold tolerance (Abbasi et al. 2004). Four CIPK PKs (*OsCIPK 2*, *OsCIPK 10*, *OsCIPK 11* and *OsCIPK 14*) also play important roles in the crosstalk between biotic and abiotic stresses (Chen et al. 2011). Another family of PKs, known as dual specificity PKs (*OsDPK*), also shows response to biotic and abiotic stresses. *OsDPK1*, *OsDPK2* and *OsDPK3* are all induced by exogenous application of ABA, drought, salinity and in response to the rice blast fungus (Gu et al. 2005). Involvement of these rice gene families in biotic as well as abiotic stress responses presents them as candidates for transgenic improvement of multiple stress tolerance.

9.7 Future Perspectives

Studies describing the effects of individual and combinatorial stresses have facilitated an initial understanding of the molecular interactions controlling plant stress responses. Plants respond to the exact set of conditions they encounter by activating both specific and non-specific stress responses. Signal specificity is achieved through the precise interplay between components of each pathway, particularly the hormones ABA, SA and JA, TFs, HSFs, ROS and small RNAs. In the past, individual plant stress factors, which trigger linear signalling pathways, have been studied in isolation. It seems that this model is no longer sufficient, as both biotic and abiotic stress pathways are inextricably linked in a network of molecular interactions.

The development of new crop varieties will depend on understanding crucial stress-regulatory networks and the potential effects of different combinations of adverse conditions. Studies of multiple stress responses in the model plants *Arabidopsis* and rice, as well as work on other species, have greatly increased our knowledge. Plant efficiency in sensing and responding to each unique set of environmental conditions means that different methods of imposing stress can lead to drastically different transcriptional profiles (Bray 2004). Commonalities between biotic and abiotic signalling pathways that have been identified may lead to their antagonistic nature. Nodes that act in both biotic and abiotic stress response systems are excellent candidates for manipulating stress tolerance (Baena-González and Sheen 2008; Miller et al. 2010). To provide a model for crop stress responses, an integrated approach should be adopted, whereby future experiments are carried out in conditions that reproduce natural or field conditions as accurately as possible (Deyholos 2010; Mittler and Blumwald 2010; Suzuki et al. 2014).

The impacts of climate change pose further challenges for plant breeding and biotechnology. Crops must be developed that can cope with multiple concurrent stresses whilst still fulfilling their genetic potential to provide maximum yields and thus ensure future global food security.

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Chapter 10
Impact of Concurrent Drought Stress and Pathogen Infection on Plants

Prachi Pandey, Ranjita Sinha, Kirankumar S. Mysore and Muthappa Senthil-Kumar

10.1 Introduction

In the field conditions, plants are constantly exposed to concurrent abiotic and biotic stresses that affect their overall growth and development (Mittler 2006; Atkinson and Urwin 2012). Plant responses to individual biotic and abiotic stresses have been well explored and a number of genes conferring tolerance to the individual stresses have been identified. Some of the genes have also been reported to impart tolerance to multiple independent abiotic and biotic stress conditions (Wang et al. 2010, 2013; Senthil-Kumar et al. 2013; Tamirisa et al. 2014). A few recent studies suggest that the combined effect of two or more abiotic stresses cause greater reduction in crop yield when compared with the losses incurred by individual stresses (Rizhsky et al. 2002, 2004; Mittler 2006; Suzuki et al. 2014). Environmental factors like drought, extreme temperature, and salinity potentially alter the occurrence and intensity of a particular disease by modulating the plant responses to pathogen (Szittya et al. 2003; Wiese et al. 2004; Achuo et al. 2006; Amtmann et al. 2008; Goel et al. 2008; Madgwick et al. 2011; Atkinson and Urwin 2012). The importance of different

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predisposing abiotic stress factors on plant–pathogen interactions has also been recently reviewed (Bostock et al. 2014).

The data from a number of individual stress studies have been analyzed using bioinformatics tools to find the common genes altered under biotic and abiotic stress conditions. For example, the response of thale cress (*Arabidopsis thaliana*, hereafter referred to as *Arabidopsis*) to a variety of abiotic and biotic stresses was studied by the comparison and cluster analysis of differentially expressed genes from publicly available microarray datasets (Ma and Bohnert 2007). Similarly, the gene expression profiles of chickpea plant under different abiotic (drought, cold, and high salinity) and biotic stress (*Ascochyta rabiei;* causal agent of blight in chickpea) conditions were compared (Mantri et al. 2010). Meta-analysis of transcriptomic data from rice (*Oryza sativa*) and *Arabidopsis* plants each exposed to independent drought and bacterial stresses revealed the commonality of 38.5 and 28.7% differentially expressed genes between two stress conditions in the respective plants (Shaik and Ramakrishna 2013, 2014). Yet, in another study, the molecular response of rice plants to multiple biotic and abiotic stress conditions was compared and genes responsive to both the stresses and to exclusively biotic stresses were identified (Narsai et al. 2013). Several other studies also support the existence of cross talk between the abiotic and biotic stress pathways (Narusaka et al. 2004; Fujita et al. 2006; Fraire-Velázquez et al. 2011). However, in all these studies, the plants were not concurrently exposed to biotic and abiotic stresses, but only the data from independently stressed plants were compared. Although the biotic and abiotic stress response pathways have common elements, plant-"tailored" responses to the actual concurrent abiotic and biotic stress cannot be predicted using the data from individual stress studies (Mittler 2006).

The physiological and molecular responses against concurrent abiotic and biotic stresses are beginning to be studied (Atkinson et al. 2013; Rasmussen et al. 2013; Bostock et al. 2014; Kissoudis et al. 2014; Prasch and Sonnewald 2014). The available literature provides evidence that plants perceive concurrent stresses as a "new stress" leading to reprogramming of their responses. Gene expression studies in *Arabidopsis* plants exposed to concurrent stress conditions like cold and high light, salt and heat, salt and high light, heat and high light, heat and flagellin, and cold and flagellin also revealed that on an average 61% of the transcripts expressed during concurrent dual stresses were not observed in the single stress treatments (Rasmussen et al. 2013). Likewise, drought and concurrent nematode infection in *Arabidopsis* plants led to the induction of 50 unique genes (Atkinson et al. 2013).

Drought is one of the most important and frequently occurring abiotic factors and can potentially alter the end result of plant–pathogen interaction. Hence, this chapter is focused on the impact of drought stress on plant–pathogen relations and the different ways through which drought modulates the plant–pathogen (fungi, oomycete, bacteria, and virus) relations. We also speculate various aspects involved in the concurrent stress-responsive signaling network of plants by reviewing recent studies.

Table 10.1 A few examples of drought-mediated modulation of plant–pathogen interaction in plants

S. No.	Pathogen	Name of the disease	Host plant	Effect on plant–pathogen interaction	References
Fungi					
	Thielaviopsis basicola	Black root rot	Tobacco	Susceptibility decreased	Bateman 1961
	Cephalosporium gramineum	Stripe	Wheat		Bruehl 1968
	Sclerotinia sclerotiorum	White mold	*Nicotiana benthamiana*		Ramegowda et al. 2013
	Sclerotinia sp.	White mold	Soybean, sunflower, canola, peanut		Markell et al. 2008
	Fusarium solani f. sp. *pisi*	Root and stem rot	Sweet pea	Susceptibility increased	Krafts and Roberts 1969
	Macrophomina phaseoli	Charcoal rot	Soybean, sorghum, cotton		Mayek-Perez et al. 2002
	Uncinula necator	Powdery mildew	Grapes		Hartman and Beale 1998
	Penicillium sp. and *Aspergillus* sp.	Seed decay	Wheat		Griffin 1966
	Rhizoctonia sp.	Stem canker	Potato		Lootsma and Scholte 1997
	Verticillium sp.	Early dying	Potato		Markell et al. 2008
	Drechslera tritici-repentis	Tan spot	Wheat		Janda et al. 2008[a]
	Ascochyta sp.	*Ascochyta* blight	Pea, lentil, chickpea		Markell et al. 2008
Oomycetes					
	Pythium sp.	Root rot	Pea	Susceptibility decreased	Kerr 1964
	Aphanomyces sp.	Root rot	Sunflower		Markell et al. 2008
	Plasmopara sp.	Downy mildew	Sunflower		Markell et al. 2008
	Phytophthora sp.	Root rots	Soybean, safflower, rhododendron, tomato	Susceptibility increased	McDonald and Cahill 1999; Duniway 1977; Blaker and MacDonald 1981; Ristaino and Duniway 1989

Table 10.1 (continued)

S. No.	Pathogen	Name of the disease	Host plant	Effect on plant–pathogen interaction	References
Virus					
	Pineapple mealybug wilt-associated virus-1	Pineapple Mealybug Wilt	Pineapple	Susceptibility increased	Sether and Hu 2001
	Maize dwarf mosaic virus	Mosaic	Sweet corn		Olson et al. 1990
	Turnip mosaic virus	Growth retardation	*Arabidopsis*		Prasch and Sonnewald 2013
Bacteria					
	Xylella fastidiosa	Pierce's disease	Vine	Susceptibility increased	McElrone et al. 2001
	Pseudomonas syringae pv. tomato 1065	Bacterial speck disease	*Arabidopsis*		Mohr and Cahill 2003
	Streptomyces scabies	Common scab	Potato		Cook and Papendick 1972
	Pseudomonas syringae pv. Tabaci	Bacterial speck disease	*Nicotiana benthamiana*	Susceptibility decreased	Ramegowda et al. 2013

[a] PEG-mediated osmotic stress
PEG Polyethylene glycol

10.2 Drought Modulates Plant–Pathogen Interaction

The net effect of concurrent drought and pathogen infection on plants depends on duration and intensity of the two stresses. Based on these factors, the combination of drought and pathogen infection can have two outcomes. In the first scenario, both the stresses when occurring concurrently can act in unison to hamper plant growth and development. For example, drought stress has been shown to aggravate many fungal (Mayek-Perez et al. 2002), bacterial (McElrone et al. 2001; Mohr and Cahill 2003), and viral (Olson et al. 1990; Prasch and Sonnewald 2013) infections in plants. On the contrary, in the second case, the drought stress has been shown to enhance the tolerance of the plants toward pathogens (Ramegowda et al. 2013; Achuo et al. 2006). The nature and outcome of plant–pathogen interaction under drought stress differs with the type of pathogens (fungi, oomycete, bacteria, and viruses) as they employ different strategies for infection. The different ways by which drought modulates plant's interactions with these pathogens are discussed. Apart from the above-mentioned two scenarios, pathogens can enhance the resistance of plants to drought (Reusche et al. 2012; Xu et al. 2008). However, this aspect is not discussed in this chapter.

10.2.1 Plant–Fungal/Oomycete Pathogen Interactions During Drought Stress

The availability of moisture is crucial for the establishment of fungal/oomycete infections on plants (Agrios 2005). The effect of concurrent drought and fungal/oomycete pathogen infection on plant growth has been fairly investigated in the past (Table 10.1). Drought stress can affect the plant–pathogen interaction by increasing or decreasing plant's propensity for infection. For soil-borne pathogens, the outcome of drought and fungal/oomycete pathogen interaction also depends on the effect of drought on the pathogen per se. So, under drought conditions, the degree of infection caused by a soil-borne fungi/oomycete on plants varies depending on whether the pathogen is favored by wet or dry soils (Cook and Papendick 1972). Drought can also influence the plant–pathogen interactions by inducing changes in the host physiology. The drought-induced changes in host physiology can be direct or indirect. The direct effects include the modulation of plant defense mechanisms against the pathogen. The indirect effects consist of changes in the nutritional status of plants brought about by drought stress.

10.2.1.1 Negative Effect of Concurrent Drought Stress and Fungal/Oomycete Infection on Plants

Fungal pathogens like *Sclerotium cepivorum* (causal agent of root rot in onions), *Streptomyces scabies* (causal agent of common scab in potato), *Fusarium* sp. (causal agent

of wilt in crop plants), and *Urocystis agropyri* (causal agent of smut on cereals), whose infections are known to be favored in dry soils, show more aggressive pathogenesis under drought conditions (Colhoun 1973). Edmunds (1964) observed that *Macrophomina phaseoli* (causal agent of charcoal stalk rot in sorghum) infection on sorghum plants under drought conditions caused more damage compared to nonstressed conditions. Drought conditions also enhanced the susceptibility of safflower and rhododendron to oomycete pathogen *Phytophthora* sp. (causal agent of root rot; Duniway 1977; Blaker and MacDonald 1981). Similarly, disease-resistant wheat plants were shown to become susceptible to *Fusarium roseum* f. sp. *cerealis* under drought stress (Papendick and Cook 1974). In all the above cases, the semidry conditions in soil apparently favored the fungal infection. The successful infection by fungal pathogens in dry soils can be possibly due to the fact that infection by these fungi depends on volatile root exudates that diffuse more rapidly through dry soil (Kerr 1964).

The altered physiology of plants due to drought stress can also favor the pathogen infection. For example, drought stress leads to nutrition deficiency in some plants and this secondary effect along with drought-induced physiological changes can aggravate the pathogen infection (Lawlor and Cornic 2002; Lawlor 2002). Drought stress-induced changes like the accumulation of osmolytes and nutrient leakage have been reported to lead to enriched nutrient supply for the pathogen. Drought stress-mediated exacerbation of infection under this category is best exemplified by pathogenesis of *Macrophomina phaseolina* (causal agent of charcoal rot in common bean) in common bean (Mayek-Perez et al. 2002). The stress-related amino acids like proline and asparagine have recently been shown to be utilized efficiently by *M. phaseolina* (Ijaz et al. 2013). The impact of drought was found to be more severe on a number of wilt and root-rot diseases. The wilt- and root-rot-causing fungi are known to interfere with the water relations of plants by colonizing the xylem vessels (Yadeta and Thomma 2013). Thus, the drought along with the pathogen imposes additional stress on plants and causes severe impact on plant growth.

10.2.1.2 Positive Effects of Concurrent Drought Stress and Fungal/Oomycete Pathogen Infection on Plants

The root-infecting oomycetes like *Pythium* sp. (causal agent of root rot in crops), *Aphanomyces* sp. (causal agent of root rot in sunflower and sugar beets), and *Plasmopara* sp. (causal agent of downy mildew) need adequate soil moisture for their survival in soil and for plant infection. Hence, the occurrence of downy mildew of sunflower and *Aphanomyces* root rot of sugar beets was less severe under drought stress conditions (Markell et al. 2008). Similar to soil-borne oomycete pathogens, less moisture in the atmosphere during drought is also shown to affect the pathogenesis of foliar fungal and oomycete pathogens. Many foliar pathogens such as those causing leaf spots are able to infect plants only when leaves are moist. Additionally, many foliar fungal pathogens produce spores that are dispersed by rain splash and germinated under high-humidity conditions. Pathogens that need rain to spread are unlikely to cause epidemics under drought conditions (Markell et al. 2008). The above-mentioned reports exemplify the effect of atmospheric water on the pathogen infection.

Drought acclimation in plants is known to combat some fungal pathogen infection during the combined stress. Ramegowda et al. (2013) showed that upon infection with *Sclerotinia sclerotiorum* (causal agent of white mold in beans), the well-watered *Nicotiana benthamiana* plants showed severe cell death, whereas the drought-acclimated plants exhibited reduced cell death. Thus, moderate drought was found to enhance plant's defense against pathogens by inducing expression of defense-related genes. The drought-mediated suppression of infection can also be attributed to the accumulation of abscisic acid (ABA). For example, drought-stressed tomato plants which showed the accumulation of ABA exhibited enhanced resistance against *Botrytis cinerea* (causal agent of grey mould in tomato; Achuo et al. 2006).

Taken together, drought can be favorable to either the pathogen or the host defense response. However, the consequences of concurrent drought on pathogen infection depend on the host, type of pathogen as well as the severity of drought stress. The ability of some fungi to interfere with the water relations of the plants and utilize the stress-induced molecules as nutrient source gives them an advantage under water stress conditions. On the other hand, plants can also fine-tune their defense responses under drought conditions to combat the pathogen infection. Thus, the modulation of plant–fungal/oomycete pathogen interaction during drought stress involves many facets, which can be interpreted by more systematic studies in this direction.

10.2.2 Plant–Bacterial Interaction During Drought Stress

Like fungi/oomycete, bacterial pathogens also depend on water for infection. The majority of the bacterial diseases are favored by the conditions of high humidity. A high water content in the apoplast facilitates bacterial growth. Incubation of plants at high relative humidity was shown to promote the growth of avirulent bacteria on plants (Freeman and Beattie 2009). Water-soaked lesions are typical characteristics of many bacterial leaf spot diseases and are known to be important for bacterial multiplication (Rudolph 1984). This reflects the importance of water in bacterial infections on plants. Thus, water scarcity should reduce bacterial infection on plants. This is true for the majority of cases. However, drought in few cases enhances plant's susceptibility to bacterial infections. Thus, drought can modulate plant–pathogen interactions for either the benefit of the host plant or the bacterium. A detailed discussion of both the scenarios is provided below.

10.2.2.1 Negative Effect of Concurrent Drought Stress and Bacterial Infection on Plants

Drought stress was found to enhance the susceptibility of grapevines to *Xylella fastidiosa* (causal agent of Pierce's disease; Thorne et al. 2006). *X. fastidiosa* has been reported to spread in plants by causing damage to intra-vessel pit membranes (Newman et al. 2003). The exposure of plants to drought conditions has also been

shown to lead to the disruption of pit membranes (Stiller and Sperry 2002). Drought stress, thus, facilitates the spread of *X. fastidiosa* in the plant. Drought-stressed *Arabidopsis* plants were found to be susceptible to an avirulent bacterial pathogen, *Pseudomonas syringae* pv. *tomato 1065* (Mohr and Cahill 2003). In this study, the susceptibility induced by drought was attributed to ABA. The exogenous ABA treatment is shown to render *Arabidopsis* plants susceptible to *P. syringae* infection by probably suppressing the salicylic acid (SA)-mediated defense responses (Mohr and Cahill 2003). Bacteria also modulate ABA-mediated responses for their infection and survival inside the plants. HopAM1, a type III effector of *P. syringae*, increases the virulence of a weak pathogen (*P. syringae* pv. *maculicola* M6 CE) under drought stress condition by enhancing the ABA-mediated suppression of basal defense responses in plants (Goel et al. 2008).

Drought stress has also been found to contribute to enhanced susceptibility of plants to vascular wilt causing bacteria. In combination with drought stress, *X. fastidiosa* (causal agent of Pierce's disease) increases the severity and progression of leaf scorch in *Parthenocissus quinquefolia* vine, reducing the total leaf area and number of nodes (McElrone et al. 2001). The dual stress caused increased reduction in stomatal conductance, leaf water potential, hydraulic conductivity, and xylem vessel length (McElrone et al. 2003) compared to individual stresses.

Another factor responsible for severe occurrence of disease under drought condition is reduction in the population of antagonistic bacteria in dry soils. For example, drought conditions are known to increase infection caused by *S. scabies* (causal agent of common scab in potatoes) in potatoes (Lapwood 1966). The decreased abundance of antagonistic bacteria in dry soil which otherwise limit lenticels infection by *S. scabies* leads to enhanced infection under drought conditions (Lewis 1970).

10.2.2.2 Positive Effect of Concurrent Drought Stress and Bacterial Infection on Plants

Moderate drought stress can enhance the tolerance of plants to bacterial infection by activating the stress response machinery. The acclimation of *N. benthamiana* plants to moderate drought stress (40–60% field capacity [FC] of soil) increased its tolerance to bacterial pathogen *P. syringae* pv. *tabaci* (causal agent of wildfire disease in tobacco) (Ramegowda et al. 2013). The degree of disease tolerance in drought-stressed plants was correlated to the extent of reactive oxygen species (ROS) accumulation (Ramegowda et al. 2013). The relation of increased ROS content to defense against bacterial infection was further substantiated by the application of methyl viologen (MV), a compound that provokes ROS production by disrupting electron transport chain in chloroplast. The MV-treated plants had high ROS and showed decreased bacterial growth (Ramegowda et al. 2013).

Drought stress can also help prevent pathogen multiplication and spread. At cellular level, water-deficit conditions help the plant to prevent bacterial survival and progression. In fact, *Arabidopsis* plants are known to promote effector-mediated signaling for localized desiccation of site of pathogen infection (Freeman and

Beattie 2009). Plants employ this effector-mediated localized desiccation possibly by one of the three ways, namely programmed cell death (PCD) of the vascular tissues, pectin-mediated occlusion of vessels, and reduction in aquaporin-mediated water exchange from xylem to surrounding tissues (Beattie 2011).

10.2.3 Plant–Viral Interaction During Drought Stress

The majority of the available reports on the effect of concurrent drought on viral infection suggest the negative impact of the concurrent stresses on plants (Olson et al. 1990; Clover et al. 1999; Sether and Hu 2001; Prasch and Sonnewald 2013). Drought stress has been shown to affect susceptibility of plants to viral infection. Moderate drought (0–15 %) increases the susceptibility of bean plants to *tobacco mosaic virus* (TMV) by fourfold (Yarwood et al. 1955). Furthermore, the simultaneous infection of *Pineapple mealybug wilt-associated virus-1* (PMWaV-1) and drought stress in pineapple has been reported to cause more loss in fruit production than that caused by the individual stresses (Sether and Hu 2001). Similarly, the concurrent drought stress and *Maize dwarf mosaic virus* (MDMV) infection in sweet corn during vegetative and reproductive stages were found to additively reduce the growth and yield of plants (Olson et al. 1990). This may be due to the fact that viral infections under drought stress can subvert plants' metabolic machinery toward viral multiplication and stress responses. Recently, Prasch and Sonnewald (2013) studied the molecular responses of *Arabidopsis* plant subjected to concurrent *turnip mosaic virus* (TuMV) infection, heat, and drought stress. The concurrent drought and viral infection led to greater reduction in biomass. However, the TuMV level was not altered in the dually stressed plant (Prasch and Sonnewald 2013). The combined stress was found to alter the circadian rhythm of plant by increasing the expression of circadian clock-associated 1 (*CCA1*) gene that is known to regulate a wide array of genes including genes involved in photosynthesis. The combination of viral infection and drought stresses down-regulated the genes involved in photosynthesis, adenosine triphosphate (ATP) synthesis, glycolysis, and tricarboxylic acid (TCA) cycle. In contrast, the expression of genes involved in photorespiration, such as glycolate oxidase and glucose–glyoxylate aminotransferase, was up-regulated. This possibly resulted in reduction in biomass (Prasch and Sonnewald 2013). Thus, the concurrent drought and viral infection possibly force plant machinery to divert its energy toward defense responses, thereby leading to the down-regulation of photosynthesis and other primary metabolic pathway genes.

Drought has also been shown to negatively affect virus translocation in plants (Liu et al. 2009). For example, drought inhibits the systemic spread of *tomato spotted wilt virus* in tomato (Cordoba et al. 1991). Moreover, in the study of Yarwood et al. (1955), increased drought intensity was found to decrease the viral infection in bean leaves. This signifies that the intensity of drought has a role to play in deciding the outcome of plant–viral interactions. Unlike bacteria, fungus, and oomycete, virus does not require nutrients for its growth, so drought-driven alleviation of viral infection apparently occurs by some other mechanisms that are not yet known.

10.3 Plant–Pathogen Interactions During Drought Stress: Current Understanding of the Underlying Molecular Mechanisms

The signaling mechanisms involved in plant responses to biotic and abiotic stress conditions have been well elucidated. Various studies in this direction have led to the identification of a number of genes that are co-regulated under abiotic and biotic stress conditions. The occurrence of cross talk between signaling pathways of abiotic and biotic stresses is well known (Fujita et al. 2006; Tippmann et al. 2006; Fraire-Velázquez et al. 2011). A couple of reports on the molecular mechanisms of plant's resistance against concurrent drought–nematode and drought–viral infection (Atkinson et al. 2013; Prasch and Sonnewald 2013) revealed the occurrence of "shared" and "tailored" responses in plants exposed to the concurrent stresses. The shared response consists of genes commonly expressed in abiotic and biotic stress conditions. The tailored response, on the other hand, implies the genes activated/repressed exclusively in response to the concurrent stress conditions. The "shared response" can be largely understood from the molecular mechanisms of plant response under independent and concurrent stress conditions. However, the inferences drawn from the individual stress studies cannot be extrapolated to explain the tailored response of plants under concurrent stresses. In this section, we describe the molecular basis of plant responses to concurrent drought and pathogen stresses based on our understanding from independent and the combined stress studies (Fig. 10.1).

10.3.1 Clues from Studies on Independent Stresses

As already stated, the abiotic and biotic stress response machinery of plants shares some common elements (Fig. 10.1a). The various elements of abiotic and biotic stress signaling are known to interact with each other leading to a cross talk between the signaling components of the two stress response pathways. Among the common elements, the most important are ROS and Ca^{2+}. Independent exposure of plants to drought and pathogen stress leads to a rapid increase in the levels of Ca^{2+} and ROS in the cells (Takahashi et al. 2011; Miller et al. 2010). The further downstream components of the signaling cascades, namely calcium-dependent protein kinases (CDPKs) and mitogen-activated protein kinases (MAPKs), are also known to play a synergistic role in drought and pathogen stress response of plants. For example, SA-induced MAPK (SIPK) is known to be activated by both SA and osmotic stress (Mikolajczyk et al. 2000; Hoyos and Zhang 2000). However, the modulation of MAPK expression also confers antagonistic effects on different stress responses (Xiong and Yang 2003; Shi et al. 2011). Also, silencing of OsMAPK5 in rice leads to constitutive up-regulation of pathogenesis-related (PR) proteins and enhanced pathogen resistance. However, these plants were sensitive to salt, cold, and drought stress (Xiong and Yang 2003).

The response of plants to drought and pathogen infection is known to be largely regulated by phytohormones. The exogenous application of drought-responsive hormone, ABA, has been shown to increase the disease susceptibility in a number of studies (Thaler and Bostock 2004; Mohr and Cahill 2003; Audenaert et al. 2002; de Torres-Zabala et al. 2007). The ABA-deficient tomato (*sitiens* mutant) plants have been found to exhibit enhanced resistance to *B. cinerea* infection due to enhanced PR proteins and repression of SA response (Thaler and Bostock 2004; Audenaert et al. 2002). The enhanced resistance to pathogen infection in ABA-deficient mutants can be attributed to reduced cuticle thickness and enhanced H_2O_2 production in response to *B. cinerea* in tomato (Asselbergh et al. 2007) and altered cell wall composition in *Arabidopsis* (Sanchez-Vallet et al. 2012). Contrastingly, the role of ABA as a positive regulator of defense has also been reported (Mauch-Mani and Mauch 2005; Melotto et al. 2006; Ton et al. 2009). ABA is shown to regulate plant defense responses against pathogens through a number of ways like modifying callose deposition, promoting stomatal closure, and regulating the expression of defense genes. For example, ABA is necessary for β-aminobutyric acid (BABA)-induced callose deposition during defense against fungal pathogens (Ton and Mauch-Mani 2008). However, it blocks the callose deposition induced by bacterial infection (de Torres-Zabala et al. 2007). ABA activates stomatal closure that acts as a barrier against bacterial infection (Melotto et al. 2006). Moreover, transcriptome and meta-analyses of gene expression profiles of *Arabidopsis* plants infected with *Pythium irregular* led to the identification of ABA-responsive element (ABRE) in the promoters of many of the defense genes (Adie et al. 2007; Wasilewska et al. 2008). Thus, ABA acts as a global switch regulating response toward biotic and abiotic stresses (Asselbergh 2008). However, the mechanism of action of ABA is still not completely deciphered. The identification of the molecular mechanisms involved in phytohormone-mediated cross talk between biotic and abiotic stress signaling needs to be done in order to elucidate the exact molecular mechanism by which different phytohormones modulate plant defense responses against different pathogens under drought conditions.

Together with the phytohormones, transcription factors (TF) like ABA-responsive element-binding protein (AREB), MYC, NAM//ATAF1/CUC2 (NAC), ethylene-responsive element-binding protein (EREB), WRKY, and coronatine insensitive 1 (COI1) are activated by pathogen challenge and drought stress (Atkinson et al. 2013). MYC2 has been found to be important in the interaction between the abiotic and biotic stress pathways. It is activated by ABA (Abe et al. 2003) and positively regulates jasmonic acid (JA)-induced defense genes, but represses the combined JA- and SA-mediated gene expression (Laurie-Berry et al. 2006; Pieterse et al. 2009). NAC and AP2/ERF TFs have also been associated with both abiotic and biotic stress signaling. NAC TFs like OsNAC6 (*O. sativa* NAC), tobacco stress-induced1 (TSI1), RD26, and botrytis-susceptible1 (BOS1) induce tolerance to both abiotic and biotic stresses, others like *A. thaliana* activating factor 1 (ATAF1) impart tolerance to either of the stresses (Mengiste et al. 2003). Apart from these, ribosome production factor 1 (RPF1), WRKY82, and WRKY85 have been shown to play roles in conferring stress tolerance to both biotic and abiotic stresses (Asselberg et al. 2008;

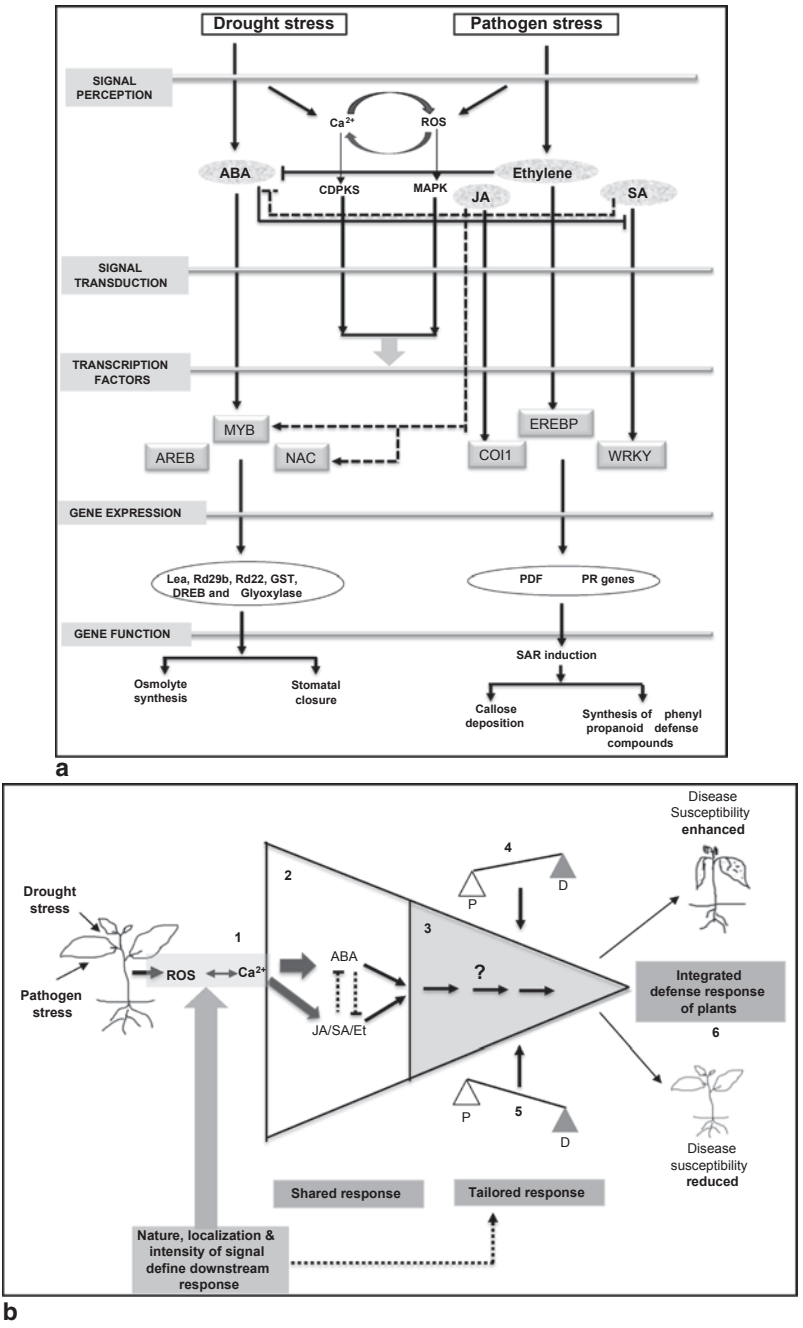

Fig. 10.1 Molecular understanding of the effect of concurrent drought on plant–pathogen interactions. **a** Schematic representation of cross talk between key players of plant defense response against concurrent drought and pathogen infection. The figure shows the signaling cascades and

Qiu and Yu 2009; Peng et al. 2011). Genes that confer tolerance to both biotic and abiotic stress can form a part of the shared response exhibited by plants under concurrent drought and pathogen infection. However, their function under concurrent stress conditions needs to be validated. The above-described independent single stress studies are not useful for understanding the tailored response. Clear understanding can be obtained only from combined stress studies.

10.3.2 Clues from Combined Stress Studies

A recent study by Atkinson et al. (2013) on concurrent drought and nematode infection revealed that in addition to the overlapping transcript changes, the combined stress treatment induced a set of genes that were not differentially regulated by either of the single stresses. This study thus points toward the activation of a tailored response which consists of unique program of gene expression in response to the combined stresses. The genes differentially expressed under combined stress included those involved in cell wall modification, carbohydrate metabolism, redox regulation, and transcriptional regulation. A characteristic down-regulation of disease-resistance genes (e.g., azelaic acid induced 1; *AZI1*) was also observed under concurrent stress treatment. This may be due the suppression of SA-mediated signaling by ABA. In order to understand the effect of concurrent stress on plants, Prasch and Sonnewald (2013) subjected *Arabidopsis* plants to concurrent drought, heat stress, and viral infection. The analyses of the microarray profiles of the stressed plants revealed the expression of 11 genes under all the stress (single, double, and triple stress combinations) conditions. These common genes are the ones encoding transcription factors like Rap2.9 and G-box binding factor 3 (GBF3), a transmembrane receptor and a lipase. The transcript analysis also showed 23 stress-specific genes that were differentially expressed in the triple stress condition. This consisted

a few representative proteins. The *dotted arrows* indicate the induction or suppression of abiotic stress response elements by the biotic stress response elements, whereas the *bold arrows* indicate the modulation by the ABA on biotic stress response elements. **b** Schematic representation of the hypothetical response of plants to concurrent stress conditions. The first line of defense in plants exposed to concurrent drought and pathogen infection presumably consists of Ca^{2+}-dependent ROS production (*1*). The nature, localization, and intensity of ROS and Ca signals can define the downstream events. The overall response of plants to concurrent stress is a combination of shared (*2*) and tailored responses (*3*) and this defines increased or decreased plant susceptibility to pathogen infections under drought stress. The question mark signifies the unexplored events of the tailored mechanism. The response (*6*) of the plants to the concurrent stress conditions depends on the intensity of the two stresses (*4/5*) as well as the nature of host and plant. The *small triangles* represent the intensity of drought stress (*D*) and the pathogen load (*P*). *ROS* reactive oxygen species, *ABA* abscisic acid, *JA* jasmonic acid, *SA* salicylic acid, *Et* ethylene, *SAR* systemic acquired resistance, *PR* genes pathogen-related genes, *CDPKs* calcium-dependent protein kinases, *MAPK* mitogen-activated protein kinase, *AREB*, ABA-responsive element-binding protein, *NAC NAM// ATAF1/CUC2*, *COI1* coronatine insensitive 1, *MYB myeloblastosis, EREBP ethylene responsive element binding protein, WRKY* stands for the first four amino acids (tryptophan [W], arginine [R], lysine [K] and tyrosine [Y] of the heptapeptide WRKYGQK, which is the hall mark of WRKY proteins, transcription factors

of three transcription factors including DREB2A, and two zinc finger proteins together with other stress-responsive proteins like cold-regulated 47, ABI5 binding protein (AFP1), a pentatricopeptide repeat-containing protein, and a universal stress protein family protein. The gene list also shows the presence of positive and negative regulators of a particular pathway. For example, AFP1 is a negative regulator of ABA, whereas *Arabidopsis* Toxicos en Levadura (ATL4) is a positive regulator. Major factors that can decide responses under concurrent stress conditions include the severity and complexity of the stresses imposed. For example, in the above study, the number of significantly regulated genes corresponding to drought alone, virus alone, and stress combinations varied and corresponded to 518, 682, and 1744 respectively (Prasch and Sonnewald 2013).

On the basis of both the cross talk and concurrent stress studies, we hypothesize a mechanism of plants response to concurrent stress conditions (Fig. 10.1b). Like the individual stress conditions, under concurrent stress conditions, the Ca^{2+}-dependent ROS production forms the first line of defense. We hypothesize a preferential role for ABA in governing the concurrent stress responses than the other hormones. However, this certainly needs to be validated and there may be exceptions. The regulation mediated by JA, SA, and ET, however, also seems to be important and this can be a key feature in the differentiation of response of plants against various pathogens (necrotrophic/biotrophic).

10.4 Conclusions and Future Perspectives

The global climate change is leading to the emergence of new and complex stress combinations and the impact of these stress combinations on crop productivity is evolving as a major concern. Considering the impact of abiotic and biotic stress conditions on crop yield, enormous efforts have been made over the past three decades, to understand the independent effect of these stress conditions on plants. The concurrent drought and pathogen infection can either increase the susceptibility of plants to the pathogen or it can suppress the pathogen infection depending on various factors like type of the pathogen, host species, and severity of drought stress. For example, drought aggravates the diseases caused by wilt/rot-causing pathogens. On the other hand, drought acclimation has been shown to confer resistance to pathogen infection in some cases. Drought environment can also affect the pathogen per se. Although a number of reports reflect on the physiological effect of concurrent drought stress on plant–pathogen interactions (Table 10.1), the understanding of molecular mechanism imparting combined stress tolerance in plants is in its infancy. As is evident from the two reports on molecular responses of plants to concurrent stresses, the combat mechanisms of plants to concurrent abiotic and biotic stresses are characterized by a combination of shared and tailored responses. Whereas the shared responses are nearly well deciphered, the molecular events leading to and explaining the tailored responses are yet to be understood. The detailed analysis of the plant responses under concurrent drought and pathogen infection is needed to

unravel the intricate regulatory network involved in plant–pathogen interactions under such conditions. The candidate genes differentially expressed under the concurrent stress conditions can be the potential targets for the manipulation in order to develop plants with improved resistance under concurrent drought–pathogen infection. These genes can also serve as important markers for selecting the concurrent stress-resistant crops.

However, the experimental evaluation of the effects of the combined drought and pathogen stress on plants is a challenging task owing to the difficulties in accurate concurrent stress imposition on plants. For example, compared to imposition of heat stress, coinciding drought stress conditions that occur gradually in soil-drying experiments with pathogen infection is difficult. The other hurdle of combined stress studies is the optimization of inoculum concentration and drought intensity that would not be lethal to the plant when imposed concurrently. These two factors are important deciding factors of the outcome of combined stresses. Owing to these complexities, physiological, molecular, and biochemical changes in plants exclusively exposed to concurrent stress conditions are yet to be identified. We need to develop standardized protocols for the imposition of drought stress and concurrent pathogen infection in order to assess the impact of drought on plant–pathogen interaction.

Effective categorization of the pathogens on the basis of their dependence on water for infection needs to be done. The pathogen which is more infective under drought conditions can be a possible threat to crops in the areas prone to drought stresses. Thus, understanding the effect of drought on pathogen can help in the prediction of emerging diseases under drought condition. This would be particularly helpful in case of predicting the effect of pathogens causing wilts and rot on plants under drought conditions. Overall, unraveling of physiological and molecular basis of plant responses to concurrent drought and pathogen infection will be a crucial step forward for the development of stress-resistant crops that can survive under the field conditions.

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Chapter 11
Combined Stresses in Forests

Patrick Mitchell, Tim Wardlaw and Libby Pinkard

11.1 What Is Stress to a Tree or Forest Ecosystem?

Environmental stress can be viewed as the physical, chemical, and biological constraints on the productivity and development of ecosystems. For plants, Grime (1977) hypothesized that stress is one of the three fundamental drivers shaping plant strategies and he defined stress as a set of external constraints limiting the rate of resource acquisition, growth, or reproduction (Grime 1977). Stress, in a broad sense, is the major force limiting species distribution and ecosystem structure and function. Forest ecosystems are maintained in a dynamic equilibrium by continuous stress-inducing factors, as well as stochastic disturbance events. For example, primary climatic stress factors can be broadly categorized as light, temperature, and water and largely explain the distribution of biomes and forest types globally (Boisvenue and Running 2006). Competitive and other biotic interactions are also important in limiting species and population distribution and function. Thus, the role of stress in triggering and shaping plant functioning is complex and can be better understood by considering responses that arise when a particular individual or population is exposed to conditions outside its normal operating range.

The impacts of stress on growth and development are evident at different temporal scales for both individuals and populations (Fig. 11.1a). At the whole-plant level, the initial stress response or period of decline in a process such as growth or photosynthesis, happens within seconds to days. Acclimation can follow the initial response and involves compensation or enhanced resistance to the initial stress

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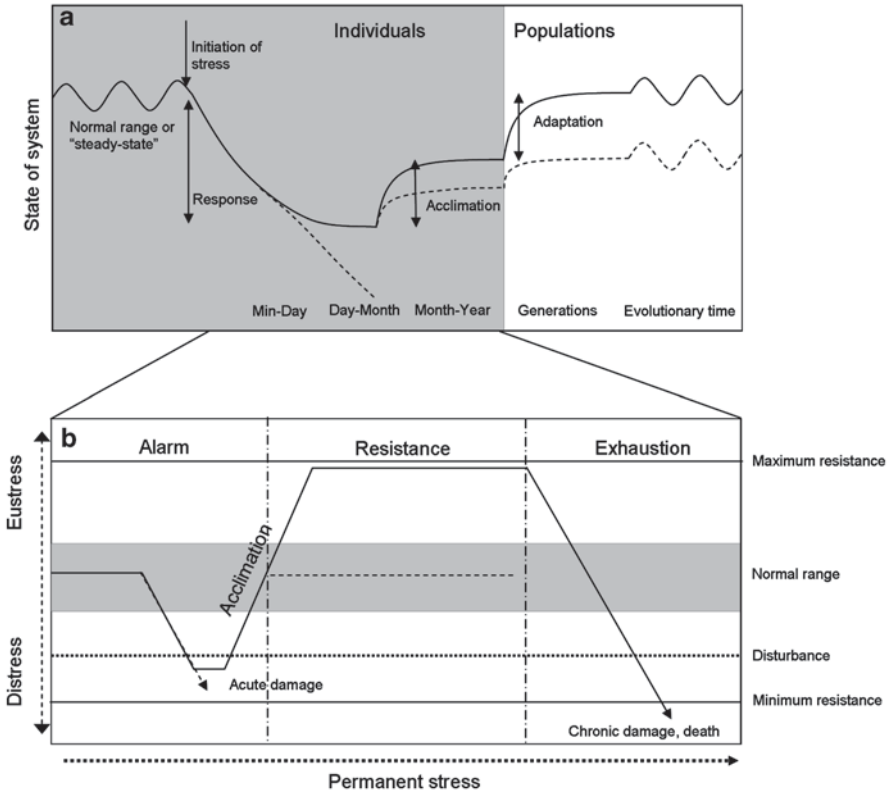

Fig. 11.1 Representation of the different scales at which stress defines the *response* of plant functioning in individuals and populations. **a** Describes changes in plant function or system state at both the individual and population level across a range of temporal scales. The *initiation of stress* in the individual results in a stress response, followed by a period of acclimation. Over generations, *adaptation* can allow further recovery from stress and some return to *normal conditions or the steady state* (*solid line*) or a new steady state (*dashed line*; modified from Lambers et al. 2008). **b** An individual exposed to a permanent stress exhibits three phases of stress as proposed by Selye (1936). The initial decline in plant function or distress can induce *acute damage* and may result in loss of biomass or plant injury (disturbance). The period of recovery, termed *eustress*, results from acclimation processes and may enable recovery back to the normal range (*dashed horizontal line*) or enhanced *resistance* to subsequent stress (*solid line*). If stress persists, *exhaustion* occurs whereby the plant shows *chronic damage or death*. (Modified from Steinberg et al. 2008)

response over days to weeks. Over longer time-scales, adaptation involves evolutionary responses arising from genetic changes in the population that can alleviate the impact of the stress (Lambers et al. 2008). Selye (1936) summarized the response of the individual to continuous or permanent stress into a three-phase stress model (Fig. 11.1b). The alarm phase is characterized by distress or a decline in physiological function. The resistance phase involves recovery to the normal range in functioning and may include a period of acclimation that increases resistance to subsequent stress. Finally, the exhaustion phase occurs if the stress continues or intensifies so

that chronic distress dominates any acquired resistance. In this generalized model of stress, improved stress resistance in response to the initial stress involves energetic costs and changes and the expression of different genes to trigger a suite of acclimation processes (e.g., heat shock proteins, osmoregulatory compounds) that enhance resistance to subsequent stress (Steinberg et al. 2008). If the stress is maintained, exhaustion eventuates, causing chronic damage and a collapse of cellular functions (e.g., membrane integrity, photosynthetic apparatus). While Selye's three-phase stress model was originally formulated to describe human physiology, it provides a simple model of how stress-defense systems might develop in individual plants.

Because tree species are long-lived, they may be exposed to multiple cycles of stress and/or various types of stress that act in concert to bring about changes in plant functioning and survival. In response to a myriad of stress combinations, trees have evolved many strategies to resist, tolerate, and recover during periods of stress. Climate change and other human influences and disturbance have the potential to introduce novel combinations of stressors that make predicting impact from multiple stressors exceedingly difficult. For example, changes in temperature and atmospheric [CO_2] will modify the range of "normal conditions" at which species will operate, which could have implications for recovery rates and effectiveness of acclimation processes during acute or chronic stress events.

To date, the study of forest stress within the fields of forest pathology, entomology, ecology, and tree physiology has taken different perspectives regarding the significance of multiple stressors. Forest pathology and entomology have sometimes assumed that epidemics of insects or fungi and the associated stress were dominated by single causal factors (Mueller-Dombois 1986). This perspective has often failed to explain the causes and consequences of major pest outbreaks in forests, because it tended to ignore other contributing factors such as stand-level dynamics and climatic variation (Mueller-Dombois 1987; Akashi and Mueller-Dombois 1995). Plant physiologists tend to explore stress by minimizing inherent complexities of stress events through careful experimental manipulation that focuses on specific responses to stressors such as drought/water deficit or salinity. These studies provide an important mechanistic basis for how plants respond and cope with stress, but are rarely of sufficient scale and design to properly consider the impact of multiple stressors and changes in their intensity, duration, or frequency. Ecologists attempt to explore the impacts of one or multiple stressors in the field through observation of natural and human-induced gradients in environmental conditions. However, these studies are often retrospective and must disentangle layers of complexity from observed impacts and scant mechanistic information. A more holistic picture of forest responses to stress involves an appreciation of the mechanistic and physiological insights within the context of complex trophic interactions, spatial and temporal variation in the landscape, and their role in triggering a hierarchy of responses within a population or ecosystem. To start gaining a deeper understanding of environmental stress and its multifaceted nature, it is important to consider these challenges using conceptual frameworks through which the system can be viewed.

Understanding changes in forest health in the face of rapid climate change presents further challenges surrounding how we utilize the wealth of climate projections

to predict potential stress dynamics and responses in biological systems such as forests (Bonan 2014). For example, projections of reduced water availability and concomitant increases in temperature might be predicted with reasonable certainty for a particular region or landscape. Yet, predicting impacts on a forest ecosystem is difficult, given that co-occurring tree species respond very differently to drought, owing to differences in factors such as rooting patterns, water management strategies, and ontogeny (Koepke 2010; Fensham and Fairfax 2007; Engelbrecht and Kursar 2003; Mitchell et al. 2008). In the case of drought and many other potential environmental drivers, the resultant physiological stress and the associated impact is not purely defined by the exposure (i.e., climatic drivers) to stress, but also by how exposure interacts with the sensitivity of the organism or system to produce an impact on the system. Sensitivity encompasses many factors, including genetic/phenotypic traits, soil conditions, and stress history for a particular site. Thus, it is important to consider physiological stress for an individual as an interaction between components of exposure and sensitivity in determining what factors are important for understanding vulnerability of forests to potential stress-inducing factors (Mitchell et al. 2013).

In this chapter, we examine how different abiotic and biotic factors combine to induce stress in trees, and its impacts on forest health more broadly. Some relevant conceptual frameworks are introduced that help to disentangle interrelations between the drivers of stress and interpret the range of impacts often described and observed in forests under stress. Examples of combined stresses are used to emphasize that physiological stress commonly arises through the joint contribution of primary, secondary, anthropogenic, and conditioning factors. The relevance of intensity, frequency, and duration of the individual and combined stress is discussed in conjunction with how they moderate physiological distress and recovery. A large focus of this chapter concerns stressors associated with global climate change, with a particular emphasis on associated increases in drought. However, insights gleaned from these examples are pertinent to many other types of stresses in natural and managed forest ecosystems.

11.2 Conceptualizing Multiple Stressors and Their Consequences for Forests

The causes and consequences of changes in forest health and condition can be viewed as a continuum of responses that are related to the temporal scale at which they impact on forest health (Fig. 11.2). At one end of this continuum lie forest declines or diebacks, which can be characterized as a protracted malfunction of tree health and a progressive decline in stand vigor and productivity over time (Mueller-Dombois 1988). Forest declines tend to occur over decades or even generations (Fig. 11.2). Forest declines tend to be driven by a combination of biotic and abiotic stressors, often involving multiple trophic-level interactions and a strong role from human influences (Jurskis 2005; Manion 1981). An example of forest decline involving complex trophic interactions is the phenomenon known as Bell miner associated dieback in Australia. This form of forest decline became common in the

Fig. 11.2 Responses of forest ecosystems to stress can be described as a continuum based on the duration over which the forest is impacted and the role of different factors in mediating the stress

early 1990s, and by the early 2000s was estimated to threaten 2.5 million ha of remnant eucalypt forests in northern New South Wales and south-eastern Queensland (Wardell-Johnson et al. 2005). Bell miner associated dieback is attributed to the exclusion of natural enemies of leaf-feeding psyllids by high densities of bell miner (*Manorina melanophrys*) populations. Dieback is triggered by increases in defoliation from psyllids that may increase the susceptibility of trees to additional biotic attack. Dieback in several eucalypt species was thought to be driven by multiple feedbacks between forest structure and site conditions, physiological responses that alter foliar chemistry, the abundance of sap-sucking psyllids, and bell miner populations (Stone 2005). At the other end of the forest stress continuum lie those event-driven changes in forest health caused by acute stress. These stress events tend to operate at much shorter temporal scales (months to years) and tend to be driven primarily by climatic factors (Jurskis 2005). As discussed in Box 1, episodic stress events such as droughts are frequently characterized by multiple climatic and biotic stressors; however, they tend to involve a less complicated set of feedbacks. It is also worth pointing out that the initial trigger for both types of responses may be quite similar, i.e., long-term drought, yet differences in the intensity, frequency, and duration of the primary driver influence the rate at which forests are impacted.

Box 1 A surge in the awareness and study of drought impacts on forests in the last decade is providing a glimpse of how multiple stressors might combine to affect tree health and survival as a consequence of global environmental change (Allen et al. 2010; van Mantgem et al. 2009). Drought-induced tree die-off events are examples of extreme stress events in forest ecosystems reflecting conditions beyond the tolerances of the affected tree species. A survey of published studies documenting episodic tree die-off events highlights the universal role that drought plays across a diverse range of forest types, including semi-arid shrub lands through to tropical rainforests (from Allen et al. 2010, Mitchell et al. 2014 and *unpublished data*). A clear pattern emerging from these studies is that these extreme drought events generally coincide with elevated temperatures and heat-wave events (Allen et al. 2010; Mitchell et al. 2014). This is a well-documented climatological phenomenon that occurs at regional and continental scales (Vautard et al. 2007; Lyon 2011). In addition to heat stress, a large proportion of drought die-off events are associated with biotic agents (Fig. 11.3). In this survey, 25 of the 67 die-off events had some evidence of biotic agents with defoliating and wood-boring insects being most common

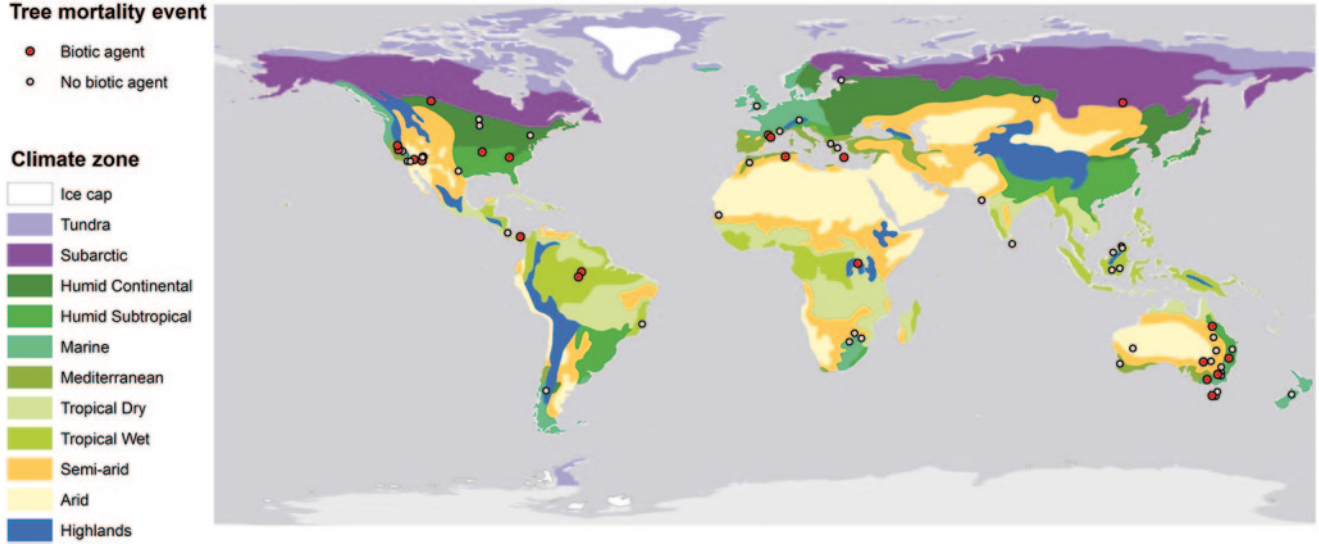

Fig. 11.3 Location of drought-induced forest die-off events across the world in relation to 12 different climatic zones. Those events associated with biotic agents, e.g., defoliating insects, stem borers are highlighted in *red*

and a small number of observations involving fungal infection (*unpublished data*). The majority of die-off events associated with biotic agents occurred at sites where mean annual temperature was <20 °C, a pattern suggesting a greater likelihood of drought–biotic agent interactions in relatively cooler environments. It is important to note that this survey only involves die-off events that were characterized as episodic; relatively sudden incidences of canopy collapse and mortality during or directly after a drought event. As we discuss in this chapter, protracted tree declines are almost always associated with biotic agents as tree health is diminished over several years or decades, exposing them to greater incidence of infection or attack.

Studies of protracted forest decline from many different ecosystems demonstrate that a complete etiology of these types of stress involves a consideration of climatic, soil, stand dynamics, physiological, genetic, and ecological factors and their distribution through time and space (Mueller-Dombois 1986; Manion 1981; Landsberg 1983). Manion (1981) presented the concept of a decline syndrome that involved three or more sets of factors to explain the complex interactions of biotic and abiotic factors. The first set of factors is termed predisposing; those long-term factors that are relatively static, such as climate, site conditions, and genotypic/phenotypic traits Manion (1981) suggested that predisposing factors weaken a plant growing in a particularly hostile location. The second set of factors, termed inciting, induces short-term stress on trees associated with events such as insect defoliation, frost, drought, or air pollution. It is assumed that these events result in drastic injury to the plant. The third set of factors is termed contributing factors, tending to operate over the long term, because they persist on the host for extended periods. While infestation by contributing agents such as canker fungi, bark beetles, or viruses is often thought to be the key agent inducing dieback or mortality, they may merely occur in association with stress caused by predisposing and inciting factors. Manion's model provides a useful approach for clarifying the contribution of different factors when there are multiple biotic agents present during protracted forest decline.

Episodic or acute stress arises from relatively discrete events that induce short- (minutes to days) and long-term (months to years) responses involving plant defenses, productivity, and survival. One prominent framework describes severe drought events that are associated with tree mortality and forest collapse (McDowell et al. 2008). McDowell and colleagues proposed two interrelated pathways through which tree water and carbon balance influence the process of tree death (McDowell et al. 2008, 2011). One physiological pathway resulting in mortality is termed hydraulic failure. This is caused by severe declines in tree water balance that produce large tensions on the water column in the xylem, and cavitation involving entry of air into the water-transport system (Tyree and Sperry 1988, 1989). The process of cavitation is well documented and is thought to result in cell death through dehydration (Brodribb and Cochard 2009). Alternatively, carbon starvation is a process involving the exhaustion of available carbohydrates. The exhaustion of carbohydrates comes about through an imbalance between carbohydrate supply from photosynthesis, and demand from growth and respiration (McDowell et al. 2008). This pathway for mortality during drought has been postulated, because water deficit can reduce photosynthesis via stomatal closure, while respiration continues to deplete

stored carbohydrates. Additional factors such as biotic agents can amplify declines in carbon balance, if carbon supply or transport is compromised through stressors such as defoliators or wood borers (Galiano et al. 2011). While trees can deplete carbohydrates during drought (Mitchell 2013; Hartmann et al. 2013; Poyatos et al. 2013), there is limited evidence for implicating carbon starvation solely for tree mortality, because trees rarely exhaust measurable stores of carbon. However, given our current knowledge of how plants store, translocate, and utilize carbohydrates during drought (Sala et al. 2012), it is likely that low carbohydrate availability can effect water transport and heighten physiological stress. This framework has helped to stimulate much research into how primary drivers such as water deficit facilitate the action of multiple stressors associated with plant hydraulics, carbohydrate dynamics, and plant defensive systems.

The other important element of McDowell's mortality framework is that it links exposure or the attributes of drought intensity and duration with the plant's life-support system (McDowell et al. 2011). For example, short and intense droughts will reduce plant water balance, rapidly leading to hydraulic failure, and have little effect on the availability of carbohydrates. Conversely, because carbohydrate utilization is rate-limited through processes such as respiration, droughts that induce extended periods of zero or negative carbon balance will deplete carbohydrates (Mitchell 2013; Poyatos et al. 2013). Elevated temperatures may not only contribute to heat stress and increased evaporative demand but also increase respiration and the rate at which carbohydrates are depleted during long duration droughts (Adams et al. 2009). This framework also highlights the need to understand the dynamics of intensity and duration in defining the mechanisms underlying the observed stress.

Both of the frameworks outlined above describe interactions of multiple stressors using different perspectives and levels of detail. So, how can we develop a more generalized picture of the triggers and relationships between different factors across the entire continuum of responses identified in Fig. 11.2? One way to view physiological stress is to partition the influence of primary, secondary, anthropogenic, and conditioning factors in influencing plant health and physiological stress (Mitchell et al. 2013; Fig. 11.3). Primary factors such as drought tend to affect a forest over large areas and at the regional scale can operate independently of other biotic and abiotic factors. Secondary factors are dependent on the occurrence of primary factors, but may be the sole source of stress or act in concert with the primary factor. These are typically biotic agents and their impact can be related to: changes in host physiology or condition, climatic conditions, disturbance events, and food web dynamics (Garrett et al. 2006). Conditioning factors include soil depth and type, the size and age distribution of the stand and the site's stress history. These factors have a large influence on the spatial and temporal patterns of stress across the landscape and can introduce considerable variation in the impacts of stress events, even within monospecific stands. For example, meteorological drought conditions across forest landscapes can be relatively homogenous, yet the magnitude of physiological stress may be greater for stands on ridge top sites, where water availability is diminished by the shallow, porous nature of the soils (Matusick et al. 2013). Over longer timescales, these conditioning factors promote adaptation within the populations. Acclimation is triggered by changes in physiological condition at a range of scales

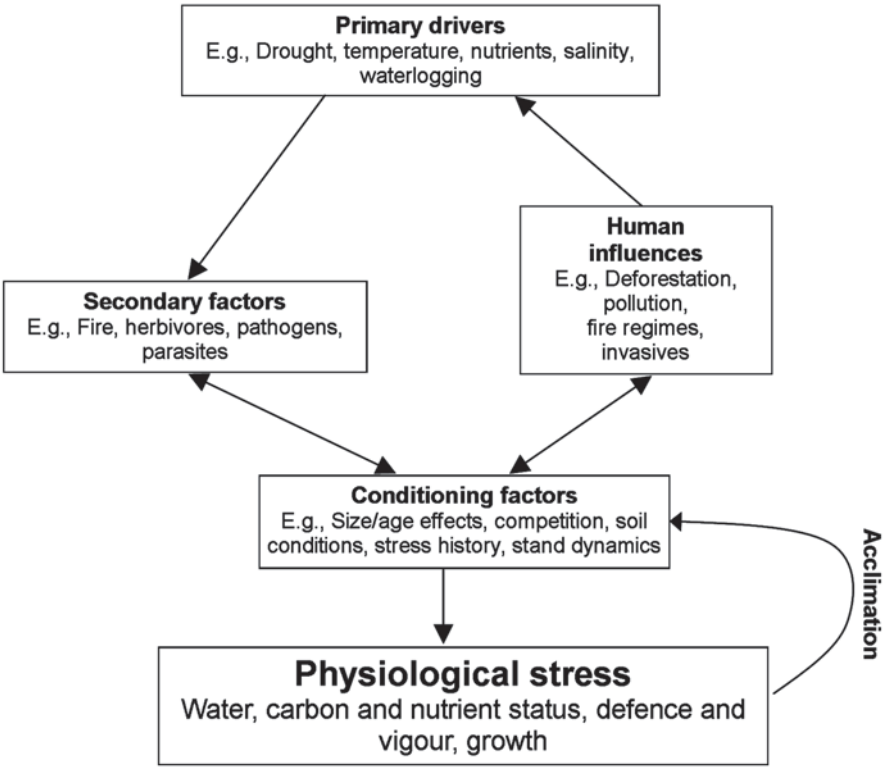

Fig. 11.4 A generalized framework for understanding the roles of *primary and secondary stressors, conditioning factors*, and *human influences* to define physiological stress. *Acclimation* represents a feedback on conditioning factors such as stress history and stand dynamics

and includes: changes in gene expression, biochemical changes in photosynthesis, changes in allocation patterns (e.g., reduction in leaf area) and an upregulation of plant defense compounds (Peñuelas et al. 2013; Breda et al. 2006). Acclimation may also induce feedbacks affecting the activity of secondary factors that can amplify the physiological stress further (see examples below). Anthropogenic factors or human influences play a role in influencing forest conditions in almost every ecosystem through deforestation, pollution, altered fire regimes, and the introduction of invasive species. These processes alter the presence and abundance of biotic agents at multiple trophic levels and play a major role in affecting the sensitivity of the forest to stress through changes in soil conditions, stand density, and structure.

The overview presented in Fig. 11.4 helps to highlight some key features in understanding the dynamics of stress in forest stands. First, unraveling the sequence of triggers for different stressors is crucial for understanding how stress comes about. Second, it is important to consider the impact of any given stress event as part of a longer-term regime of stressors that continually shape the sensitivity of the forest stand to subsequent stressors (Dreesen et al. 2014). High severity events that reduce stand density through mortality or canopy collapse can have a stabilizing effect on

forests and reduce the impact of future drought through acclimation and reductions in competition for soil water (Lloret et al. 2012). Patterns in stress response and recovery reflect the current state and conditioning of the system thereby influencing the severity of future stresses (Loehle and LeBlanc 1996; Niinemets 2010). Third, the contribution from primary, secondary, conditioning, and anthropogenic factors will vary according to their magnitude (intensity, frequency, and duration) and how they overlap in time and space. Some relevant examples are presented below that highlight how plants respond to different combinations of the primary, secondary, anthropogenic, and conditioning factors presented in Fig. 11.4.

11.2.1 Combinations of Primary Factors

At their extreme, low air temperatures can cause freezing injury including cell burst, damage to foliar and stem tissues, and death (Clements and Ludlow 1977). At sublethal levels, low temperatures capable of causing frosts inhibit rates of photosynthesis through limiting the rates of the biochemical reactions of photosynthesis. There can also be a light-dependent decrease in photosynthetic efficiency termed cold-induced photoinhibition, which can amplify the impacts of frost (Davidson 2004). Successive sublethal frost events reduced photosynthesis of *Eucalyptus globulus* and *E. nitens* saplings growing in southern Australia between 9 and 17%. High early morning light conditions following a frost event contributed to photosynthetic reduction via photoinhibition, but only before midmorning (Davidson 2004). Many tree species can acclimate to frosts which reduces photoinhibitoin effects (Long et al. 1983), although the effectiveness of acclimation varies between species. For example, photosynthesis of cold-acclimated *E. nitens* recovered within a day following a frost event, whereas in *E. globulus* it took 3 days to recover to pre-frost levels (Davidson 2004).

The combined stressors of waterlogging and salinity are common to many regions where disturbance has led to an increase in dryland salinity from increased water tables or increases in soil sodicity and reductions in infiltration (Barrett-Lennard and Shabala 2013). Waterlogging restricts plant growth by inducing hypoxia in the roots resulting in diminished carbon metabolism and nutrient supply (Trought and Drew 1980). Responses to salinity involve osmotically mediated changes in water status and toxic effects associated with salt accumulation in tissues (Munns and Termaat 1986). Under waterlogging and saline conditions, hypoxia exacerbates these toxic effects and affects plant K^+ nutrition (Barrett-Lennard and Shabala 2013). The co-occurrence of waterlogging and salinity can induce similar or larger reductions in gas exchange in eucalypt species depending on species tolerances to either of these stressors (van der Moezel et al. 1989).

The increase in atmospheric [CO_2] is thought to increase water-use efficiency during drought due to decreases in stomatal conductance, a common response observed in tree species exposed to elevated [CO_2] (Ainsworth and Rogers 2007). However, this leaf-level response may be negated where increases in leaf growth and vegetation cover under favorable conditions enhance stress impacts during adverse conditions. This has been demonstrated at the stand-level, where those stands

of *Liquidambar styraciflua* exposed to elevated [CO_2] experienced larger declines in leaf area index during water deficit than stands exposed to ambient conditions (Warren et al. 2011). These patterns presumably arise because gains in plant growth and tree size realized under elevated [CO_2], increases inter-tree competition for water during drought. Experiments assessing the interactive effect of drought, elevated temperature, and [CO_2] conclude that elevated temperature and [CO_2] can ameliorate the effects of potentially stressful water deficits from higher temperature at moderate drought intensities (Duan et al. 2013) but does not influence leaf water relations and time to mortality when water deficit is extreme (Duan et al. 2014).

11.2.2 Primary and Secondary Factor Interactions

Drought affects multiple physiological pathways in trees that can influence both the attractiveness of the host to particular pest species (e.g., increases in tissue carbohydrate and nitrogen concentration) and constitutive and induced defense mechanisms. Moderate water stress can promote defense through increases in secondary metabolites in foliage (Ayres and Lombardero 2000), while severe water stress can result in tougher foliage that reduces defoliation damage (Steinbauer 2001). However, acclimation to water stress can also enhance folivory activity in some species. Rivas-Ubach et al. (2014) concluded that increased production of compounds associated with osmoprotection (potassium, sugars, and antioxidants), a response that increases the tolerance of a low water potential in *Quercus ilex,* promoted more severe attack from defoliating insects, highlighting the complexity of drought–pest interactions (Fig. 11.5).

Moderate water stress can also promote secondary metabolite production in stems (Jactel et al. 2012), resulting in increased resistance to damage from pests such as stem borers and fungi (Fig. 11.6). Under severe water stress, the capacity of the host to divert carbohydrates to production of defense compounds decreases (Rouault et al. 2006), thereby reducing resistance to pests. For example, drought reduces the capacity of *Eucalyptus globulus* to produce bark exudates as a defense against the stem borer *Phorocantha mastersi* (Pook and Forrester 1984). Severe water deficit can result in increased concentration of compounds favoring fungal development, such as glucose which has been shown to stimulate growth of *Armillaria* spp and enable them to grow in the presence of normally inhibitory phenols (Wargo 1996).

11.2.3 The Significance of Conditioning Factors

An important conditioning factor affecting the sensitivity of plants is stress history. Dreesen et al. (2014) examined the impacts of one-off and repeated periods of drought and/or heat stress in herbaceous plant assemblages. Drought and heat treatments reduced leaf survival to a larger extent than either heat or drought alone, and the occurrence of successive drought and heat treatments with a low recovery window (2 weeks) increased the leaf sensitivity to the combined stress treatment.

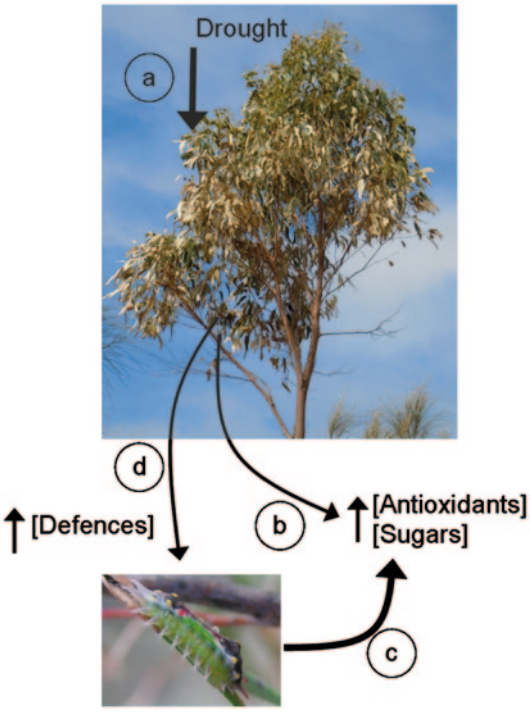

Fig. 11.5 Summary of how acclimation to *drought* stimulates increases in folivory activity. An increase in *drought* stress triggers (**a**) a shift in the foliar metabolome and a concomitant increase in the concentrations of *antioxidants*, nitrogen, and *sugars* (**b**). These changes in foliar chemistry promote higher folivory activity on drought-stressed trees (**c**). Increases in folivory can also stimulate an upregulation in the concentration of plant defensive compounds such as terpenes and phenolics (**d**). (Modified from Rivas-Ubach et al. 2014)

Interestingly, treatments that had longer recovery times (3.5 or 6 weeks) did not affect leaf and whole-plant survival (Dreesen et al. 2014). Studies in woody species show that the impact of repeated drought on photosynthetic capacity is dependent on the intensity and frequency of the drought regime (Liu et al. 2010). Incomplete recovery in some species was only observed after the third severe drought cycle and was attributed to stomatal limitations on photosynthesis (Liu et al. 2010). In another study, the increasingly incomplete recovery of photosynthesis was associated with a reduction in the maximum quantum efficiency relative to control plants, pointing to significant metabolic impairment of the photosynthetic apparatus (Liu et al. 2010; Gallé and Feller 2007).

Soil conditions can exert a strong control on the development of stress from primary climatic drivers by controlling how plants match water uptake with demand. While a mismatch in water uptake and demand is characteristic in plants experiencing drought stress, plant water uptake can be impeded in frozen soils. In boreal forest ecosystems, a delay in soil warming in frozen soils compared with air warming at the end of winter can result in plant water deficit even when soils are wet (Repo et al. 2005, 2008). The disjunct between root dormancy (or slowed metabolism) and increased shoot growth can induce xylem cavitation (the process of air filling and blocking xylem conduits), and lead to reductions in tree growth, photosynthetic efficiency, and plant water potential (Larsen 1993). Thus, rapid climate change in boreal forests that alters patterns in frost and/or soil and air temperatures has the potential to introduce stress combinations via delays in thawing events and/or earlier starts to spring-time growth.

11.2.4 Contribution from Human Influences

The effects of pollution such as nitrogen deposition can play a significant role in forest declines in the northern hemisphere. High rates of tree mortality in Japanese red pine (*Pinus densiflora*) forests were found to be correlated with early phenological development in south-facing stands and exposure to extremely low air temperatures (Shan 2000). Acid rain played a crucial role in reducing frost hardiness, thereby increasing the sensitivity of foliage that had developed early in the growing season (Shan 2000).

Trees in highly disturbed agricultural landscapes may succumb to stress from a variety of sources. Landsberg and Wylie [25] proposed a conceptual model of the initiation and development of rural dieback in *Eucalyptus* spp. Factors controlling leaf nitrogen, populations of leaf feeding insects or defoliation can directly promote dieback (Landsberg 1983). Changes in these factors arise from a variety of different sources including: climate extremes, salinity, excessive nutrients, changes in conditions for insects and their predators, soil compaction, and increased competition with agricultural crops. Interestingly, they showed that the nitrogen concentration of resprouting foliage produced by weakened defoliated trees made them more attractive to leaf feeding insects (White 1984), leading to a cycle of defoliation and incomplete recovery, progressive dieback, and sometimes mortality. The case of rural dieback in Australian eucalypts demonstrates the multifaceted nature of some tree declines and the problems with treating single causal factors when attempting to manage 170 fragmented and highly disturbed forests and woodlands.

11.3 How Does the Contribution from Different Stressors Affect the Magnitude and Direction of the Stress Response?

It is helpful to view the impact of multiple stressors in terms of whether the combination of two or more stressors produces antagonistic, additive, or synergistic outcomes for plant function such as growth or photosynthesis. Antagonistic stress combinations produce a response that is less than would be expected from adding the impact of two hypothetical stressors, stress A and stress B (the additive response). Synergistic stress combinations result in a response that is greater than the additive response, implying an amplifying effect from the interaction of stress A and stress B. Manipulative experiments that simulate two common stress combinations, drought and defoliation, show that growth responses can range from antagonistic through to synergistic. In fast-growing *E. globulus,* treatments involving 50% defoliation at a low water availability enhanced growth relative to that of plants in the undefoliated, low water stress treatment (Pinkard et al. 2011). One might expect defoliation to reduce water loss under mild drought, thereby reducing the decline in water deficit and plant growth. However, the interaction of defoliation of *Quercus robur* and *Pinus pinaster* (85 and 50% defoliation, respectively) and water deficit tends to produce additive or synergistic growth outcomes (Gieger and

Thomas 2002; Jacquet et al. 2014). These conflicting results suggest that the host physiology, conditioning factors such as tree age, and the simulated intensity of the primary and secondary stressors can determine the magnitude and direction of the response.

The intensity of the primary stressor has the potential to modulate the impact of additional secondary and conditioning factors. As a primary stressor such as drought progresses, cell expansion and growth are often the first casualties of water deficit, followed by a decline in the rate of photosynthesis (Hsiao et al. 1976), and the eventual breakdown of water and sugar transport (Hölttä et al. 2009). The addition of a secondary stressor at a given drought intensity, can act to preserve or further disrupt changes to these processes. Of the few studies to test for interactions between stress type (drought and simulated herbivory) and intensity (moderate and high), Bansal et al. (2013) reported a larger impact on growth in *Pinus sylvestris* in the combined stress treatment than in the single stress treatments at the moderate intensity (Bansal et al. 2013). However, under high intensity stress, the reduction in growth was largest in the drought treatments, regardless of levels of herbivory

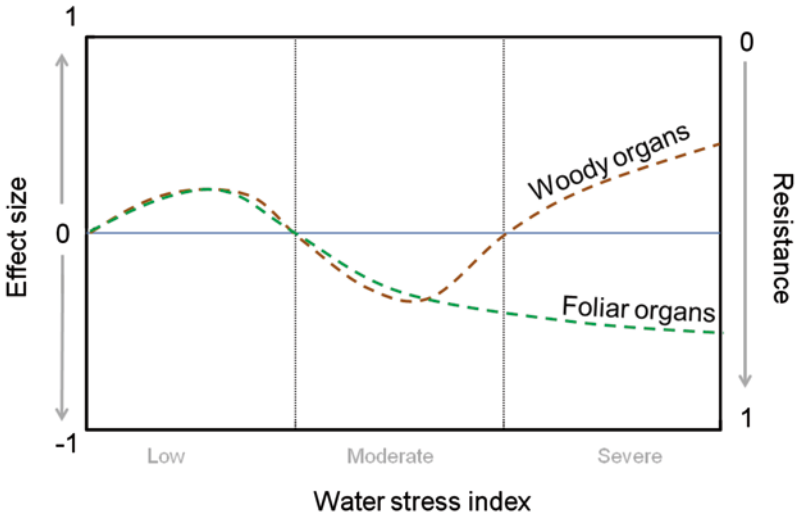

Fig. 11.6 Relationship between the level of damage (*effect size*) associated with pests of *woody organs* or foliage and a species-specific index of water stress (see text). For *woody organs*, conditions of low water stress may promote allocation of carbohydrates produced by photosynthesis to growth rather than defense; the host may demonstrate less resistance to pests of woody organs under these conditions. Many studies show that under moderate levels of water stress, resistance to pests of *woody organs* increases, but severely water-stressed trees are less likely to be resistant to these pests. For *foliar organs*, low levels of water stress induce nutritional changes in the foliage that can leave trees less resistant to defoliation pests. Under moderate levels of water stress, the host resistance to foliar pests may increase, due to the production of defense compounds in the leaves. Under severe water stress conditions, while production of defense compounds may be more limited, physical changes such as increases in leaf toughness may promote resistance. (Adapted from Jactel 2012).

(Bansal et al. 2013). Recently, Jactel et al. (2012) conducted a meta-analysis of drought studies that involved damage by insects and fungi. The study defined the intensity of plant water deficit using a species-specific index of water stress using plant water status and the stem hydraulic resistance to cavitation. They concluded that the impact on tree growth or survival (derived using an effect size method) increases with the intensity of water stress for foliar pests and pathogens, whereas the effect from pests on woody organs was dependent on whether the insect or fungal species was a primary (develop on healthy trees) or secondary (develop during physiological stress) agent Jactel et al. (2012). Figure 11.6 summarizes some of the key findings of the meta-analysis of Jactel et al., and shows how risk of damage is strongly determined by the relationship between intensity of water stress and changes in the resistance of different tree organs (foliar or woody).

Intensity-dependent responses may also arise because a highly sensitive process such as growth ceases at a threshold water deficit (Mitchell et al. 2014) so that any further reductions in water deficit combined with injury from biotic attack have minimal effect on growth-related responses. Figure 11.7 illustrates a hypothetical

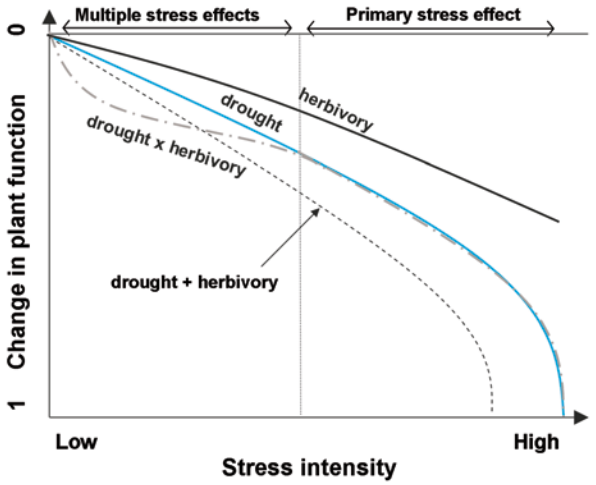

Fig. 11.7 A hypothetical scenario illustrating how the importance of primary, secondary, and multiple stressors changes across different stress intensities. The two stressors, *herbivory* (*solid black line*) and *drought* (*solid blue line*), and their interaction for any given intensity are represented by the *drought* × *herbivory line* (*gray, dot-dash*). The trajectory of the additive impact of the two stressors, i.e., *drought* + *herbivory* is given as a reference (*dashed line*). At high intensities, drought may reduce plant functioning to zero (death), whereas herbivory has less impact on function. The positioning of the drought x herbivory line below the additive impact line indicates a synergistic effect (multiple stress impact is greater than the sum), and above the additive impact line indicates an antagonistic effect (multiple stress impact is less than the sum). In this scenario, the stress impacts of *drought x herbivory* on tree vigor are determined by "multiple stress effects" at low to moderate intensities up until some threshold value (*vertical dashed line*). Beyond this threshold, the *drought x herbivory* line converges on the drought stress line, indicating tree function to be solely determined by the "primary stress effect" (drought in this case; after Mitchell et al. 2013). Copyright Oxford University Press.

response of a tree species to the combined stressors of drought and herbivory, and demonstrates why predicting the impacts from stress combinations under changing intensities is notoriously complex and difficult. The impact of combined stress on some element of plant function, e.g., growth, tends to be significant at low-to-moderate intensities of the stress, whereas as drought stress intensifies, the primary stressor becomes the dominant impact on plant function. This example helps to emphasize why consideration of the appropriate physiological thresholds related to water and carbon balance will help to differentiate between the effects of single and multiple stressors. Thus, it is critical that researchers can measure and report stress intensity using parameters such as soil and/or plant water potential, percentage change in leaf area or leaf temperature.

11.4 Recovery from Multiple Stressors

An essential component of characterizing the complete response to stress is defining recovery. Recovery can be defined as the ability of an individual to resume prestress function, such as the return to some mean growth rate, canopy structure, or level of productivity. Recovery from stress is often not considered in the study of stress tolerance in herbaceous species, but for long-lived woody species, assessing recovery from stress can provide a powerful insight into the resilience of a species or forest ecosystem. Time to recovery can be a useful metric to understand the severity of the stress event, and tends to increase with increasing impairment of plant functioning, i.e., hydraulic dysfunction (Brodribb and Cochard 2009). Tracking the trajectory of growth or carbon gain beyond their initial decline or distress period, helps to reveal the degree to which the stress was transient, delayed, or sustained. It can allow us to elucidate the key physiological recovery mechanisms involved, such as remobilization of carbohydrates, hydraulic repair, recovery of leaf biochemistry, or the action of heat shock proteins (Peñuelas et al. 2013). Mechanisms of recovery are often associated with metabolic costs that delay a return to prestress levels. Brodribb et al. (2010) found that the recovery of gas exchange via the restoration of hydraulic conductance tracks the growth of new xylem tissues, suggesting that the recovery imposes significant carbon costs after drought. Recovery strategies such as resprouting, enables a canopy suffering severe damage from dieback or herbivory to be rebuilt, and for the re-establishment of prestress rates of growth and carbon gain. While many species such as eucalypts, draw on a large store of nonstructural carbohydrates for rapid recovery (Pinkard et al. 2011), canopy recovery in taxa such as *Pinus* spp. is often limited due to a smaller pool of stored carbon (Galiano et al. 2011; Mitchell et al. 2014), particularly following multiple stress events. Contrasting recovery strategies are reflected in differences in life history, ontogeny and resource-use, and will have important consequences for water and carbon balance during and after stress. For example, the ability to resist and recover from repeated drought events was influenced by plant size in the resprouting *Quercus ilex,* presumably due to differences in rooting depth and the size of the carbohydrate store

(Lloret et al. 2004). Bansal et al. (2013), as well as others (Jacquet et al. 2013), have shown that recovery is hampered to a greater extent by the presence of a primary stressor such as drought, rather than the addition of a secondary agent such as herbivores.

11.5 How Do We Predict Responses and Impacts from Multiple Stressors?

As highlighted in the preceding sections, forest responses to stresses are complex and extrapolation of experimental results to situations outside experimental conditions may be inappropriate. Models can play an important role in quantifying the impacts of stress under a range of conditions or scenarios. Some progress has been achieved in simulating the impacts of single stress-related disturbances such as fire and harvesting on net primary productivity in Canadian boreal forests (Li et al. 2003), spruce bark beetle outbreaks in Norway (Jönsson et al. 2012) and drought impacts on biomass stocks in the Amazon (Rammig et al. 2010). However, even single stresses are, in general, poorly represented in models, reflecting the difficulty in representing complex, nonlinear responses to stress, a lack of mechanistic understanding of response processes and a lack of data for model validation (Jönsson et al. 2012; Pinkard et al. 2011). The importance of incorporating physiological responses into modeling the impacts of biotic attack is demonstrated by a recent study into carbon exchange in a conifer forest. The magnitude of change in ecosystem carbon fluxes during spruce beetle infestation was influenced not only by the final rates of mortality in the two dominant species but also by carbon losses incurred during the period in which tree growth and gas exchange were declining (Frank et al. 2014). Thus, plant physiological responses are foundational to the prediction of broader ecological outcomes during stress.

The challenges in modeling stress are exacerbated when considering multiple stress dynamics arising from interplay between primary, secondary, conditioning, and anthropogenic factors. A comparison of the capacity of six models, ranging in scale from tree to globe, to simulate drought mortality in pinyon pine-juniper woodlands, found that none of the tested models dealt well with multiple stress interactions such as biotic agents and drought. The authors concluded that the models were useful for defining key processes rather than quantifying impacts (McDowell et al. 2013). There is always a trade-off in models between the need to represent complex systems appropriately and making the models so complex it is difficult to parameterize them. McDowell et al's study illustrated that models of varying complexity can be effective in determining the likely direction of change in a system. However, quantifying the level of change at the ecosystem-level will require modeling frameworks that consider a hierarchy of stress responses and interactions distributed across the forest landscape. While there are few if any examples of models that can achieve this, synthesizing data from a diversity of sources, such as controlled experiments, environmental drivers (climatic, pest dynamics, soils), stand-level

responses, and process-based/spatially explicit models could be achieved using approaches such as hierarchical Bayesian models (Cable et al. 2009; Metcalf et al. 2009; Ogle and Barber 2008).

11.6 Conclusions

The extent and magnitude of impacts from multiple stressors in forests are likely to become larger in response to future shifts in climate and land-use intensification. Building on existing perspectives of stress dynamics, we have presented a conceptual framework that allows us to generalize about the nature of stress interactions with regard to a range of impacts, such as protracted forest declines and episodic forest collapse. This perspective highlights some key issues in understanding the mechanisms underlying stress in forest ecosystems: (1) The specific sequence of triggers for different stressors is crucial for defining their physiological response and impact; (2) any single event needs to be viewed as part of a longer-term regime of stressors that continually shapes the sensitivity of the forest stand to subsequent stressors; (3) the contribution from primary, secondary, conditioning, and anthropogenic factors will vary according to their magnitude (intensity, frequency, and duration) and how they overlap in time and space. Increasing intensity of any given stress can lead to threshold-type responses that exacerbate or diminish effects from other stressors; and (4) recovery patterns are facilitated by changes in water and carbon balance and can inform overall levels of stress that are not necessarily apparent during the period of distress. While there is much progress to be made in translating our mechanistic understanding of multiple stressors into models for predicting broad-scale impacts, this chapter raises some pertinent issues for researchers dealing with combined stress in complex ecosystems.

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Chapter 12
The Interactive Effects of Drought and Herbivory on Ecophysiology of Trees

Sheel Bansal

12.1 Introduction

The impact of drought or herbivory on tree growth and physiology can range from moderate and recoverable to severe and lethal depending on the intensity of either stressor. When these two stressors occur simultaneously, their combined impact on tree performance is assumed to be synergistic, i.e., greater than expected based on simple additive effects from either stressor alone (Niinemets 2010). This assumption is fueled from repeated observations of massive forest dieback following insect outbreaks during years with extreme drought (Ayres and Lombardero 2000; Mattson and Haack 1987). Drought affects a broad set of physiological processes such as transpiration and photosynthesis, hydraulic conductivity, and carbohydrate utilization, while herbivory elicits a number of carbon- and nitrogen-expensive defense mechanisms (Taiz and Zeiger 2002). Thus, the two stressors complement their negative impacts on tree ecophysiology. However, tree responses to either stressor may trigger physiological adjustments that protect against the effects of the second stressor (Fujita et al. 2006), thereby leading to antagonistic (less than expected) responses to co-occurring drought and herbivory. There are thousands of published studies on the effects of drought or herbivory, yet very few have simultaneously considered their combined impacts on tree performance (Bansal et al. 2013; Trowbridge et al. 2014). Unfortunately, studies on multiple stressors frequently show non-additive effects (i.e., synergistic or antagonistic), and therefore the combined effects cannot be predicted based on results from single-stressor studies. Given that both drought events and biotic stressors (e.g., insect outbreaks) are expected to occur with increased frequency and intensity with climate change (Mitchell et al. 2013), research on the effects from these combined stressors on tree growth and physiology is critical for predicting future forest health and productivity.

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12.2 Drought Alone

The environmental condition known as "drought" can be defined simply as the condition when soil moisture falls below a minimum threshold causing reduced plant growth (Bréda et al. 2006). However, under natural scenarios, "drought" can be episodic or chronic, moderate or severe, and caused by warm temperatures, low precipitation, high vapor pressure deficit, intense solar radiation, salt accumulation, and/or freezing soils (Larcher 2003). Also, the effects of drought on plant ecophysiology are a function of innate adaptive plant traits to conserve water, current plant health and vigor, and competitive interactions to acquire limited soil moisture supply. Consequently, the impacts of drought on growth and physiology are context-dependent on a number of external and internal factors. Nevertheless, the physiological responses of trees to drought have many similarities across species. This topic has been well reviewed, particularly in recent years, because the traditional mechanisms assumed to cause drought-induced tree mortality are being questioned and expanded upon (McDowell et al. 2008; McDowell 2011; Ryan 2011; Sala et al. 2010).

The fast-acting responses of trees to drought occur at the genetic, biochemical, and cellular levels (Fig. 12.1; Osakabe et al. 2014). Soil drying and desiccation of root tissues trigger the expression of drought-induced genes that synthesize various hormones, in particular abscisic acid (ABA; Fig. 12.2; Chaves et al. 2003).

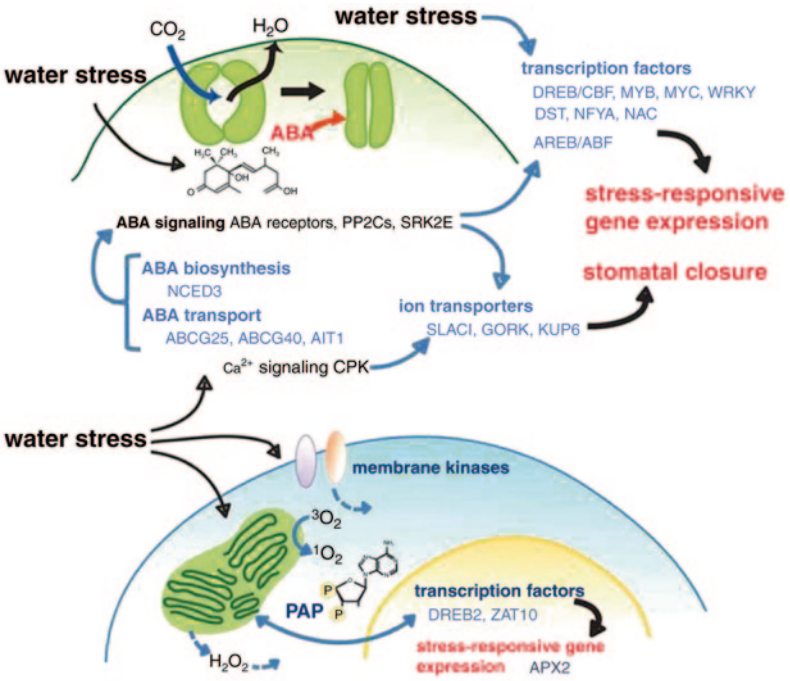

Fig. 12.1 Genetic, biochemical, and hormonal signaling factors in stomatal closure and retrograde signaling during water stress. (Figure from Osakabe et al. 2014)

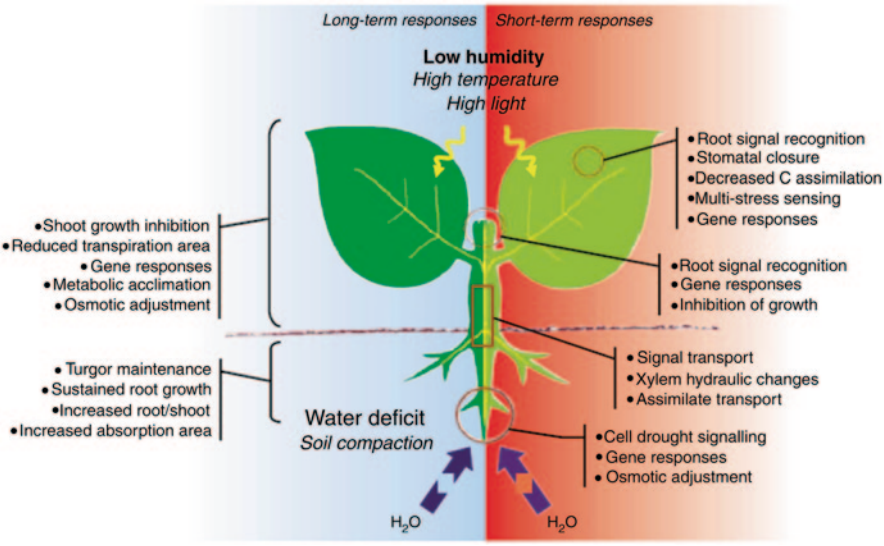

Fig. 12.2 Whole-plant responses to drought stress. *Left*, long-term or acclimation responses; *right*, short-term response. (Figure from Chaves et al. 2003)

ABA is a ubiquitous plant hormone that activates several physiological processes in response to environmental stress. In the case of drought stress, ABA is translocated to leaf tissues from roots, and there it binds to the plasma membrane of stomatal guard cells (Taiz and Zeiger 2002). The result is a flux of ions across the cell membrane, leading to rapid osmotic adjustments, shrinkage, and closure of the stomatal guard cells. ABA-induced stomatal closure is a key physiological mechanism to quickly limit water loss and to increase water-use efficiency, particularly for isohydric species. However, stomatal closure may not be 100% effective at constraining water vapor loss ("leaky stomata"), and water vapor also diffuses through the cuticular membranes that enclose leaf tissues (cuticular transpiration; Burghardt and Riederer 2003; Kerstiens 1996; Schreiber and Riederer 1996). Moreover, stomatal closure comes at significant costs of reduced CO_2 uptake for photosynthesis, thus limiting the production of carbon assimilates that are needed for growth, maintenance, reproduction, and/or the production of defense compounds against herbivores (Kempel et al. 2011).

There are many longer-term morphological adjustments that occur in response to drought stress. At the leaf level, reduced turgor pressure in cells decreases growth rates, in particular the process of cellular elongation (Meier et al. 1992). Reduced elongation is evident in droughted plant leaves as a decrease in the ratio of leaf area to leaf mass (i.e., lower specific leaf area; Abrams et al. 1994). Lower specific leaf area has adaptive value for plants exposed to chronic moisture stress because there is less leaf area for water loss through stomatal or cuticular transpiration (Grace 1990; Bansal et al. 2014). Unfortunately, lower specific leaf area also

reduces leaf area for light interception and CO_2 uptake, which has negative impacts on photosynthetic carbon assimilation rates (Oren et al. 1986).

At the whole-plant level, large-scale redistribution of carbon assimilates can be used to further minimize water loss and to increase soil moisture uptake. Specifically, plants typically undergo an increase in the ratio of root-to-shoot biomass, an increase in rooting depth and root density, and leaf shedding or abscission in response to drought (Larcher 2003). While these changes may be critical for plant survival during periods of extreme drought stress, they also come at a severe cost to carbon uptake and assimilation.

If drought conditions persist, even extreme physiological and morphological adaptive responses may not adequately prevent dysfunction of basic processes necessary for survival (Sevanto et al. 2014). If water loss continues via cuticular transpiration, even after water uptake by roots has diminished, tension builds up on the transpiration stream in the xylem (i.e., more negative xylem water pressure), particularly for trees because of their high transpirational areas and long distances to transport water (Taiz and Zeiger 2002). With increasing tension, hydraulic conductance of water to leaves from roots is eventually disrupted by cavitations and embolisms of air bubbles into the xylem stream. These breaks in the water column can quickly lead to 100% loss of hydraulic conductivity, although the extent that plants are vulnerable to cavitations under conditions of negative xylem water pressure differs greatly among plant taxa (Cochard 1992; Maherali et al. 2004; Tyree and Ewers 1991).

Hydraulic failure has been the traditional mechanism assumed to cause mortality in trees exposed to frequent and severe drought events. However, as described above, many of the ecophysiological responses to cope with drought stress reduce carbon assimilation, which have led to the development of a newer "carbon-starvation" hypothesis regarding drought-induced tree mortality (McDowell et al. 2008; Sala et al. 2010). While hydraulic failure is expected to cause tree mortality relatively quickly, carbon starvation is hypothesized to take place over longer periods of time in which plants experience negative carbon balances (i.e., greater carbon use than carbon gain). As trees become depleted in carbohydrates, they are unable to meet metabolic demands for basic functioning, or to biosynthesize carbon-rich defense compounds necessary against biotic agents (Fig. 12.3; Gutbrodt et al. 2011; McDowell 2011). Clearly, these consequences of carbon starvation have direct implications for tree–herbivore relationships.

12.3 Herbivory Alone

Herbivory can be defined as the consumption of plant material, often occurring on living plants, but not always lethal (Ohgushi 2005). However, this simple definition is one of the only ubiquitous generalizations that can be made regarding the impact of herbivory on plant performance. The reason being that the effects of herbivory are context-dependent on a number of factors, including the herbivore func-

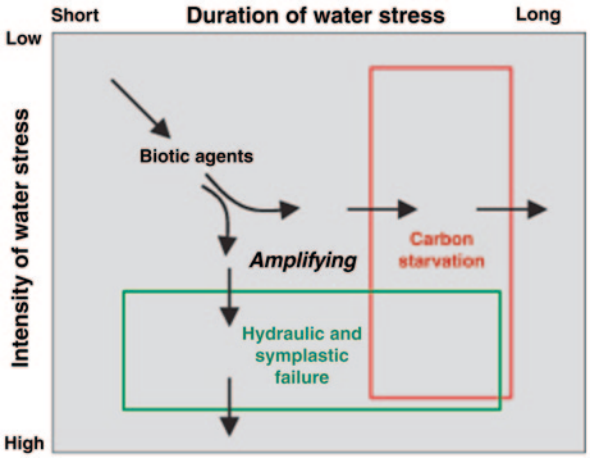

Fig. 12.3 Theoretical relationship, based on the hydraulic framework, between the temporal length of drought (duration), the relative decrease in water availability (intensity), and the three hypothesized mechanisms underlying mortality. Carbon starvation is hypothesized to occur when drought duration is long enough to curtail photosynthesis longer than the equivalent storage of carbon reserves for maintenance of metabolism. Hydraulic failure is hypothesized to occur if drought intensity is sufficient to push a plant past its threshold for irreversible desiccation before carbon starvation occurs. Biotic agents, such as insects and pathogens, can amplify or be amplified by both carbon starvation and hydraulic failure. (Figure from McDowell et al. 2008)

tional group (e.g., insects, mites, mammals), feeding behavior (e.g., defoliators, phloem-feeders, cell-content feeders), and stage of host physiological development (e.g., seed, juvenile, adult; Agrawal 1998; Karban and Myers 1989). In addition, the impacts from herbivory may be confounded by hitchhiker pathogens such as parasites, bacteria, fungi, and viruses that are often introduced during feeding (Hatcher 1995; Trapp and Croteau 2001). Among herbivores, trees are probably most affected by phytophagous insects, and entire forests have been decimated from beetles, moths, weevils, budworms, and caterpillars (Ayres et al. 2014). Herbivore-induced plant responses include the production of secondary metabolites, physical deterrents, compensatory physiology and growth, and tissue abscission (Agrawal 1998; Strauss and Agrawal 1999). Many of these responses come with high carbon costs (Dungan et al. 2007), similar to the impacts from drought stress.

Like drought, plant responses to herbivory begin at the subcellular level (Fig. 12.4; Wu and Baldwin 2009). Wounding of plant tissues from feeding or the injection of foreign compounds from herbivores initiates the release of hormones such as jasmonic acid (JA), elicits defense-related genes, and increases the production and modification of secondary metabolites (Karban and Myers 1989; Kessler and Baldwin 2002). The ultimate goal of these defense compounds is to reduce the preference for the host plant or the performance of the herbivore. Secondary metabolites are generally categorized as terpenoids or phenolics, which are carbon-rich allelochemicals such as flavonoids, tannins, and lignins, or as nitrogen-containing

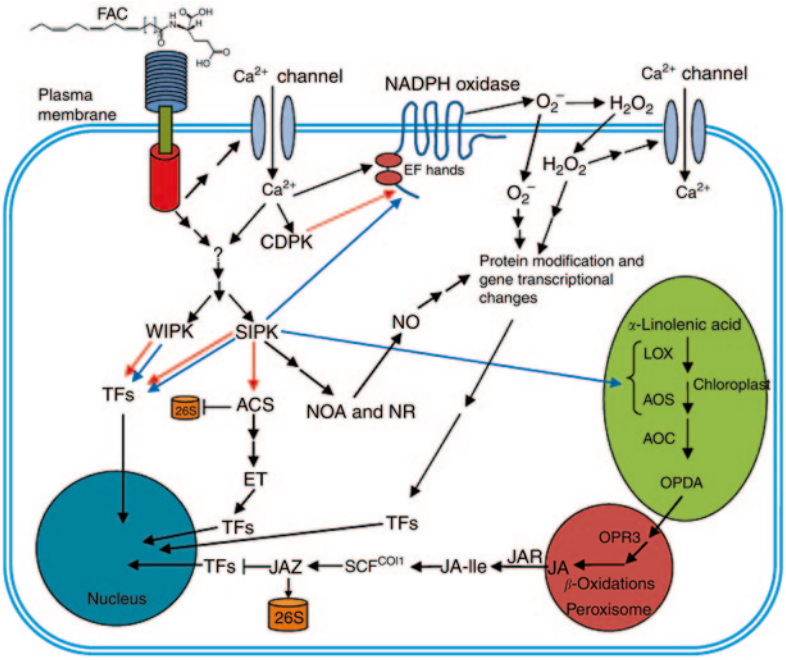

Fig. 12.4 A model summarizing early signaling events in herbivore-attacked plants. After herbivore attack, herbivore elicitors (here FAC??) bind to putative receptors on plasma membranes and activate further responses. Through an unknown mechanism, Ca^{2+} influx is initiated, which depolarizes cell membranes. Increased Ca^{2+} (likely together with a CDPK) greatly enhances NADPH oxidases located in cell membrane and leads to ROS production. MAPKs (at least SIPK and WIPK) are quickly activated; they transcriptionally regulate many genes involved in JA and ethylene biosynthesis, as well as NADPH oxidase and WRKY transcription factors (TFs). SIPK is likely also involved in NO production; both ROS and NO modify amino acids in proteins and induce transcriptional changes of various defense-related genes. A yet to-be-indentified pathway triggers JA biosynthesis. JA is further converted to JA-Ile by JAR; binding of JA-Ile to SCF^{COI1} initiates the degradation of JAZ proteins that negatively regulate JA-responsive genes. Without phosphorylation, ACS is degraded through 26S proteasome pathway; after being phosphorylated by SIPK, it gains higher stability and enhances ethylene biosynthesis. *Red arrows* represent phosphorylation; *blue arrows* represent transcriptional regulation. *AOC* allene oxide cyclase, *AOS* allene oxide synthase, *CDPK* calcium-dependent protein kinase; *FAC* Fatty acid chains; *JAZ* jasmonate ZIM-domain, *LOX* lipoxygenase, *OPDA* 12-oxo-phytodienoic acid, *OPR3* OPDA reductase 3, *NO* nitric oxide, *NOA* NO-associated protein, *NR* nitrate reductase, *ROS* reactive oxygen species, *SCF* Skp, Cullin, F-box, *SIPK* salicylic acid-induced protein kinase, *WIPK* wound-induced protein kinase. (Figure from Wu and Baldwin 2009)

compounds, which include alkaloids, cyanogenic glycoside, and lectins (Taiz and Zeiger 2002). Because these compounds are carbon and nutrient expensive to plants, they are only produced when necessary, especially those that have no apparent function toward growth or development (Langenheim 1990; Poorter and Villar 1997).

Conifer trees utilize volatile monoterpenes as a primary defense against insect herbivory and exude oleoresins following wounding, which constitute large carbon investments into compounds that are not recycled and ultimately lost to the environment (Croteau and Johnson 1985; Trapp and Croteau 2001; Trowbridge et al. 2014).

Plants also undergo defensive, morphological adjustments to protect against herbivory (Hanley et al. 2007). These changes can include the production of external thorns, prickles, spines, and hairs (Myers and Bazely 1991) or an increase in epicuticular waxes, cutins, and suberins (Eigenbrode and Espelie 1995). In addition, plants can cope with herbivory through repair of wounded tissues, abscission of infected tissues, or compensatory regrowth of lost tissues (Neely 1970). Like chemical defenses, morphological changes comes at large carbon investments for the host plant, which increase survivorship but at the cost of reduced growth, reproduction, and carbohydrate storage (Fig. 12.5; Agrawal 2011; Dungan et al. 2007; Orians et al. 2011).

Insect herbivores have additional impacts on plant carbon balance beyond the induction of plant chemical and morphological defense responses. Consumption of leaf tissues by defoliators reduces the amount of leaf area available for photosynthetic carbon assimilation, while phloem-feeders (such as weevils or beetle larvae) directly consume phloem sap sugars as they are transported through the stems from leaves to roots (Karban and Myers 1989). Both of these impacts from herbivory can have tremendous consequences on carbohydrate reserves that are needed for growth, reproduction, and metabolic functions, thus reducing plant vigor and ultimately leading to mortality.

12.4 Drought Combined with Herbivory

When trees experience more than one stressor simultaneously, complimentary impacts on related physiological processes may turn an otherwise recoverable situation into catastrophic dysfunction and mortality. From the above descriptions on the impacts of drought stress or herbivory alone, it is clear that there are several ecophysiological mechanisms that would be negatively impacted if both stressors co-occurred. Herbivory tends to increase carbon demands, while drought stress decreases carbon gain, making it easy to assume that the two stressors combined will have a synergistic, negative effect on plant carbon balance. Thus far, very few studies have empirically tested the interactions of drought and herbivory on plant performance, particularly for trees (Bansal et al. 2013; Trowbridge et al. 2014).

One study that explicitly tested for synergistic, additive, or antagonistic effects from drought combined with herbivory (simulated phloem-feeding weevils in this case) on ecophysiology of *Pinus sylvestris* seedlings found, contrary to expectations, that many traits were affected antagonistically (Fig. 12.6; Bansal et al. 2013). Specifically, gas exchange and growth rates were sharply reduced when both stressors co-occurred, although the total, combined effects were less than expected based on additive effect of either stressor alone. While these findings were unantici-

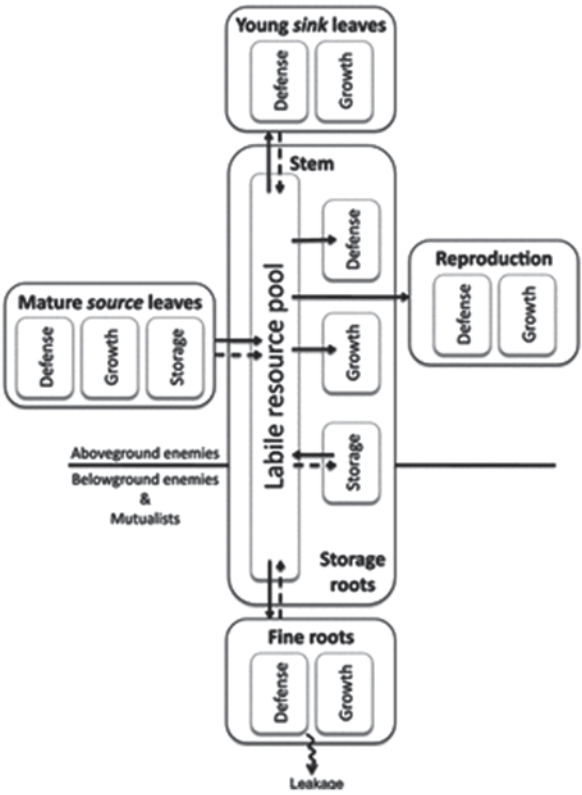

Fig. 12.5 Conceptual model for resource flows in plants. The labile resource pool is derived from newly captured pools of carbon and nutrient pools or from remobilized storage reserves. The labile carbon pool is generated from photosynthesis, primarily by mature source leaves. The labile nutrient pool is obtained from roots. The resulting labile resource pool can then be allocated to support the growth of sink tissues (roots, leaves, or reproductive tissues), to defense traits, and to storage tissues. Herbivore-induced export of resources from leaves or from fine roots (*dashed arrows*) into stems and storage roots functions to sequester resources in tissues inaccessible to the respective herbivores but may incur opportunity costs if resources allocated for storage limit growth and reproduction or ecological costs if other enemies specialize on these storage tissues. (Figure from Orians et al. 2011)

pated, there are a few biological mechanisms that could explain the results. First, exposure to drought stress *or* herbivore wounding may have triggered a series of stress-induced genes and physiological responses that "primed" or protected the trees from the second, co-occurring stressor (Bowler and Fluhr 2000; Fujita et al. 2006; Leshem and Kuiper 1996; Rennenberg et al. 2006). For example, some studies have shown a short-term increase in resin acid concentrations in plants exposed to moderate drought stress (Turtola et al. 2003), which could facilitate wound healing and monoterpene emissions to cope with herbivory. *Pinus taeda* showed an

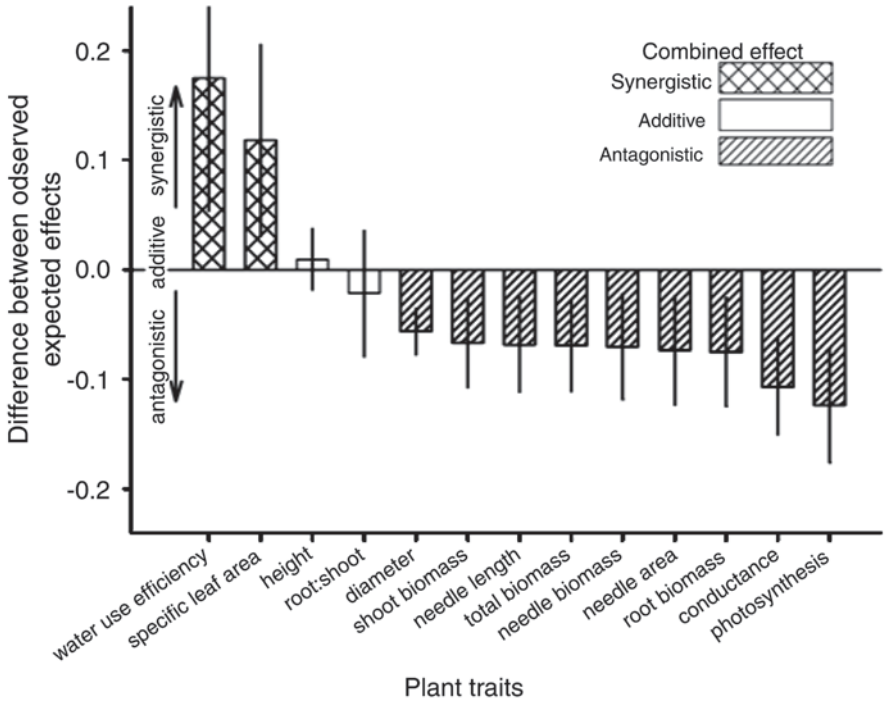

Fig. 12.6 The combined impacts from drought and herbivory on various plant traits were synergist, additive, or antagonistic (greater than, equal to, or less than expected effects, respectively, based on single stressor effect sizes). The *bars* represent the overall effect size difference (mean ±95% CI) between the observed and expected additive effects from combined drought and herbivory on morphological traits at final harvest and second-year physiological traits of *P. sylvestris* seedlings. The *zero line* represents the expected additive effects from combined stressors. When the means (and their 95% confidence limits) were greater than or less than the zero line, they were considered synergistic or antagonistic, respectively. (Figure from Bansal et al. 2013)

increase in defense resin synthesis in response to soil moisture stress despite a decrease in growth rates (Lorio and Sommers 1986). This phenomenon of interacting responses to multiple stressors has also been documented for the combined effects of fire and herbivory on *Pinus radiata,* in which tree exposed to fire had increased antiherbivore resin defenses that provided protection against subsequent bark beetle attacks (Lombardero and Ayres 2011).

A second possibility for antagonistic effects from drought and herbivory on tree ecophysiology was that the impact of drought stress overrode the effects of herbivory. A study conducted in situ that monitored monoterpene emissions of *Pinus edulis* found the influence of soil moisture was relatively strong compared to herbivory during the midsummer drought (Trowbridge et al. 2014). However, they also showed how herbivory played a dominant role in affecting plant defenses during periods of the growing season with higher soil water availability, thus dem-

onstrating the importance of stressor severity and temporal variability on the interaction of drought and herbivory.

Even though Bansal et al. (2013) generally found antagonistic effects of drought and herbivory on tree physiology and growth, there were two key functional traits, specific leaf area and water-use efficiency (ratio of carbon gain to water loss), that exhibited relatively strong, synergistic effects from the combined impact of the two stressors (Fig. 12.6). These two traits are particularly important to resource-use efficiency, carbon gain and allocation, and survival (Reich et al. 1997). Droughted seedlings had decreased shoot biomass and needle size, thus reducing water demands disproportionately to water supply. Those morphological adjustments allowed the seedlings to maintain relatively high water saturation for individual needles, which in turn led to an increase in specific leaf area in response to drought. The increase in water-use efficiency of droughted seedlings was driven by stomatal closure in response to decreased soil moisture. Unlike with drought, the mechanisms leading to an increase in specific leaf area and water-use efficiency from herbivory were less clear, as were the mechanisms driving the synergistic effects from both stressors combined. Regardless of the mechanisms involved, the synergistic increase in specific leaf area increased light-capturing area for photosynthetic carbon gain, while the increase in water-use efficiency decreased carbon gain but conserved relatively more water under droughted conditions. Consequently, these two synergistic effects aided in establishing seedlings and coping with multiple stressors.

Intensity-dependent effects from multiple stressors have rarely been explored. In Bansal et al. (2013), stressor intensity played an important role in determining the impact of drought or herbivory alone but also affected how the two stressors interacted. For example, the cumulative effects from the two stressors on height, diameter, and shoot biomass were stronger (synergistic or additive) when both stressors were of moderate intensity, but were antagonistic when either stressor was severe. This suggests that co-occurring stressors at lower intensity could have a disproportionate, negative impact on seedling growth (Mitchell et al. 2013). In contrast, the combined effects of drought and herbivory were stronger on needle length and gas exchange when drought stress was severe, irrespective of herbivore intensity, thus demonstrating how the effects of multiple stressors are also trait-dependent.

12.5 Drought Effects on Herbivores and Plant–Herbivore Interactions

Clearly, drought and herbivory has many overlapping consequences on tree ecophysiology. However, from an ecological perspective, drought also has a direct effect on herbivorous insect populations. In addition, the changes in plant chemistry that occur from drought can affect herbivore feeding preferences, thus altering plant–insect interactions (Gutbrodt et al. 2011; Mattson and Haack 1987).

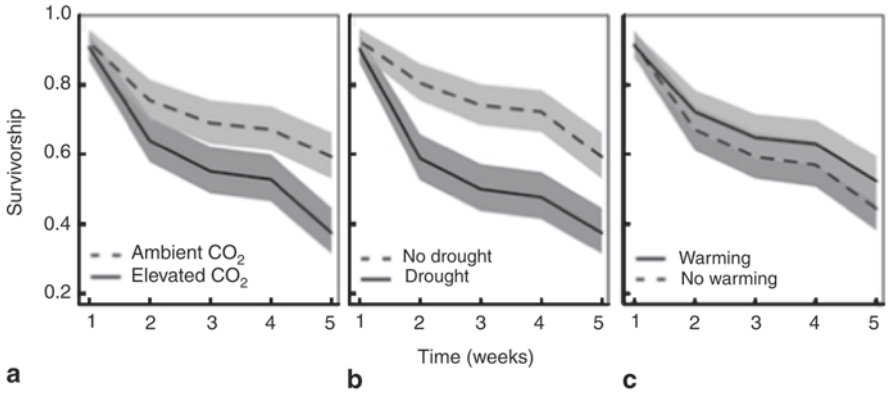

Fig. 12.7 Kaplan–Meier survivorship of *Lochmaea suturalis* larvae over time for plots with elevated treatments ($N=24$ per time point, *solid lines*) and ambient plots ($N=24$ per time point, *broken lines*). *Gray shaded areas* show 95 % confidence intervals of the Kaplan–Meier estimator. Significance of interactions with time: **a** $P=0.057$ and **b** $P<0.0001$. **c** Warming was only significant in a three-way interaction with CO_2 and drought ($P=0.019$). (Figure from Scherber et al. 2013)

Drought can have either negative or positive impacts on herbivore populations, depending on the severity and duration of the drought. Moisture stress typically has negative impacts on the fitness of developing larvae (Fig. 12.7; Scherber et al. 2013). However, drought is frequently associated with warmer temperatures, which accelerates insect metabolism, leading to faster growth, consumption, and developmental rates (Jamieson et al. 2012). Warmer winter temperatures in particular tend to enhance insect overwintering survival (Bale et al. 2002; Bentz et al. 2010), as well as induce earlier emergence and phenological development (Parmesan and Yohe 2003). Consequently, the effects of drought on herbivorous insect populations are not unidirectional and context-dependent.

The direct effects of drought on herbivore populations are confounded by indirect effects of drought on their forage quality, i.e., on host plant ecophysiology (Bauerfeind and Fischer 2013; Koricheva et al. 1998; Rouault et al. 2006). There has been a long-standing "plant stress hypothesis" which states that plants under abiotic stress have lower defensive capabilities and are therefore more suitable as a food source for herbivorous insects (Mattson and Haack 1987; White 1974; White 1984). Temporal correlations between drought events and insect outbreaks support this hypothesis (Hart et al. 2014). Alternatively, the "plant vigor hypothesis" predicts that insects will preferentially feed on faster-growing, healthier plants that have higher nutritional content and lower defense compounds (Price 1991). A suite of meta-analyses, modeling, and manipulative studies have shown that drought stress does not consistently lead to increased or decreased insect consumption, and is often dependent on species, feeding guild, and specialization of the herbivores (Bauerfeind and Fischer 2013; Grinnan et al. 2013; Haynes et al. 2014; Huberty and Denno 2004; Larsson 1989; Rouault et al. 2006). A unique study conducted by Gutbrodt et al. (2011) elegantly demonstrated how the effects of drought stress on plant tissue

moisture content (health) and secondary metabolite concentrations (defense) led to differing feeding preferences for a generalist compared to a specialist herbivore. These changes in feeding behavior that occur on drought-stressed plants will likely impact subsequent insect population dynamics, and further influence plant vigor and chemistry, thus creating a feedback system and further complicating the interaction of drought and herbivory.

12.6 Conclusions

In the environment, the co-occurrence of multiple environmental stressors is the rule rather than the exception (Chapin et al. 1987; Niinemets 2010; Vierling and Kimpel 1992). Global climate change is expected to increase the frequency and intensity of drought events and herbivorous insect outbreaks (Allen et al. 2010; Bale et al. 2002; Vinebrooke et al. 2004; Williams and Jackson 2007), thereby increasing the probability that the two will co-occur in the future. Moreover, expected increases in other stressors, such as extreme heat events and wildfires, could exacerbate conditions beyond a critical threshold of plant tolerance. Consequently, forests worldwide are at increased risk of extreme dieback. For drought and herbivory in particular, their combined, negative impact on tree carbon balance has important implications for forest productivity and carbon sequestration at global scales. Therefore, improving our understanding of the interacting effects of multiple stressors on tree growth and physiology is crucial (but poorly investigated) for managing future forests.

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