Narendra Tuteja
Sarvajeet Singh Gill *Editors*

Crop Improvement Under Adverse Conditions

Springer

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Professor Arturo Falaschi
(January 21, 1933–June 1, 2010)

Prof. Arturo Falaschi was born in Rome and graduated in Medicine in 1957 from Milan University. He undertook post doctoral trainings with J. Adler and Har Gobind Khorana (Nobel Prize in 1968 for deciphering the genetic code) in Wisconsin, USA (1961–1962), and later with Arthur Kornberg (Nobel Prize in 1959 for his studies on DNA replication) at Stanford (1962–1965). His main field of research was in the field of DNA replication and his contributions in this field are significantly important. His scientific work featured in the most prestigious international journals. Prof. Falaschi remains one of the few international researchers whose scientific activity is

documented throughout almost fifty years (from 1962 to 2010). Prof. Falaschi was responsible for the establishment of several research institutes and was a strong believer in the internationalization of science. Prof. Falaschi was very articulate and convinced several governments in the developed and the developing world to establish a 3-component International Centre for Genetic Engineering and Biotechnology (ICGEB), with one component in New Delhi, India, one in Trieste, Italy and one in Cape Town, South Africa. All these centres are devoted to research and training of young researchers from the developing world (www.icgeb.org). One of the focuses of ICGEB, New Delhi component is the development of crops resistant to various stresses. Prof. Falaschi was the mind and driving force in the founding and development of ICGEB, where he served as the Director General from 1989 to 2004. From 2004 to 2010, he worked as ICGEB Distinguished Scientist and Professor of Molecular Biology, Scuola Normale Superiore SNS, Pisa, Italy.

This book is dedicated to the memory of Prof. Arturo Falaschi as a token of our appreciation and respect for him and his achievements.

Foreword

Plants are fundamental to life on Earth and they have been harnessed by humans for food, feed, fibre, fuel and fun. The need to increase crop production is becoming more urgent due to increasing population and diversion of crops to biofuels production. Furthermore, this needs to be done sustainably with reduced inputs and in the face of global environmental change. It is also notable that one-third of the world's food production is estimated to grow under irrigation—much of this irrigation is unsustainable, using water supplies that are overexploited and under threat from changing weather patterns resulting from global climate change. It is estimated that to meet the recent Declaration of the World Summit on Food Security target of 70 % more food by 2050, an average annual increase in production of 44 million metric tons per year is required, which is a 38 % increase over historical increases in production.

The gap between potential yield and actual yield is primarily due to the effects of abiotic stresses on crop production. It is therefore an imperative to improve our ability to maintain crop production in environments with suboptimal conditions, such as low water or nutrient supplies, or high salinity. This is, of course, required in addition to improving the efficiency of delivery of existing technologies into developing countries through improved education and outreach.

As such, the book edited by Dr. Narendra Tuteja and Dr. Sarvajeet Singh Gill provides a useful and timely compilation of up-to-date overviews of advances in the important area of plant sciences, "Crop Improvement Under Adverse Conditions". In this volume, a range of papers have been brought together which address both the technologies required to understand mechanisms of abiotic stress tolerance and the biological questions to which those technologies need to be applied. An understanding of the molecular and physiological aspects of plant function is provided in this book, and the emphasis on contributors from developing countries is very valuable—delivery of improved technologies and improved varieties of crops in such regions will have the greatest relative impact on global food production.

The editors and contributors are to be congratulated on their efforts, and readers are recommended to use this volume for a long time to come.

Mark Tester
Adelaide, Australia

Preface

Plant development and productivity are negatively regulated by various environmental stresses. Abiotic stress factors such as heat, cold, drought, salinity, wounding, heavy metals toxicity, excess light, flooding, high speed wind, nutrient loss, anaerobic conditions and radiations etc. represent key elements limiting agricultural productivity worldwide. The loss of productivity is triggered by a series of morphological, physiological, biochemical and molecular stress-induced changes. Such an unfavourable situation is in contrast with the increasing global food demand. World population is increasing at an alarming rate and is expected to reach more than nine billion by the end of 2050, whereas, plant productivity is being seriously limited by various abiotic stresses all over the world. Global climatic pattern is becoming more unpredictable with increased occurrence of drought, flood, storm, heat waves, and sea water intrusion. It has been estimated that abiotic stresses are the principal cause for decreasing the average yield of major crops by more than 50 %, which causes losses worth hundreds of millions of dollars each year. Therefore, to feed the world population maintaining crop productivity even under unfavourable environment is a major area of concern for all nations. Developing crop plants with ability to tolerate abiotic stresses is need of the day which demands modern novel strategies for thorough understanding of plant's response to abiotic stresses. Molecular breeding and genetic engineering have significantly contributed to expand the basic knowledge of the cellular mechanisms involved in stress response, suggesting new strategies to enhance stress tolerance.

In this book "Crop Improvement Under Adverse Conditions", we present a collection of 17 chapters written by 55 experts in the field of plant abiotic stress tolerance and crop improvement. It is a timely contribution to a topic that is of eminent importance. The chapters provide a state-of-the-art account of the information available on abiotic stress tolerance and crop improvement. In this book, we present the approaches for crop improvement under adverse environmental conditions. Chapter 1 deals with the research, development, commercialization, and adoption of drought- and stress-tolerant crops, where the factors affecting adoption of stress-tolerant crops by farmers are explored which includes complementary technologies, competing technologies, appeal to first-time users, distribution and timing of benefits to users, and social perceptions of the technology. Chapter 2 uncovers the

impact of extreme events on salt-tolerant forest species of Andaman and Nicobar Islands. Chapter 3 deals with greenhouse gases emission from rice paddy ecosystem and their management. The plant development path of mitigating greenhouse gases (GHG) from agriculture cropping systems is not yet well established. Therefore aggressive research strategies and field validations are needed for establishing 'plant development' as a sustainable tool for GHG mitigation in agriculture sector. Chapter 4 covers remote sensing applications to infer yield of tea in a part of Sri Lanka. Chapter 5 deals with the polyamines contribution to the improvement of crop plants tolerance to abiotic stress, where, mechanism of action of polyamines to protect crop plants from challenging environmental conditions has been discussed. Chapter 6 discusses the overlapping horizons of salicylic acid in different stresses. In this chapter, the indigenous accumulation and overlapping roles of SA under different environmental and physiological conditions highlighting its recently updated roles and regulations in plants is discussed. Chapter 7 focuses on the effects of oxidative stress within the nuclear compartment where DNA becomes the main target of the highly toxic reactive oxygen species (ROS). Chapter 8 deals with a fast and reliable approach for crop improvement through in vitro haploid production. This chapter will act as a guide to prospective scientists working in the area of haploid production intended for crop improvement. Chapter 9 discusses the strategy for the production of abiotic stress-tolerant fertile transgenic plants using androgenesis and genetic transformation methods in cereal crops. Chapters 10 and 11 deal with the control and remedy of plant diseases through nanotechnology and the scope and potential of nanobiotechnology in crop improvement. The use of mutifunctionalised nanoparticles as plant transgenic vehicle for developing disease and stress resistant transgenic plants is discussed. Nanotechnological approaches on plants allow more efficient and sustainable food production by reducing the chances of disease and pest incidence in plants. In Chap. 11, thorough studies and reliable information regarding the effects of nanomaterials on plant physiology and crop improvement at the organism level are discussed. Chapter 12 deals with the role of nematode trapping fungi for crop improvement under adverse conditions. Chapter 13 uncovers the role of sugars as antioxidants in plants. This chapter discussed that soluble vacuolar carbohydrates (e.g. fructans) may participate in vacuolar antioxidant processes, intimately linked to the well-known cytosolic antioxidant processes under stress. All these insights might contribute to the development of superior, stress-tolerant crops. Chapter 14 deals with chromium toxicity and tolerance in crop plants, where, the mechanism of phytotoxicity and phytotolerance under Cr stress is discussed. Chapter 15 deals with boron toxicity and tolerance in crop plants, where, attempts to improve crop yields under boron-toxic soils is discussed. Chapter 16 deals with the approaches for stress resistance and arsenic toxicity in crop plants. Chapter 17 uncovers the mechanism of cadmium toxicity and tolerance in crop plants.

The editors and contributing authors hope that this book will include a practical update on our knowledge of "Crop Improvement Under Adverse Conditions" and lead to new discussions and efforts to the use of various tools for the improvement of crop plants under changing environment.

We are highly thankful to Dr. Ritu Gill, Centre for Biotechnology, MD University, Rohtak for her valuable help in formatting and incorporating editorial changes in the manuscripts. We would like to thank Prof. Mark Tester for writing the foreword and Springer Science+Business Media, LLC, New York, particularly Editor, Plant Sciences, Amna Ahmad and Developmental Editor/Project Manager, Daniel L.A. Dominguez and Andy Kwan, for their support and efforts. We have dedicated this book to Prof. Arturo Falaschi, the mind and driving force in the founding and development of ICGEB.

Narendra Tuteja
Sarvajeet Singh Gill

Contents

The Editors

Narendra Tuteja was born in 1955. Currently, Dr. Tuteja is working as a Senior Scientist in Plant Molecular Biology Group, International Centre for Genetic Engineering and Biotechnology (ICGEB), New Delhi. Dr. Tuteja obtained his M.Sc., Ph.D and D.Sc. in Biochemistry from the Lucknow University in 1977, 1982 and 2008, respectively. He is fellow of the Academies of Sciences: FNASc. (2003), FNA (2007), FASc. (2009), FNESA (2009) and FTWAS (2012).

Dr. Tuteja has made major contributions in the field of plant DNA replication and abiotic stress signal transduction, especially in isolating novel DNA/RNA helicases and several components of calcium and G-proteins signaling pathways. Initially he made pioneer contributions in isolation and characterization of large number of helicases from human cells while he was at ICGEB Trieste and published several papers in high impact journals including EMBO J. and Nucleic Acids Research. From India he has cloned the first plant helicase (Plant J. 2000) and presented the first direct evidence for a novel role of a pea DNA helicase (PNAS, USA, 2005) in salinity stress tolerance and pea heterotrimeric G-proteins (Plant J. 2007) in salinity and heat stress tolerance. Dr. Tuteja has reported the first direct evidence in plant that PLC functions as an effector for Gα subunit of G-proteins. All the above work has received extensive coverage in many journals, including Nature Biotechnology, and bulletins all over the world. His group has also discovered novel substrate (pea CBL) for pea CIPK (FEBS J. 2006). He has already developed the salinity tolerant tobacco and rice plants without affecting yield. Recently, few new high salinity stress tolerant genes (e.g. Lectin receptor like kinase, Chlorophyll a/b binding protein and Ribosomal L30E) have been isolated from Pisum sativum and have been shown to confer high salinity stress tolerance in bacteria and plant (Glycoconjugate J. 2010; Plant Signal. Behav. 2010). Recently, very high salinity stress tolerant genes from fungus Piriformospora indica have been isolated and their functional validation in fungus and plants is in progress. Overall, Dr. Tuteja's research uncovers three new pathways to plant abiotic stress tolerance. His results are an important success and indicate the potential for improving crop production at sub-optimal conditions.

Sarvajeet Singh Gill was born on January 21st, 1979. Dr. Gill obtained his B.Sc. (1998) from Y.D. College, Kanpur University and M.Sc. (2001, Gold Medalist), M. Phil. (2003) and Ph.D (2009) from Aligarh Muslim University. Presently, Dr. Gill is working as Assistant Professor in Centre for Biotechnology, MD University, Rohtak, Haryana.

Dr. Gill's main area of research includes Genetic Engineering, Stress Physiology and Molecular Biology (Development of abiotic stress tolerant crop plants, the physiological, biochemical and molecular characterization of agronomically important plants under abiotic stress factors, involvement of mineral nutrients and other biotechnological approaches in the amelioration of abiotic stress effects in crop plants, use of a combination of genetic, biochemical, genomic and proteomic approaches to understand the responses of various components of antioxidant machinery to abiotic stress and stress signaling and stress tolerance in crop plants. Dr. Gill has several research papers, review articles and book chapters to his credit in the journals of national and international repute and in edited books. He has edited four books namely Sulfur assimilation and Abiotic Stress in Plants; Eutrophication: causes, consequences and control; Plant Responses to Abiotic Stress, Omics and Abiotic Stress Tolerance and Improving Crop Resistance to Abiotic Stress, published by Springer-Verlag (Germany), IK International, New Delhi, Bentham Science Publishers and Wiley-VCH, Verlag GmbH & Co. Weinheim, Germany, respectively. Dr. Gill is a regular reviewer of National and International journals and grants. He was awarded Junior Scientist of the year award by National Environmental Science Academy New Delhi in 2008. With Dr. Tuteja, Dr. Gill is working on heterotrimeric G proteins and plant DNA helicases to uncover the abiotic stress tolerance mechanism in rice. The transgenic plants overexpressing heterotrimeric G proteins and plant DNA helicases may be important for improving crop production at sub-optimal conditions.

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Chapter 1
The Research, Development, Commercialization, and Adoption of Drought and Stress-Tolerant Crops

Gregory Graff, Gal Hochman and David Zilberman

1 The Importance of Stress-Tolerant Crops

Global crop production tripled over five decades from 1960 to 2010 (Fig. 1.1). In the next four decades from 2010 to 2050, global crop production must double yet again if supply is to keep up with expected growth in demand. For not only is global population growing—with basic food requirements thus expanding proportionately—but the burgeoning middle classes of Asia, Latin America, and Africa are consuming ever more livestock products and processed foods, thus amplifying those populations' demand for basic crop commodity output (Rosegrant et al. 2002). There is also growing demand for crops to produce biofuels, with numerous countries legislating ambitious renewable fuel standards (Rajagopal et al. 2007). These and other pressures have manifested in recent upward trends in agricultural commodity prices (Trostle 2008). Limited supplies and higher prices of food inevitably impact most the poorest and most food-insecure members of the human population, the billion or so who live on the equivalent of one or two dollars a day and spend a majority of their income on food, resulting in malnutrition, hunger, poor health, stunted growth, and entrapment in poverty.

The greatest challenge in further increasing agricultural production, it is generally argued, is that agriculture already operates at or beyond the limits of available resources—including arable land, fresh water, energy inputs, carbon emissions, and the loading of excess nutrients and agrochemicals onto neighbouring and downstream ecosystems. Further expansions in agricultural production are not feasible,

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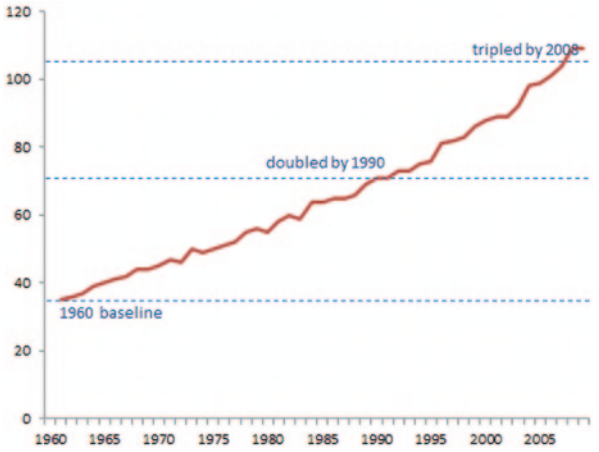

Fig. 1.1 Five decades of world crop production: 1960–2010 (Gross Production Index Number (2004–2006 = 100). (Source: FAOStat 2011)

not economical, or increasingly likely to cause irreversible environmental impacts, such as species extinction and climate change (Tilman et al. 2002). Climate change, moreover, threatens to further complicate the challenge of a sustainable increase in agricultural production—given increasing temperatures, shifting rainfall patterns, increasing variability, and greater frequency and severity of extreme weather events.

Such Malthusian views, however, are based on a linear conception of agricultural productivity growth and a static conceptualization of the nature of resource constraints. In the long run, the resource-use efficiency of agricultural production—the amount of land and water required per ton of harvested yield—has proven to be quite dynamic, and has improved markedly when measured over decades (Alston et al. 2010). Constraints of land or water use shift and recede before changes in technology, cropping, and cultural practices. Productivity growth is driven by several factors, and the most important include: (i) The quantity and the quality of the natural capital employed, particularly land and water, (ii) other physical capital employed, including mechanical equipment and irrigation infrastructure, (iii) suitability of other inputs and technologies employed, such as fertilization and pest control, (iv) the genetic traits and yield potential of the crops sown, and, finally, (v) the efficiency of allocations of agricultural inputs and outputs under the management of farmers and in response to price signals from markets. Moreover, all these factors can interact in complex, sometimes mutually reinforcing ways to improve agricultural productivity and, ultimately, its sustainability.

Of these factors, one of the potentially most significant for future increase in productivity will be the genetic improvement of crops to maintain yields under suboptimal conditions—such as drought or chronic water deficit, excessive salinity, extreme hot or cold temperatures, or other kinds of environmental stress or abiotic stress (Araus et al. 2008). Such conditions characterize—indeed, they define—those lands that are considered to be ‘marginal’, whether currently cultivated lands achieving lower and unstable yields or currently uncultivated lands, including those abandoned due to soil degradation by previous agricultural practices. Such yield-

limiting conditions could even come to characterize some of today's prime agricultural lands, depending upon how scenarios of climate change unfold in the future.

1.1 Background of Stress-Tolerant Crop Genetics

Historically, tolerance of adverse environmental conditions was not a primary characteristic favored in the domestication of crop species and subsequent genetic selections in traditional agriculture. Indeed most domesticated crop species are largely incapable of prolonged survival in the wild (Gepts 2004). Many traits that enhance chances of plant survival under adverse environmental conditions—like small stature and large root systems, photosynthetic reductions, or accumulation of defensive proteins—come at physical cost of lower production of harvestable output under optimal growing conditions (Cattivelli et al. 2008). Selection and replanting by farmers has tended to trade-off between plants that apportioned more of their energy to harvestable product versus survival. Recalling also that, for most of history, subsistence agriculture was quite diversified and spatially diffused, it stands to reason that, all other factors being equal, as long as a better-yielding variety did not fail too often, farmers would favor its higher expected yields, in any given season, over other varieties with lower yields but better assurance against failure in the uncertain event of a poor season. Moreover, diversification across multiple crops and livestock, often supplemented with other food sources like hunting and gathering, meant that, if a harvest did fail, other sources could be depended upon. Moreover, in most regions over most of history, if soils in one location tended to hinder growth of favored varieties, they would be passed over or abandoned in favor of other areas with more suitable soils (thus, effectively relegated to the category known as 'marginal' lands). Crop survival mechanisms were not, in short, the primary genetic characteristics for which subsistence farmers were selecting historically.

With the advent of scientific breeding in the last century, this trade-off was, if anything, further accentuated, at least initially. Evidence shows that early Green Revolution varieties with greater yield gains, also experienced greater yield variances (Traxler et al. 1995). While improved varieties yielded significantly better than existing varieties under optimal growing conditions, under adverse conditions they did not necessarily perform better—and may have performed worse—than existing (usually locally adapted) varieties. The benefits from an improved variety's increased yield potential were realized by maintaining optimal growth conditions with other inputs, such as irrigation and nitrogen application, thus creating incentives for farmers to procure these complementary inputs. Especially where these complementary inputs were available at economical or even subsidized rates, Green Revolution farmers favored varieties with higher mean yields, not necessarily higher survival rates under stressful conditions. This trade-off, thus, lies at the root of contentions that the Green Revolution did not equitably help all farmers, particularly neglecting to benefit those farmers who are relegated to cultivating marginal lands (Hazell et al. 2002).

However, the trade-off between higher yield and yield stability is not a rigid one, and post-Green Revolution, public breeding programs as well as commercial breeding programs have met with some success in achieving greater yield stability while breeding for higher yields (Cattivelli et al. 2008; Traxler et al. 1995). A 1998 survey to assess gene pool enrichment in the US summarized the objectives of 280 breeding projects, largely by public-sector breeders at the State Agricultural Experiment Stations (SAES) associated with the state Land Grantre search universities and the US Department of Agriculture's (USDA) Agricultural Research Service (ARS) in the US (Frey 1998). Out of the total number of breeding projects surveyed, 33 projects—representing just 12 %—reported an objective related to abiotic stress tolerance. Of these 33 stress-tolerance projects, seven had not produced successful results and five more were still uncertain. Considering breeding projects by type of crop, emphasis on stress-tolerance objectives was highest among temperate fruit and nut crops, with 14 out of 44 projects (32 %) related to improving cold hardiness, winter hardiness, heat tolerance, or drought-tolerance—with these concentrated primarily in blueberry, strawberry, and grape. Emphasis on stress-tolerance objectives was also higher in forage grasses, where four out of 29 projects (14 %) were breeding for drought and salt tolerance. By contrast, in grains, only four out of 52 (8 %) of reported projects related to stress tolerance; all of which were in wheat. In the majority of crop categories—including fiber crops, forage legumes, root crops, and oilseeds—stress tolerance objectives were all but absent. While stress tolerance has been targeted in breeding programs, it has been a minor emphasis relative to other types of objectives, and more difficult to achieve.

1.2 Recent Advances at the Molecular Level

Over the last two decades, rapid advances in plant molecular biology have opened up new opportunities to enhance stress tolerance while also increasing or at least preserving mean yields. The tools of molecular biology have enabled the identification of hundreds of genes involved in plant stress response and elucidated plants' complex stress response mechanisms as well as the interrelationships amongst them. This rapidly expanding knowledge base has enabled molecular breeding programs and transgenic strategies for drought and stress tolerance. Such knowledge can be used in molecular breeding programs to identify and bring multiple genes involved in stress response into elite germ plasm (Araus et al. 2008; Cattivelli et al. 2008; Liu and Chen 2010; Salekdeh et al. 2009; Sinclair 2011; Jenks et al. 2007). The molecular breeding approach has, for example, resulted in new varieties of hybrid maize released for the North America 2011 growing season that are marketed specifically as 'drought tolerant', including Syngenta's *Agrisure Artesian* maize, released in Colorado, Kansas, and Nebraska (Syngenta rolling out drought-tolerant corn 2011), and Pioneer Hi-Bred's*Optimum AQUAmax* maize, released in Colorado, Kansas, Nebraska, and Texas (Bennett 2011).

Alternatively, transgenic strategies allow individual stress response genes to be discretely added to high-yielding varieties, without compromising the transformed variety's yield potential as well as being stacked together with other transgenic traits—such as herbicide tolerance or insect resistance—that are already widely desired by farmers. Transgenic drought-tolerant maize varieties are in advanced field trials and being reviewed by regulators in the United States, Europe, Australia, and several other countries, including Monsanto's and BASF's jointly—developed MON 87460 event, with the cold shock protein B (*CspB*) from *Bacillus subtilis*. The same transgene is being adapted to African maize varieties by the Water Efficient Maize for Africa (WEMA) initiative, coordinated by the African Agricultural Technology Foundation (AATF), and consisting of a publicprivate partnership between Monsanto and several public sector agricultural research institutions in Africa, with funding from the Bill and Melinda Gates Foundation and the Howard G. Buffett Foundation.

1.3 Research and Development and the Commercialization of New Crop Varieties

Regardless whether a breeding or a transgenic approach is taken to improve crop genetics, doing so involves a research and development process followed by the commercialization of the successfully developed crop variety or other related technology. Today, these processes constitute the primary pathway by which fundamental knowledge of plant biology is translated into human benefit.

It is important to clarify that *any* release of a new crop variety—whether done by a company or a public-sector organization—can be understood as the commercialization of that variety for that is the point at which it leaves the controlled environment of the laboratory, greenhouse, or test plot and enters the much less controlled environment of human commerce. Moreover, varietal release occurs in the context of markets in virtually all cases. There is little dispute this is the case when a company makes the release. Indeed, however, it is exceedingly rare that seeds are freely handed out directly by governments or non-profit organizations to farmers. Even in those cases when they are, the released seeds are intended as inputs for (essentially commercial) agricultural production, or may be sold on the secondary market. In the more common case of a 'public' release of a new variety by a publicsector breeding program, the new variety is still very much entering commerce. Typically it is taken up by local seed companies or nurseries, either to be crossed into local varieties or simply to be multiplied and sold to farmers. Even when small scale or small profit margins dissuade companies from taking on this role, farmers themselves will grow and sell their surplus seed to other farmers for use in subsequent seasons. Finally, the very act of any farmer taking up or adopting a new crop variety is an economic decision, taken with due consideration of its economic implications for that farmer's production and household.

1.4 Roles of the Public Sector and the Private Sector

Both publicsector (government or non-profit) and private-sector (business) entities—as well as publicprivate partnerships between the two—are deeply engaged in R&D for crop improvement and in the commercialization of new varieties. The history of public support to crop breeding is primarily due to the genetic nature of such innovations. Seeds or other planting materials historically have been easily replicable, and therefore entrepreneurs have had little assurance, regardless of crop, that a genetic innovation they might introduce would not become widely copied at merely the cost of reproduction and transportation. Such imitators, because they invest nothing into R&D or breeding programs, can undercut the prices that the innovative entrepreneur must charge to recoup the value of his initial R&D investment plus interest (Baumol 2010).

Even when private entrepreneurs can manage to appropriate at least some returns above the marginal costs of reproducing and reselling a crop variety, they inevitably calibrate their levels and types of R&D efforts based only upon their expected profits from those appropriated returns: They are thus not induced to take into consideration any other benefits that their innovations might bring to others within the society. Such R&D can include the benefits of improved nutrition or food security that accrue to food consumers, improvements in environmental quality or public health, or technology spillover effects improving and accelerating the innovation efforts by other breeders or seed companies. For these reasons governments, non-profit philanthropies, and aid agencies have taken the lead and intervened in the missing or underperforming markets for innovation in agriculture (Sunding and Zilberman 2001).

The first real increase in private sector involvement in crop genetics began in the 1930s with changes initiated by the development of hybrids. The fact that hybrids would not breed true variety, introduced a physical mechanism of appropriability into the market for improved seeds. Breeders would only release seed for the hybrid progeny on the market while holding their foundational or parental breeding lines as trade secrets. As hybrid corn became commonplace in the 1930s and 1940s, private investment in corn breeding and the improvement of hybrid corn genetics took off.

At roughly the same time, in 1930, a new legal mechanism was introduced in the US: the '*plant patent*'. It was intended to enhance the appropriability of genetic innovations in asexually propagated crops, in order to encourage more privatesector investment in varietal improvement. Later, by the 1960s, yet another form of intellectual property over crop genetics—plant variety protections (PVP) or plant breeders' rights (PBR)—began to be developed in Europe and elsewhere, and was later coordinated internationally under the International Union for the Protection of New Varieties of Plants (UPOV) convention (Lesser 2007).

Much more significant privatization of crop genetics has come with the rise, since the 1980s, of recombinant DNA, cell and tissue culture, and plant transformation technologies. With the tools of biotechnology, the cost of making genetic improvements increased, while at the same time the potential value of (at least some)

new traits increased too. Costs increased due to the much greater perceived needs for translation, testing, and regulatory oversight to control for potential bio-safety risks. At the same time, the primary factor that has made the large investments by the private sector economically feasible has been the adaptation of patent law so that patents can be used to protect inventions in crop genetics. With patents, the value of a much wider range of genetic improvements can begin to be appropriated in a way that resembles the hybrid varieties, and thus the private sector is much more likely to invest. Moreover, tools of molecular biology made it technically much easier to detect and enforce breaches of trade secrets, patents, and other means keeping breeding lines and other genetic materials proprietary, as was vividly illustrated in a 1994 US federal case between Pioneer hi-bred and Holden foundation seed (Pioneer Hi-Bred International v. Holden Foundation Seeds Inc 1994).

Despite the extent to which private involvement in crop genetic development has increased, it is most accurate to describe today's relationship between the public sector and the private sector as one of interdependence. The public sector continues to make significant contributions, especially in the more basic areas of biological research, but it is typically not able to justify the dedication of resources necessary for advanced testing, commercial scaling, and market deployment, including, in the case of transgenic varieties, the costs of obtaining regulatory approvals. The private sector has a comparative advantage in managing the riskier financial arrangements needed for these processes.

The resulting interdependence between the public and private sectors and the role of patents in inducing follow-on private investment in development and commercialization is reflected in the newer process of technology transfer between public sector agricultural research and the private sector. Today, patents or PBRs are often taken out by universities and government agencies when they make potentially useful inventions in crop genetics. The purpose of such intellectual property protection taken by public sector organizations is not to provide incentives to public sector research in the first place nor to generate financial support for them by way of royalties earned but, rather, its purpose is primarily to induce follow-on private investment of the magnitude necessary to further test the viability of inventions that have resulted from publicly funded research, and for those that prove feasible, to bring them the rest of the way through the R&D pipeline to commercialization. Thus, while the private sector depends upon the public sector as a source of new ideas, the public sector depends upon the private sector as a source of capital and expertise for development and commercialization.

2 The Stages of the R&D Process

The research and development process always starts from new knowledge—whether a newly identified trait, the characterization of a promising new collection accession, or an insight about a better way of achieving an outcome. The R&D process, if completed successfully, results in the introduction of a new innovation, whether a

product or a proces, to a marketplace. The R&D process is not necessarily linear—i.e. proceeding from basic science, to applied research, and on to development—and may even begin with users of the technology. Yet, it typically does follow a causal sequence of operational steps. The term 'R&D pipeline' is commonly used in industry to describe the full set of candidate innovations currently being worked on and therefore likely to be forthcoming from the R&D process in the foreseeable future. A typical characterization of stages in the R&D pipeline for the crop breeding and biotechnology industry includes: (a) discovery, (b) proof of concept, (c) early development, (d) advanced development, and (e) regulatory submissions, and (f) market launch. Of course, not all of these steps are necessary under both the breeding and transgenic approaches.

The 'discovery' stage includes identification of potentially desirable genes or plant characteristics with methods such as high throughput screening, model crop testing, or participatory breeding. Potentially useful identifications can be made in university, government, and industry laboratories. Basic research can be very important for generating such discoveries, particularly in agriculture. Indeed, innovations are one set of important byproducts of basic research. Yet, discoveries also may arise from the watchful eyes of farmers who grow diverse varieties, such as land races cultivated near the center of origin for a given crop.

Next, the genetics underlying a trait, whether from a land race or another type of organism, must be moved into breeding material. This occurs in the 'proof of concept' and 'early development' stages, in which crosses or crop transformations are made. Particularly when the approach is transgenic, additional work is required to evaluate the viability of the transformation event, improve expression, and test performance in greenhouse and controlled field conditions. The 'advanced development' stage includes combining (or stacking) the new trait(s) with other valued traits, field testing, agronomic evaluation, and, for transgenic varieties, generation of necessary regulatory data. When a significantly novel trait, or a transgenic trait, is involved, bio-safety or environmental impact may be the concerns. In order to comply with legal requirements controlling the environmental and market release of novel traits and transgenes, submissions are made in the 'regulatory' stage for review and approval by regulators.

Finally, at the 'market launch' stage, a successful launch can depend upon integration into production and distribution systems and a sufficient quantity of stocks in preparation for distribution and expected sales. Often an initial release is done in a smaller, controlled manner in regional test markets, in order to collect market data to guide a subsequent full rollout, as well as to minimize losses in the event the crop fails to perform as expected. Other work after commercialization includes marketing, the informing of potential adopters about the new variety and its characteristics. For more novel traits, additional work may need to be done together with growers to help them learn how to manage the crop with the novel trait.

The notion of an unimpeded flow, as suggested by the image of a 'pipeline', is perhaps misleading. Better metaphors still capturing the notion of a dynamic flow might be an 'R&D funnel' or an 'R&D sieve'. The R&D process—whether in crop breeding or biotechnology or, really, any field of technology—consists of progres-

sive acts of selection. The initial large set of potential innovations considered early in the R&D process is, throughout the process, continually winnowed, filtered, and narrowed down.

2.1 Managing R&D Risks

Decisions are routinely made by scientists, managers, and their fund-providers—both in the private sector and in the public sector—as to whether to proceed with, modify, or terminate particular innovations at each successive stage of R&D. Those who are responsible for making such decisions—again, in both private and public sectors—are essentially engaged in an exercise of calculating the expected net benefits (expected benefits minus expected costs, to all relevant stakeholders) of moving the innovation one more step closer to commercialization. As a result, making a decision, either way, involves risk. At a minimum, if the decision is made to terminate, potentially large future private and social returns may be foregone (had the commercialization of the innovation succeeded). On the other hand, if the decision is made to proceed further, those further investments may be lost (if the innovation is later terminated or its commercialization does not succeed). In addition, liabilities may be incurred if the innovation somehow causes damages or losses to others.

Typically, the degree of uncertainty confronted is greatest early in the R&D process and decreases as the innovation progresses towards market and more is learned. There may be significant value gained in moving an innovation one step closer to market, precisely because of the learning that results in thereby reducing risk, sometimes referred to in the business world as ‘buying down the risk’ of a larger stream of future investments. There may also be value in simply ‘buying time’ for an innovation and keeping options open. This is particularly true if stopping and re-starting an R&D project and the associated redeployments of key personnel and physical resources, is costly or unfeasible. Finally, it should be noted that, at any given point in time, risk calculations about whether to further invest in or to terminate an R&D project, are made looking forward and considering future risks and opportunities for the innovation. R&D managers should not regard the size of prior investment that has already been made to bring the innovation to its current stage. These are ‘sunk’ costs. Of course, without them, the innovation would not have been brought this far, but having done so, they should no longer factor into how much further there is to go. Nor do past investments factor into the uncertainties faced further down the pipeline.

2.2 Four Types of Uncertainties

There are four primary types of uncertainties to be managed in crop genetic R&D: technological, intellectual property, regulatory, and market. Properly managing

each of these requires specialized expertise and tends to be the purview of different types of professionals. All four, however, interact extensively, thus requiring ultimately a comprehensive management strategy by those ultimately responsible for the R&D and commercialization of a new variety.

2.2.1 Technological Uncertainty

The first is technological uncertainty, not knowing whether the genetic innovation would work under increasingly realistic conditions and achieve desired performance parameters. Since this is the primary expertise of the molecular biologists, breeders, and agronomists at the core of any agricultural research organization or seed company, it is typically well addressed. Of course, until technical uncertainties are overcome, the R&D process does not progress, but once targeted technical issues are resolved, technological uncertainty can be greatly diminished in the later stages of R&D, unless IP or regulatory issues arise that require changes in the technology. New technological uncertainties can arise once a variety is commercialized and is being grown under more diverse field conditions and farming practices.

2.2.2 Intellectual Property Uncertainty

The second form of uncertainty involves intellectual property (IP) rights. The most fundamental concern whether a new variety—or a genetic component incorporated into a variety—might infringe another's IP rights and the likelihood that the owner would seek to enforce his IP rights against the infringing variety. If so, R&D or commercial use of the variety may need to be terminated, resulting in a loss of investments and potential benefits. However, it may be possible to negotiate a license to use the protected technology, but the outcomes of such negotiations, such as the terms of license and the cost of royalties, can be uncertain (Cahoon 2007; Nilsson 2007; Satyanarayana 2007). On the other hand, uncertainties also arise when seeking to obtain one's own IP rights over new technologies invented or new varieties developed or to enforce them. There are questions as to costs of pursuing IP rights, when or whether an application will be granted, as well as questions about the strength and enforceability of an IP right even if granted, as this can depend upon a range of legal issues (Livne 2007). Moreover, IP rights are country-or jurisdiction-specific. (Some regions, such as Europe, have multi-country patent offices). IP issues that arise in one market may not be relevant in another (Yin et al. 2007). Yet, such variations in IP coverage, can potentially affect international trade in agricultural products resulting from protected varieties (Binenbaum et al. 2003). Intellectual property uncertainty is of little concern in the early stages of R&D, particularly in the public sector, and, as such, many breeders and geneticists engaged in the discovery and proof-of-concept stages, tend to give it little consideration; however, IP becomes progressively more important as decisions are made to move a genetic innovation closer to market, particularly when larger investments are required to

do so (Fenton et al. 2007). Moreover, public sector innovators may not have the legal expertise or resources needed to manage IP uncertainty, whether to thoroughly examine the extent to which an innovation enjoys freedom to operate with regards to others' IP rights (Krattiger et al. 2007), to make effective applications to obtain IP rights to support further private investment in a genetic innovation, or to assure public access to a new genetic innovation (Nelsen et al. 2007) depending upon the goals of the R&D organization.

2.2.3 Regulatory Uncertainty

The third type of uncertainty is *regulatory* uncertainty: This means not knowing when or whether an innovation will be approved by regulators for market release, or what costs may be incurred to meet the testing and data required for regulatory review. There also may be penalties imposed or liabilities incurred in the event of a regulatory violation. In one example, in 2001 the French-German company, Aventis, incurred hundreds of millions of dollars in fines, damages, and recall costs for releasing a transgenic *Bt* corn variety '*StarLink'* in the US market without having all the required regulatory approvals (Taylor and Tick 2001). Most countries regulate transgenic crops carefully, addressing bio-safety concerns such as environmental, human health, animal health, plant pest, as well as economic concerns such as value of exports (given that trade partners, also regulating such crops, may not accept some as imports). In the countries that have not yet adopted and implemented a functioning bio-safety regulatory framework, the presumption is effectively universal that no transgenic crops should be grown until the regulations can be implemented and transgenic varieties approved. Crop varieties developed using a breeding approach, confront far less regulations, thus greatly reducing if not eliminating regulatory uncertainty (and costs). In some countries, such as Canada, bio-safety regulations are being applied to novel traits resulting from other, non-transgenic methods such as mutagenesis.

2.2.4 Market Uncertainty

The fourth and ultimately most important type of uncertainty confronted in managing R&D is the one that arises from the market, reflecting a range of unknown factors that the resulting innovation will face once commercialized. In the marketplace, a new crop variety is subjected to the independent decisions of thousands of farmers. They ultimately are the decision-makers who decide whether or not the variety is appropriate for their farming operations. These decisions by farmers depend upon a host of technical, economic, legal, and other considerations that can only be partially anticipated during the controlled pre-market stages of R&D. How well will the variety actually perform? What will growing conditions be like? What will the weather be like? What competing varieties are in the market? The market exerts very real selective pressure of its own, whereby those varieties that prove unfit in

an economic sense do not survive, while those that prove to be well adapted to their new commercial environment, flourish.

Different types of crop traits confront different types of market uncertainty. The market for pest control or disease resistance traits depends upon the prevalence of the targeted pest or disease problems and the relative costs of other technologies, such as chemicals, for controlling them. Output or product quality traits such as nutritional content, controlled ripening, or reduced all ergenicity will each target very specific user segments and create value one or more steps down the vertical chain of markets between farmers and consumers. Markets for drought and stress tolerance traits, depend upon yet other considerations, to which we turn next.

2.3 Sources of Market Uncertainty: Factors Influencing Farmers' Decisions to Adopt Drought- and Stress-Tolerant Crops

Those investing in the development of drought- or stress-tolerant crop genetics must consider what factors will influence the likelihood of adoption of such crop varieties. Questions of adoption—by whom, where, and when—are central to the success and impact of new crop varieties on agricultural productivity. Extensive literature on technology adoption clearly shows that the extent to which a new technology—such as a new crop variety—is taken up by potential users is strongly influenced by several key factors, including: (i) the degree of complementarity between the new technology and other already existing technologies or (ii) the degree of substitution between the new technology and other already existing technologies, (iii) the appeal of the new technology to potential first-time adopters (i.e., its potential for adoption on the extensive margin), (iv) the distribution and timing of benefits actually realized by adopters of the technology, and (v)social perceptions of the new technology. Each of these market factors will be considered for drought and stress tolerant crop varieties in turn.

2.3.1 Interaction with Other Complementary Technologies

The first factor that influences the adoption of a technology is the degree of complementarity between that technology and other parts of the production system to which it is to be added. When two technologies in a production system are complementary, adding or improving upon one increases the returns to using more of the other—whether by improving its efficacy or efficiency. As such, the extent to which one technology is adopted is not an isolated decision; rather it becomes part of a more complex set of decisions about the production system as a whole package of technologies.

In particular, there is complementarity in the farming context between risk reducing and yield-enhancing technologies. The complementarity principle can be illustrated in a simple mathematical model of crop production. Let actual yield be

designated as Y, which can be calculated as the potential level of output, f-which is a function of farming inputs, Z, such as fertilizer-times1 minus the share, D, by which yield is typically reduced as a function of pest damage, j, times the contribution to yield, h, made by the level of rain, r, and irrigation, I, received:

$$Y = f(Z)(1 - D(j))h(r, i).$$

Farmers, who typically lose some of their potential yield to pests or drought, can, when they know pest damage or drought damage can be controlled, increase profits by using more fertilizer per acre. The higher expected yield and the lower likelihood of losing the crop altogether, means that each unit of fertilizer applied to the crop will have higher likelihood of efficacy and conversely an investment in fertilizer will have a lower likelihood of being wasted.

This effect can be seen in the yield gains realized by actual farmers, who adopt transgenic crops, typically being higher than the controlled yield gains observed in field trials (Sexton and Zilberman 2011). This happens because farmers are not only changing the genetics of the variety being grown, but, at the same time, are increasing their use of other complementary inputs. The cumulative effect can be seen in greater productivity growth in those top producing countries that have adopted transgenic crop varieties. Among the countries where transgenic crops have not been adopted, crop productivity has not grown significantly in recent years. This is partly because of the fact that farmers in these countries will have held back on adopting other complementary inputs, in addition to not having adopted the improved transgenic varieties.

This effect of complementarity can also be seen in the much greater yield gains from adoption of transgenic varieties over non-transgenic varieties realized on an average in developing countries relative to developed countries (Qaim and Zilberman 2003). Again, because yields with the transgenic variety are expected to be higher due to reduced pest damage, farmers in developing countries find it worthwhile to apply greater amounts of complementary inputs such as fertilizer, or to adopt complementary technologies such as irrigation systems upon adopting a variety with significantly higher yield potential. Since farmers in developed countries were already using high levels of these complementary technologies, like fertilizer and pest control, when they adopted transgenic varieties, their yield gains consist only of the gain from the transgenic alone.

Based on these observations, it is reasonable to expect that adoption of drought- or stress-tolerant varieties will, as they increase expected yields, lead to investments in pest control and chemical fertilizers, from which additional yield gains will also be realized. Conversely, however, to the extent that access to these other inputs is constrained, adoption of drought and stress-tolerant varieties may indirectly be constrained.

2.3.2 Competition with Potential Substitute Technologies

The second factor influencing adoption is the degree to which a new technology may substitute for—and thus compete against—another existing technology. The

broad question is whether the characteristics of the new crop variety could serve as a replacement for any other technology that is or that might alternatively be used in production. And, if so, how closely do the new and the existing technologies compare in terms of efficacy and price?

A clear example of substitution from biotechnology can be seen in insect resistant *Bt* crop varieties, which substitute for chemical pesticides and, potentially, organic or integrated pest control methods. A transgenic insect resistance trait, like*Bt*, however, is not a perfect substitute for these other solutions to pest damage. Factors such as timing, insect specificity, monitoring requirements, and more will vary between these different pest control options. There can also be economic differences in how the crop can be sold afterwards. Under these conditions of what economists call 'imperfect' substitution, the extent to which the new biotech option is adopted is, thus, not an isolated decision; rather the decision is strongly influenced by comparisons with other technologies for which it provides a substitute.

Drought tolerance may, at first sight, appear to be a fairly novel technical option, without real substitute. However, upon closer inspection, it is clear that irrigation technologies (providing greater efficiency of water access, storage, and delivery) are at least a partial substitute. Tolerance to salinity or metal toxins may not have many substitutes, and therefore may be more readily adopted as solutions in those areas where such problems are endemic. Cold or frost tolerance will substitute, to some degree, for techniques to control frost damage—such as spraying or fans or greenhouses. Of course, crop varieties developed through a breeding approach and varieties developed through a transgenic approach for a similar trait, such as drought tolerance, will be very close substitutes and can be expected to compete.

2.3.3 Appeal to First-Time Adopters of the Technology (Adoption on the 'Extensive' Margin)

The third factor is the degree to which a technology is likely to be adopted by first-time users—that is, farmers who do not already have a closely comparable solution in place for managing crop stress problems. The decision by new users to adopt a new technology for the first time is a very different process than the decision of those who are effectively upgrading or switching from an existing technology.

The cumulative effect of decisions to adopt or not to adopt a new technology across a population of first-time users can be modelled as the diffusion curve of that technology. Diffusion is typically S-shaped over time. It begins at the point of commercial release and increases slowly at first with a few per cent of early adopters taking up the technology. Among farmers, early adopters are typically better-capitalized and better-educated and more willing than others to take risks (Feder et al. 1985). The new technology then comes to its most critical test: will the percentage of adopters take off by appealing to 'mainstream' adopters. If adoption does take off, the diffusion curve can rise rather quickly. Eventually, the market approaches 'saturation' as diffusion levels off at the maximum percentage of the market that

has adopted the technology. And over a period of time, a given technology will eventually become obsolete, or become superseded by a yet newer version, and the percentage of the market using that technology begins to 'decline', thus completing that technology's 'life cycle'.

Diffusion rates may differ by country or by region. It can be observed that some farmers in some regions adopt a technology earlier than others. And sometimes a new technology can lead to the expansion of cultivated acreage into 'marginal' areas not previously utilized for growing crops, thus the technology is truly pioneering, tipping the balance between being able to viably farm in an area versus not.

Modern irrigation is one example. As a technology, it is land-quality augmenting, and, as such, it tends to be more quickly adopted on low-quality lands, much of which could not previously be cultivated. Different irrigation technologies have different efficiencies. The water-use efficiency of flood irrigation is typically 0.2–0.6 depending on soil conditions. Upgrading to sprinkler irrigation increases efficiency into the range of 0.5–0.8. Drip irrigation may reach efficiencies as high as 0.95. The more efficient irrigation technologies, however, are also more expensive. They are more likely to be adopted on very poor soils, such as sandy soil types or steeper grades, or when water prices or output prices are high. Drip irrigation has, in California, expanded the acreage of high-value perennial crops such as avocados and wine grapes into previously uncultivated marginal lands in foothills and desert areas.

Transgenic herbicide-tolerant soybeans are another example. In Argentina, the area sown to soybeans has tripled since the introduction of herbicide-tolerant varieties (Trigo et al. 2009). And, apart from the rise in acres, the mean yield per acre has also increased. The technology has enabled farmers in Argentina to grow soybeans by addressing an end-of-season weed problem. Agricultural commodity analysts had been concerned by the late 1990s, about the effect that growing demand from China would have on the global price for soybeans, but the increased production in Argentina resulting from adoption of herbicide-tolerant soybeans, has been able to meet China's demand.

Based on these observations, drought- and stress-tolerant crop varieties are likely to increase cultivation acreage. There is significant acreage globally where rainfall is at the margin of viability under current rainfall patterns (300–400 mm per year). To the extent that drought-tolerant varieties can enhance the probability of crop survival and increase average yields in these marginal uncultivated lands, they can become arable cultivated lands.

The adoption of drought- or stress-tolerant varieties will not necessarily be driven by an increase in mean yields, but rather by the reduced probability of detrimental losses. For example, suppose the threshold yield—the yield giving a farmer just enough revenue to meet costs—is 0.6 tons per acre. Suppose then that adopting a drought-tolerant variety increases mean yield from 0.6 to 1.1 tons per acre, and it also reduces the probability of falling below 0.6 tons per acre in any given year from 40 to 10 % probability. The increase in mean yield matters less than the decreased probability of failure. The main differences are in the lower tail of the yield distribution, and this is the primary factor that will drive adoption on the extensive margin.

Indeed, the mean yield may be even less, only 0.8, as long as the probability of falling below the threshold yield is diminished, farmers may still favor this variety, particularly on lands that could not previously be cultivated.

One important implication of this, which could arise under certain circumstances, would be that aggregate average yields reported for a particular crop or region may actually decrease as a result of adoption of drought- or stress-tolerant varieties. This could arise when adoption rates are relatively high on the extensive margin, characterized by newly cultivated marginal lands with mean yields lower than the region's previous average but still above the threshold, making cultivation economically-viable for poor farm households. In such areas, it may be important to report cropping statistics by smaller aggregate units in order to disentangle effects of switches to the new variety, at the higher-yielding intensive margin versus expansion on the lower-yielding extensive margin.

2.3.4 The Distribution of Benefits to Adopters of the Technology Over Time and Different Locations

The fourth factor affecting adoption of a new technology is the timing of its impacts and differences in its impact in different locations. A given technology's performance within the given production contexts—particularly in farming where there can be significant variations in land quality, water availability, labour, and other inputs—can vary significantly across the population of potential adopters of that technology.

The most effective way for a farmer to arrive at a decision about adopting a new technology is by learning from experience—such as trying the technology on a portion of one's land or watching a neighbour try the technology, and thus experiencing the results firsthand. Yet, learning-by-doing will encourage adoption only if the technology reliably yields a perceptible benefit each season. But, depending upon weather patterns, a drought-tolerant variety may produce a benefit only occasionally, resulting in a less predictable 'stochastic' benefit stream (Lybbert and Bell 2010). For example, if drought occurs only 20 % of the time, the learning-by-doing farmer does not detect any impact in four out of five years. Here, adoption is expected to be limited if benefits are delayed and uncertain. This can be seen, for example, in farmers' limited willingness to pay for crop insurance. Similarly, if the drought impacts one region but not another, farmers in the affected region are more likely to have experienced the benefits of a drought-tolerant variety and in the following year to plant that variety. The distribution of impacts over time and across space, can therefore be, expected to strongly affect the rate and pattern of adoption of this technology across markets.

Thus, the timing of commercial release of the new variety in each location will be critical. Studies of past droughts suggest that droughts represent periods of significant change in farming practices. It is, therefore, reasonable to expect that adoption of drought-tolerant varieties may be observed in a given region only after it experiences substantial drought.

For example, consider the lessons of California's response to severe drought in 1988–1992. One major lesson was that water storage matters. Water storage facilities enabled California to survive the three early years of the drought with minimal impact or changes to cropping practices and then to survive the later years with only moderate impact. Another important lesson was the importance of being able to make multiple responses to reduced water supply. During the California drought, water deficit was addressed primarily through three different responses: One-third of the deficit was made up from groundwater pumping; another third of the deficit was accomodated by letting land lie fallow; and the remaining one-third of the deficit was compensated for by conservation measures, including the adoption of high-efficiency irrigation technologies such as drip irrigation. Indeed, more efficient technologies made a real difference in conservation. By 1992, at the end of the drought, more than 50 % of the tree crops in California used drip irrigation and more than 40 % of the cotton and alfalfa in major growing areas used sprinkler irrigation. So, similar impacts could be expected for adoption of drought tolerant varieties.

The situation is somewhat different for crops resistant to non-transient stresses embodied in soil, such as salinity, pH, or metals. In such cases, the benefits will be observable as long as the soil problem persists. Side-by-side demonstration plots in affected regions may be enough to encourage many farmers to adopt. However, for those traits that confer tolerance to transient stressors, such as drought or freezing, an important implication that follows from this reasoning is that adoption to a level that takes full advantage of the social benefits of the technology, may require subsidies and public action.

2.3.5 Perceptions Are More Important than Reality

The fifth factor that influences adoption decisions is the general perceptions of a technology across society. How do various stakeholders, including the general public, perceive the new technology? This depends greatly upon the nature of the technology, and how it affects major social concerns such as the environment, health, and poverty.

In the field of marketing, this phenomenon is called reputation building, and it has to do both with the effects of informal interpersonal sharing of information about a new technology as well as broad coverage and commentary about the technology in the media, amongst activists, and throughout the policy community. Reputation building is often the initial process by which potential adopters are informed about and have their first impressions of a new technology. But it is also the main avenue by which members of the public form their (often simplistic) opinions about the acceptability or desirability of a new technology (Brossard et al. 2007). Reputation thus can weigh heavily upon the disposition of policy-makers and regulators towards a new technology (Graff et al. 2009). It can thus influence the extent to which the technology is approved and becomes available to farmers to grow. It can also influence farmers' interest in adopting the technology if there are concerns about

how it may affect their ability to sell the crop or affect the price they will receive for their harvest. A poor reputation can reduce consumers' willingness to pay and thus the price of crops produced using a technology (e.g. transgenic versus conventional crops), just as much as a good reputation can enhance consumers' willingness to pay and thus the price of crops (e.g., organic versus conventional).

There may be a significant gap between public perceptions of a technology and the reality of how it performs. The popular public perception is that the first generation of transgenic crops—those consisting of *Bt* and herbicide tolerance pest control traits—have been damaging to the environment, have not increased yields for farmers, and have not helped the poor. In reality, the poorest farmers have not adopted transgenic varieties to any great extent except for small-scale cotton growers in India. However, the lack of adoption by poor farmers has been mostly due to regulators; transgenic varieties have not been available to the poor primarily due to the decisions of their governments (Paarlberg 2008). In reality, transgenic varieties, when made available, have largely increased yields and incomes, especially for small subsistence farmers in developing countries (Pehu and Ragasa 2007).

Current popular perceptions of transgenic drought- and stress-tolerant varieties are, however, considerably better: That they are yield-increasing and poor-friendly varieties. Of course, the reality is that their impacts are yet to be observed beyond test plots, since the varieties have not yet been commercially released. However, economic analyses of the potential impacts of drought-tolerant varieties indicate that while they should be significant (Rovere et al. 2009), they are likely to be less than the impact of insect-resistant varieties (Lybbert and Bell 2010). Even though drought-tolerant varieties eliminate drought loss, the expected gains from pest control is generally greater than the expected gains from drought tolerance under realistic models of pest damage and drought risks. This is largely due to the fact that pest damage is basically a more frequent threat than drought across a larger range of growing regions.

Drought-tolerant varieties are being developed and promoted because public perceptions of the technology are generally favorable, even when using the transgenic approach. While pest control traits likely would have greater impact on the economic welfare of farmers and consumers in most developing countries, political constraints are leading to the introduction of drought-tolerant varieties first. And this is occurring despite our prediction that the technology will require extra support to encourage its adoption.

3 What Is in the Global R&D Pipeline?

Given the attendant dynamics and uncertainty of the R&D process, not to mention the strategic concerns of many of those engaged therein, it should not be surprising to find that it can be quite difficult to collect very reliable information about what exactly is in the R&D pipeline at any point in time. Yet, it is possible to get a

rough first look, at least retrospectively, for a particular field of technology that is detailed enough to develop some understanding of the major contours of activity, the major players, and possibly the timelines involved. Several types of data can serve as 'R&D indicators' in crop genetics, and here we will draw upon four. First, we seek to collect the full literature of scientific articles, and look at what topics are being written about, by whom, where, and when, to give us an idea of what is going on at the earliest stages of the R&D pipeline. Second, we collect the full set of patents from around the world, to look at what inventions are being protected, by whom, where, and when, shedding light on activity a bit further into the R&D pipeline. Third, we collect all field trial data available from governments around the world, and look at what transgenic crop varieties with stress tolerance traits are being field tested, by whom, where, and when, to give us an idea of trends at the advanced development stages of the R&D pipeline. Fourth, we collect all regulatory submissions we can find seeking commercial release of transgenic stress resistant crops. What we seek is an empirical sketch of the trends of the industry, the interplay between public sector and private sector innovators, and at least a rough sense of where innovation stands at this point in time. What we do not observe yet, are any transgenic drought-tolerant varieties released in the market. But, based on the picture that emerges below, we find that they are indeed imminent, even if their uptake by farmers and the resulting impacts are uncertain.

3.1 Activity at the Earliest Stages of R&D: Trends in Scientific Publications

The quantity of publications coming out in the scientific literature, roughly represents the amount of activity going on in basic research, and thus the size of the public corpus of 'pre-competitive' knowledge that can be useful in advancing R&D. While very difficult to separate from research that is purely public in nature, it also reflects the applied research activities being conducted in the early stages of R&D, including 'discovery', 'proof of concept', and 'early development'. There are two important advantages of utilizing scientific articles as an R&D indicator. First, the view they provide is not restricted by geography or nationality: The work of authors from any country can and will be represented in the international scientific literature, with language differences of increasingly diminished importance. Second, the view they provide is not restricted by field or technology. In particular, both breeding and transgenic approaches are described within the scientific literature.

To identify the publications specifically related to the biology of stress response or tolerance in plants, the Web of Science database was queried using several carefully constructed search strings utilizing water stress, salinity stress, temperature stress, oxidative stress, environmental stress, and abiotic stress terms while at the same time using plant-specific terms to narrow the queries down from biology of stress response in all other types of organisms. Only records designated as 'research articles' (as opposed to 'reviews', 'notes', 'conference proceedings', etc.) were re-

Table 1.1 Top 20 journals by number of research articles on plant stress topics. (Data source: Web of Science, Thomson Innovation 2011)

Rank	Journal name	Articles
1	Plant Physiology	903
2	Plant Molecular Biology	517
3	Journal of Experimental Botany	462
4	Plant Journal	458
5	Plant Science	389
6	Planta	362
7	Journal of Plant Physiology	336
8	Physiologia Plantarum	333
9	Plant and Cell Physiology	304
10	Plant Cell and Environment	300
11	Plant Cell	248
12	Proceedings of the National Academy of Sciences of the USA	193
13	Plant Physiology and Biochemistry	190
14	Theoretical and Applied Genetics	176
15	Plant Cell Reports	176
16	Journal of Biological Chemistry	152
17	Crop Science	143
18	Euphytica	109
19	New Phytologist	106
20	Biochemical and Biophysical Research Communications	105

tained, under the assumptions that they are more likely to represent discrete novel contributions to the field, to conform to certain norms and standards, and to meet a certain threshold of research significance. A total of 13,583 research articles published from 1991 to 2010 were found.

Preliminary assessment of the publications dataset confirms that the majority consists of technical plant genetics and physiology articles, but a minority covers more general articles on the biology of stress response across organisms (but presumably by including plants in the discussion or references, given the search strings used). The journals most frequently represented are the leading journals in plant biology (listed in Table 1.1), but do include a couple of more general science journals. Together, these 20 journals account for 44 % of the articles in the dataset.

Diagnostic queries within the publications dataset illustrate the relative frequencies and interrelationships of research on different types of plant stress (Table 1.2). These indicate that temperature and water stress are the most frequently addressed topics and that they co-occur in 1833 out of the 8538 (or 21 % of the) articles that include either or both sets of topics. The greatest degree of co-occurrence between topics, however, is between water stress and salinity stress (33 %). The more general terms of abiotic stress occur less frequently in the literature, but overlap relatively equally with all others. Thus, the literature appears to be fairly self-specialized within each major type of stress, with some degree of overlap between stresses, as should be expected (Mittler 2006).

Table 1.2 Numbers of research articles responding to query terms for different types of stress. (Data source: Web of Science, Thomson Innovation 2011)

	Temperature stress	Water stress	Salinity stress	Oxidative stress	Abiotic stress
Temperature stress	*5,622**	1,833	1,131	890	664
Water stress		*4,749**	2,043	746	716
Salinity stress			*3,578**	640	644
Oxidative stress				*2,813**	375
Abiotic stress					*1,654**

* Number of articles responding to each set of query terms alone lie along the diagonal

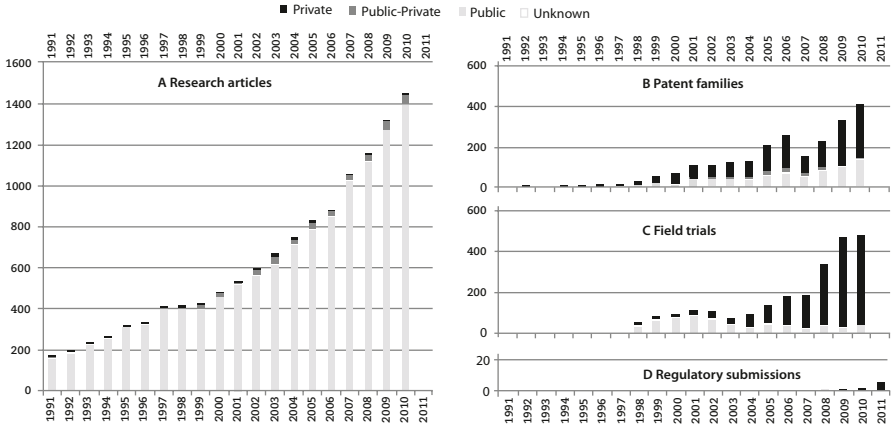

Fig. 1.2 The dynamics of the drought and stress tolerance R&D pipeline

Growth in the publication of scientific articles has been rapid, expanding exponentially from about 400 articles a year in the late 1990s to about 1400 articles a year by 2010 (Fig. 1.2a). This rate of increase reflects the significant investments made, primarily by governments, in research on drought and stress tolerance over the last decade (likely more than $ 1 billion according to Lybbert and Bell 2010). This research publication activity has been almost entirely done by authors at public sector research institutions. Authors at private institutions are found in less than five per cent of research articles in any year (Fig. 1.2a).

The research effort in the biology of plant stress—and its growth—has not been limited to any one country or part of the world, but is markedly international (Fig. 1.3). In the early 1990s, the United States was the leading locus of research, and while publications from US-based authors have continued to grow steadily, they have been eclipsed by publications from other regions in both growth rate and absolute numbers. Europe emerged by the mid-1990s as the leading region, but the output of publications from other developed countries has grown at least as quickly, especially in recent years. Yet, by far the most remarkable growth has been in China and other developing countries, and largely since 2000. By 2010, for example the

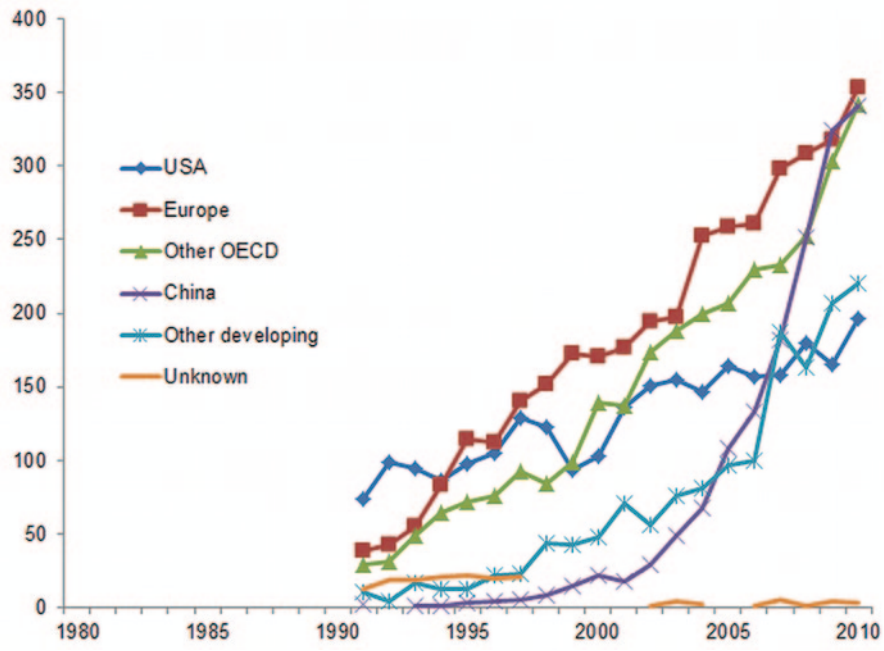

Fig. 1.3 Numbers of scientific articles on biology of drought and stress tolerance published per year, by country of main author affiliation. (Data source: Web of Science, Thomson Innovation 2011)

number of scientific publications from authors based in China rivalled the number from authors in all of Europe.

It is clear from the dynamics at the discovery stage, as revealed in the rates of scientific publication, that the early stages of the crop genetics R&D pipeline are rich with candidate traits, technologies, and potential breeding stock that should be advancing towards commercialization over the next few years. The rate at which this advance might be expected to occur, however, is suggested by trends observed using the other R&D indicators.

3.2 Activity in Early-Stage Development: Patenting Trends

Patents, as described above, are sought to protect against the copying of an invention by others, in order to maintain some degree of appropriability of the value of that invention by the patent holder. Inventions eligible for being patented must be truly inventive (not just an obvious extension of the current state-of-the-art), they must be novel (i.e., not already published), and they must be shown to be of some utility in human commerce or industry. Patents are not granted for discoveries of

natural phenomena (Although inventive uses or modifications of natural phenomena, including isolated DNA and transgenic organisms, are patentable in some jurisdictions). Finally, inventions must be, to a certain extent, 'reduced to practice' to be patent eligible. As a result, patents best represent activity at the 'proof-of-concept' and 'early development' stages of the R&D pipeline.

One complication in using patents as an R&D indicator is the fact that patents are national or, at best, regional. An invention may be represented by a single patent application and granted patent in just one national or regional jurisdiction (such as the United State Patent and Trademark Office or the European Patent Office), or it may be represented by multiple patent applications and grants in multiple jurisdictions. The extent and scope of filing depends upon the applicant and their choice of where to seek protections. The set of patent documents in one or more jurisdictions, claiming a common invention and the same (set of) priority date(s), is called a 'patent family', and is catalogued by INPADOC, an international patent data service of the European Patent Office. It is useful to track patent families rather than individual patent documents, if one is interested in the actual rate at which inventions are being made in the R&D pipeline.

Two advantages of using patents as R&D indicators are that patent documents are fairly standardized, and thus represent reasonably comparable quantum of knowledge, and that they can be observed across countries. However, patents are to some degree, less universal and more biased—geographically, institutionally, and technologically—than are scientific articles. Patents are, after all, historically a commercial instrument that arose from Western (Italian and English) legal traditions. Thus, patents will tend to over-represent inventions from higher income countries, by private sector inventors, and in molecular or transgenic technologies. Innovations at a comparable point in the R&D pipeline from lower income countries, being developed by public sector institutions, and using a breeding approach are less likely to show up in the patent literature. Nevertheless, in seeking to understand the commercialization processes, patents, especially when queried randomly, do provide a representative cross section of what is in the R&D pipeline.

To identify just those patents (and thereby underlying inventions) related to the biology of stress tolerance in plants, the Derwent World Patent Index database was queried using the same keywords used to search the scientific literature, in combination with specific International Patent Class indices to narrow the search to plant biology. A total of 13,213 patent documents from 60 different countries were identified, which altogether represented 2,769 patent families. In this dataset, patent families consist of 1 to 153 patent documents (with an average of 2.5).

The top jurisdictions in which patents are being filed are listed in Table 1.3. The United States has the most patent documents filed, but also the most patent families represented among its filings. Notably, quite a number of the patent families represented in the dataset, at least 1,650 out of the 2,769, are unique to a single jurisdiction. (For example, the United States had 804 such inventions, largely corn and soybean varieties which are patentable in the US but not elsewhere.) The remainder, of no more than 1,120 patent families, represents the subset of stress-tolerance-related in-

Table 1.3 Number of total patent documents and count of patent families by jurisdiction. (Data Sources: Derwent World Patent Index and INPADOC, Thomson Innovation 2011)

	Country	Total patent documents in the jurisdiction	Patent families represented in the jurisdiction	Patent families unique to the jurisdiction
US	United States	3,759	1,553	804
WO	WIPO applications	1,858	893	73
EP	Europe	1,432	568	4
AU	Australia	1,190	647	59
CN	China	958	660	358
CA	Canada	731	515	2
JP	Japan	545	347	19
DE	Germany	316	165	2
MX	Mexico	290	269	103
BR	Brazil	232	166	7
AR	Argentina	209	182	0
KR	Korea	208	150	26
IN	India	188	188	187
AT	Austria	173	127	0
ZA	South Africa	133	100	1

ventions that are protected in more than one jurisdiction. Of these international patent families, only a few hundred involve multiple patent filings across many jurisdictions.

The rate of invention is represented in Fig. 1.2b. The annual rate of new patented inventions has grown significantly since 2005. There was a notable dip in new inventions in 2007 and 2008, but the growth recovered in 2009 and 2010.

We can also note that the magnitude of patenting is significantly smaller than that of publishing, at a ratio of around one quarter to one third. This largely reflects the selection or winnowing of the R&D process: Not all research findings translate into patentable inventions. The ratio of patenting to publishing observed here is somewhat lower than the norm observed across all fields of technology. (As a benchmark, in 2008 the US patent office granted 80,048 patents while the Web of Science reported 202,037 articles published; exhibiting a ratio of 0.4). This also suggests that a significant share of the knowledge generated and published remains in the public domain.

The involvement of the private sector is much greater in patenting in publishing (comparing panel b with panel a in a Fig. 1.2). Further investigation of those to whom these inventions are assigned (Fig. 1.4) shows that the private sector accounts for 66 % of patent families in the dataset, while the public sector accounts for 29 %. The ratio between public and private deviates significantly from the norm, given that all fields of technology the public sector accounts for is less than 3 % of patenting activity. Prior analysis has shown the public sector accounted for about 25 % of the patents in the field of plant biotechnology up through 2000 (Graff et al. 2003).

The degree of concentration of patent holdings in the private sector is also apparent in Fig. 1.4. The top five patent assignees own 44 % of the stress-tolerance related inventions. Recall, however, that 809 (or fully 29 %) of the patent families are

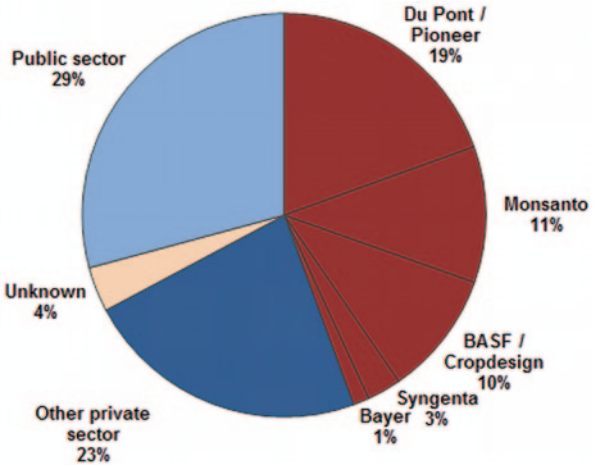

Fig. 1.4 Ownership of drought and stress-tolerance genetics globally (N = 2,769 patent families)

soybean and maize cultivars patented only in the US and the portfolios of the two top assignees, Du Pont-Pioneer and Monsanto, account for a large portion of these.

3.3 Activity in Late-Stage Development: Field Trial Trends

It is required by virtually all governments to obtain permission to conduct open field trials of transgenic crops not yet approved for commercial cultivation. In the R&D process, open field trials are useful for testing varieties under more realistic field conditions after they have passed through early development stages in the laboratory and greenhouse. As such, open field trials are a good indicator of activity at the advanced development stage of R&D. Fortunately, most governments make basic information on such field trials available to the public.

The greatest advantage of field trial data is the specificity of the information (in most cases), identification of the crop, the trait, and the organization doing the trial. The most obvious disadvantage of field trial data is that it covers only varieties that are under development using a transgenic approach. Varieties developed by the breeding approach are not required to be registered in the same manner, and therefore are not systematically available.

Field trial data were obtained from online postings by relevant government authorities in the United States (http://www.aphis.usda.gov/brs/status/BRS_public_data_file.xls), Europe (http://gmoinfo.jrc.ec.europa.eu/gmp_browse.aspx), Canada (http://www.inspection.gc.ca/english/plaveg/bio/pbopnte.shtml), Australia (http://www.ogtr.gov.au/internet/ogtr/publishing.nsf/Content/ir-1), Japan (http://www.bch.biodic.go.jp/english/lmo.html), and India (http://igmoris.nic.in/field_trials.asp). Field trial data were located for Mexico (Juarez and Branson 2011), but none pertained to drought or stress tolerance. Secondary reports on a handful of field trials

Table 1.4 Registered field trials of transgenic crops with drought- and stress-tolerance traits (cumulative totals through mid-2011)

Country/Region	Stress tolerance field trials
United States	1,386
Canada	1,003
Australia	17
India	9
Japan	7
Europe	14
Spain	6
France	3
Germany	2
United Kingdom	1
Sweden	1
Hungary	1
Africa	11
South Africa	6
Egypt	3
Uganda	1
Kenya	1

of drought-tolerant crops were available for African countries. The most obvious deficiencies, given the rates of publication and patenting by country, observed in the preceding sections were due to our inability to access field trial data from China (Huang and Wang 2002) or Brazil (http://www.ctnbio.gov.br/index.php).

Table 1.4 tallies the numbers of field trials in each country of transgenic varieties involving stress-tolerance traits. The immediate contrast that stands out is between the number of stress tolerance trials in the US and Canada versus all other countries, suggesting that late stage development activity in North America is two orders of magnitude greater than in most other parts of the world. Again, however, we are not observing field trials of stress-tolerant crops developed by breeding programs, nor are we seeing transgenic field trials conducted in China, Brazil, and possibly other countries where they may be significant.

Field trials of stress-tolerant crops really began in 1998, with an initial surge of field trials largely driven by the public sector (Fig. 1.2c). If we decompose the annual field trials into type of stress trait being tested (Fig. 1.5a) and type of crop being tested (Fig. 1.5b), we see that most of those early 1998–2003 field trials were designated as general stress tolerance in forage plants. Indeed, most of that early surge was driven by just one program, at the University of Guelph (Guelph, Ontario). More recently, since 2004 there has been significant growth in field trials of stress-tolerant crops, almost all occurring in the private sector (Fig. 1.2c) largely focused on drought tolerance (Fig. 1.5a) and mostly in grains, but with a very recent surge in 2009 and 2010 in oilseeds (Fig. 1.5b).

Examining numbers of field trials of different types of crops tolerant to different types of stress, conducted by different types of organizations (Table 1.5) shows that efforts in the public sector are relatively spread out across traits and crops, while corporations are relatively focused on drought tolerance in grains and oilseeds. We see, perhaps surprisingly, entrepreneurial companies more diversified than corpora-

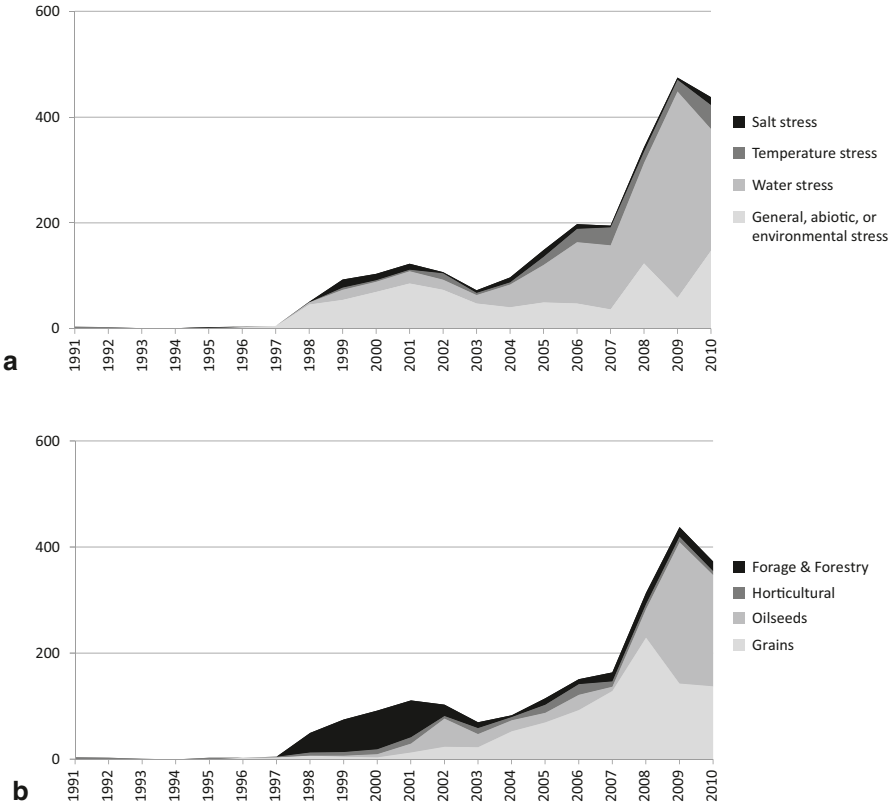

Fig. 1.5 Trends in transgenic field trials. **a** by type of stress traits; **b** by type of crops

tions, with some entrepreneurs conducting a significant share of field trials in select areas. Two companies, Performance Plants (Kingston, Ontario) and MS Technologies (Ames, Iowa), are working on stress tolerance in canola and soybeans respectively, largely licensing traits to major corporations for final integration and commercialization. Others are focused in areas apparently of no interest to corporations, like horticulture and forestry. For example, a Canadian winery, Chateau des Charms (Niagara-on-the-Lake, Ontario), is developing cold-tolerant grape vines,and Arbor-Gen (Summerville, South Carolina) is developing cold-tolerant eucalyptus hybrids.

3.4 Activity at the Regulatory Stage: Regulatory Submissions

At the regulatory stage, only a small trickle of activity has begun in the last few years (Fig. 1.2d), with the first regulatory filings, for approval of drought-tolerant maize, being made 10 years after field trials related to stress tolerance were first conducted. As an R&D indicator, regulatory filings share the same obvious disadvantage as transgenic field trials data, in that they cover only transgenic varieties.

Table 1.5 Types of crops and stress tolerance field tested by different types of R&D organizations

		Public institutions	Entrepreneurial seed & biotech companies	Corporations
Grains	General, abiotic, or environmental stress	53	14	156
	Drought stress	35	18	704
	Temperature stress	11		55
	Salinity stress	2	12	5
Oilseeds	General, abiotic, or environmental stress	116	107	146
	Drought stress	15	2	320
	Temperature stress		5	24
	Salinity stress	1		18
Horticultural	General, abiotic, or environmental stress	42	22	2
	Drought stress	40	7	4
	Temperature stress	30	3	7
	Salinity stress	10	4	
Forage & Forestry	General, abiotic, or environmental stress	226	1	
	Drought stress	56	12	
	Temperature stress	8	64	
	Salinity stress	49	1	

Again, varieties being developed by a breeding approach are not regulated, and therefore are not systematically visible in this data.

Regulatory filings and approvals for stress tolerant biotech crops, were searched on international lists kept by the OECD (BioTrack Product Database 2011), the Center for Environmental Risk Assessment (GM Crop Database 2011), and the International Service for the Acquisition of Agri-Biotech Applications (ISAAA) (GM Approvals Database 2011). These data sources appeared to exhibit some lag in reporting. So, again, regulatory filings were sought directly from relevant government authorities in the United States (http://www.isb.vt.edu/search-petition-data.aspx), Europe (http://ec.europa.eu/food/dyna/gm_register/index_en.cfm), Canada (http://www.hc-sc.gc.ca/fn-an/gmf-agm/appro/index-eng.php; http://active.inspection.gc.ca/eng/plaveg/bio/pntvcne.asp), Australia/New Zealand (http://www.foodstandards.gov.au/consumerinformation/gmfoods/), Japan (http://www.mhlw.go.jp/english/topics/food/), India (http://igmoris.nic.in/commercial_approved.asp), Mexico (Juarez and Branson 2011), and Brazil (http://www.ctnbio.gov.br/index.php/content/view/12492.html). Recent data on regulatory approvals could not be located for China (Huang and Wang 2002). Amongst these sources, effectively only two transgenic plants were found to have reached the regulatory stage. These two are the subject of about a dozen regulatory submissions observed across six different countries and regional authorities to date (Table 1.6). The two are the drought-tolerant maize varieties developed by Monsanto and a cold-tolerant eucalyptus developed

Table 1.6 Regulatory submissions for commercial release of drought- and stress-tolerant transgenic crops

Year	Country	Agency	Regulatory type	Crop	Event/Gene	Trait	Applicant
2008	United States	US Department of Agriculture	Deregulation	Eucalyptus	C-Repeat Binding Factor (CBF2)	Fertility Altered, Freeze tolerant	ArborGen
2009	United States	US Department of Agriculture	Deregulation	Corn/maize	Cold Shock Protein B	Drought tolerance	Monsanto Company
2009	European Union	European Food Safety Authority	Food & feed safety (Import only)	Corn/maize	MON 87460	Drought tolerance	Monsanto Europe S.A.
2010	Australia	Food Standards Australia New Zealand	Food safety	Corn/maize	MON 87460	Drought tolerant	Monsanto Australia Ltd.
2010	United States	U.S. Food and Drug Administration	Biotechnology Consultation	Corn/maize	MON 87460	Drought tolerance	Monsanto Company
2011	Japan	Ministry of Health, Labour and Welfare	Food safety	Corn/maize	MON 87460	Drought tolerant	Monsanto Japan Ltd
2011	Japan	Ministry of Health, Labour and Welfare	Food safety	Corn/maize	MON 87460 × MON 89034 × MON 88017	Drought tolerant, Insect Resistant, Herbicide tolerant	Monsanto Company; Forage Genetics
2011	New Zealand	Food Standards Australia New Zealand	Food safety	Corn/maize	MON 87460	Drought tolerant	Monsanto Australia Ltd.
2011	United States	US Department of Agriculture	Deregulation	Eucalyptus	–	Fertility Altered, Freeze tolerant	ArborGen
2011	Canada	Health Canada	Food safety	Corn/maize	MON 87460	Drought tolerance	Monsanto Canada Inc.
2011	Mexico	CIBIOGEM	Human consumption (Import only)	Corn/maize	MON 87460	Drought tolerant	Monsanto

by ArborGen. Thus, all regulatory stage activities to date involve private sector organizations.

4 Conclusion

The success of development and commercialization of drought- and stress-tolerant varieties will depend on developing the right genetics for the right crops, and releasing them in the right markets at the right time. The historical lessons of irrigation technologies should be a warning, for the adoption of higher efficiency sprinkler and drip systems was delayed by attempts to commercialize them in the wrong crop and at the wrong locations.

Understandably, the desire is to introduce drought-tolerant varieties to help the poor. But technology diffusion tends to trickle down from commercially high-value uses to lower-value uses. This is consistent with the threshold model of adoption, and it is consistent with experience: Golf courses were the first to use and refine modern irrigation technologies. So, while drought-tolerant varieties are being targeted at crops typically grown by poor farmers, the successes may end up to be that ones that start with high value crops. Therefore, because the costs of crop losses are greater, the incentive for adopting stress- and drought-tolerant varieties is greater. Again, looking at the precedents set by irrigation technology, drip irrigation system was first designed for cotton, but the technology was actually first adopted on a widescale in farming tomatoes and avocados, which have much higher values on a per area basis than does cotton.

The ability to survive dry periods suggests a heightened demand for drought-tolerant crops in more extreme environments, effectively expanding the climatic range of crops. Again, incentives will be higher for doing so with higher value crops. Drip enabled expansion into marginal semi-arid lands in California, cotton was not the crop that was chosen for this resulting area expansion, instead tree nuts and grape vines, again, both higher value on a per area basis. An expansion of range would be desirable in many parts of the world, even without the impending challenges of climate change, because it can open up more opportunities for lowincome farmers. Importantly, because they will assure a higher frequency of realized benefits, the likelihood of adoption in more extreme locations is more assured.

Drought-tolerant varieties present challenges. Not only are they difficult to develop technically, but they will vary in importance across seasons and years and across regions once implemented. This variability in benefit may require tailor-made strategies for introduction region by region. The drought-tolerance trait may in the end be most useful as a mechanism to introduce other complementary traits or technologies. The greatest value to be realized from drought-tolerant varieties is likely to be found among high-value crops and in regions with more extreme conditions.

As we have seen, incentives for the development of stress- and drought-tolerant varieties vary significantly, depending upon the appropriability conditions shaped

by the biological, legal, and strategic nature of the technology, as well as farmers' willingness (or simply ability) to pay for the trait and society's perceptions. As such, perhaps it is not surprising to observe that, despite a robust research base, progression of drought-tolerant varieties through the R&D pipeline has taken more than a decade. And the varieties poised in the later stages of R&D to reach market in the next few years are generally not of high value crops, but are predominately grains and oilseeds and perhaps some forages. It may thus be complementarity with other transgenic traits that will serve to be the driver of commercialization for drought- and stress-tolerance traits. Time will tell.

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Chapter 2
Impact of Extreme Events on Salt-Tolerant Forest Species of Andaman and Nicobar Islands (India)

Alok Saxena, P. Ragavan and Mani Saxena

1 Introduction

Andaman and Nicobar Islands situated in the Bay of Bengal off the eastern coast of India (Fig. 2.1) are endowed with very dense forest exhibiting rich diversity of plant species. Luxuriant mangroves constitute one of the most important forest types that exist in these islands. Mangroves are the salt-tolerant plants that are found mainly in the tropical and sub-tropical intertidal regions of the world (www.mangroveindia.org). They are defined as tree or bushes growing between the level of high water of spring tide and level close to but above mean sea level (Macnae1968) or type of coastal woody vegetation that fringes muddy saline shores and estuaries in tropical and sub-tropical regions (Blasco et al. 1975). Duke (1992) has defined a mangrove as "a tree, shrub, palm or ground fern, generally exceeding one-half metre in height, and which normally grows above mean sea level in the intertidal zone of marine coastal environment, or estuarine margins". A term often used while describing mangroves is 'mangal'. Macnae (1968) suggested using the term 'mangal' to refer the mangrove ecosystem and the term 'mangrove' for referring the individual plant species. The mangrove forest ecosystem comprises the intertidal flora and fauna in the tropics and sub-tropics and dominated by evergreen sclerophyllous broad-leaved trees with stilt roots or pneumatophores and viviparous seedlings (UNESCO 1973).

There have been different estimates on global distribution of mangroves. According to McGill (1959), mangroves cover approximately 75 % of the World's tropical coastline between 25° N and 25° S latitude. There have been different estimates of area under mangroves in the world varying from 10 million ha to 24 million ha. It is estimated at 16.2 million ha by Saenger et al. (1983), 10 million ha by

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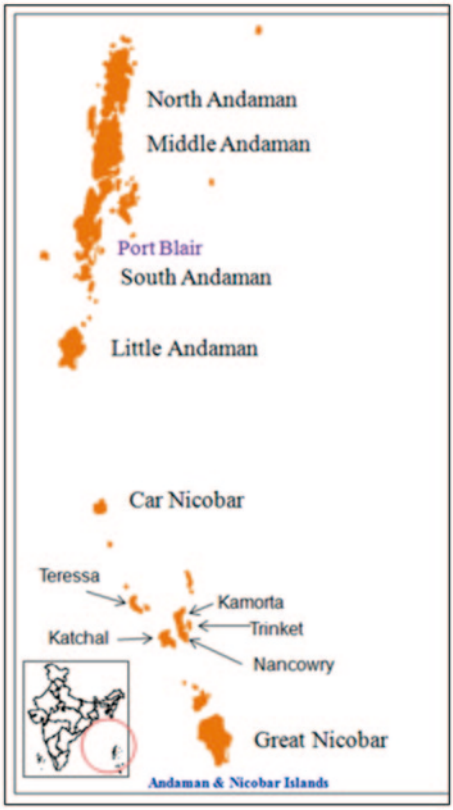

Fig. 2.1 Location of Andaman and Nicobar Islands

Bunt (1992), 24 million ha by Twilley et al. (1992), 14–15 million ha by Schwamborn and Saint-Paul (1996) and above 18 million ha by Spalding (1997). The most recent estimate by Wilkie and Fortuna (2003) suggests that mangroves cover an area of about 14.65 million ha. In India, distribution of mangroves spreads across all the nine coastal states, Union Territories (UTs) of Pondicherry, Daman-Diu and Andaman and Nicobar Islands (ANI). Figure 2.2 shows State/UTs-wise mangrove availability in India. There have been different estimates of mangroves by different authors. Khan (1957) estimated area under mangroves of Indian coast as 0.55 million ha, Sidhu (1963) as 0.68 million ha and Blasco (1977) as 0.36 million ha. These estimates did not include mangroves of Daman-Diu, Goa, Karnataka, Kerala and Pondicherry. Forest Survey of India (FSI), an organisation under the Ministry of Environment and Forests, Government of India, is assessing the forest cover of the country including that of mangroves on biennial basis since 1987. These assessments are based on interpretation of satellite data followed by limited ground verification. According to the latest estimate of the FSI (2009), total area under mangrove vegetation in India is 4,639 km^2. Out of this, 615 km^2 area (i.e. 20 % of the total mangrove area of the Indian territory) is in ANI. In Andaman district, the area under mangroves is 612 km^2, while in Nicobar district mangroves occupy

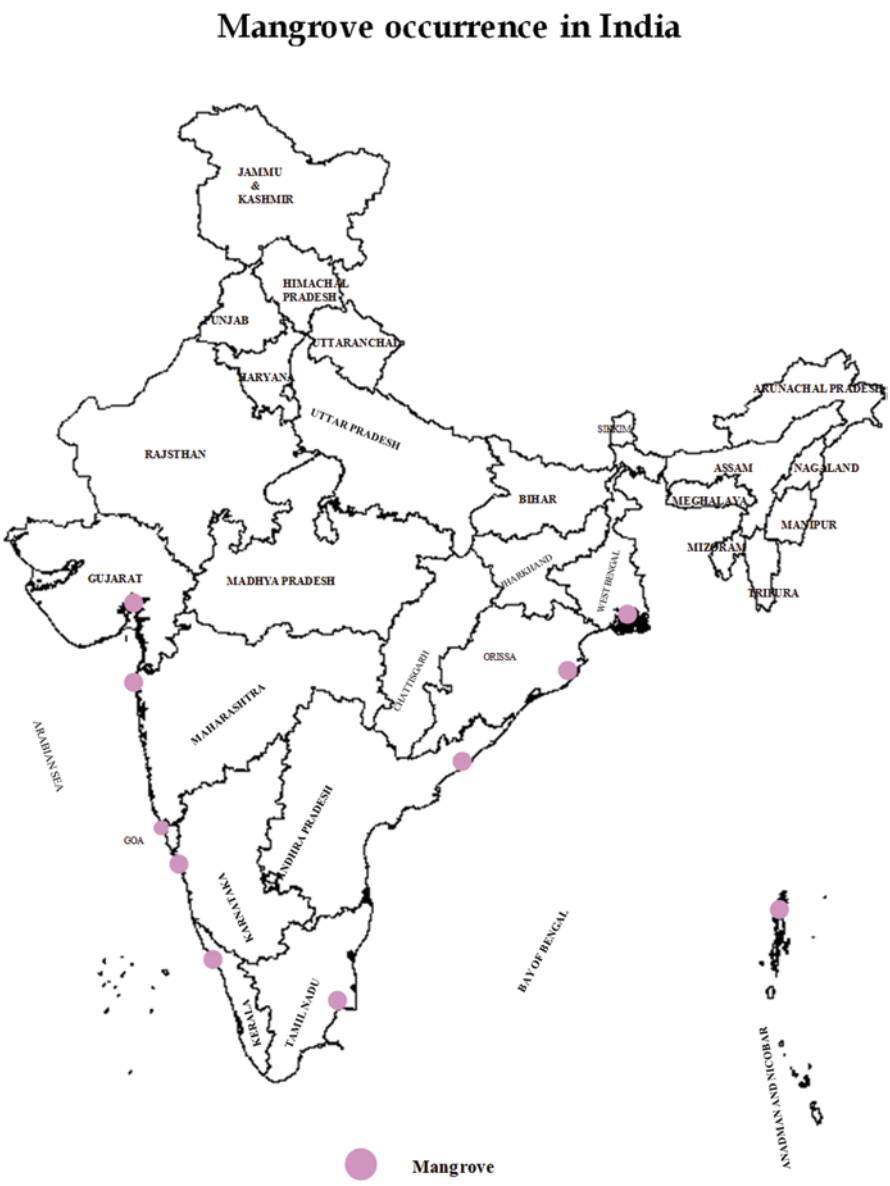

Fig. 2.2 Locations of availability of mangroves in India

only 3 km^2. Area wise, mangrove cover in ANI ranks third in the country after West Bengal and Gujarat, but as far as density and growth are concerned mangroves in the ANI are perhaps the best in the country (Fig. 2.3). It is evident by the fact that approximately 89 % of the mangrove cover in ANI falls under the category of either very dense mangroves (with more than 70 % canopy density) or moderately dense mangroves (with canopy density between 40 and 70 %). As many as 34 exclusive

Fig. 2.3 Luxuriant mangroves of Austin Creek, North Andaman

species distributed among 17 genera and 13 families are reported from ANI (Dagar et al. 1991). Mangroves in these islands mostly fringe the creeks, backwaters and muddy shores. Luxuriant mangroves are seen in Shoal Bay in South Andaman, Yerrata in Middle Andaman and Austin Creek, Kalighat Creek and Cadell Bay in North Andaman (Dam Roy 2003).

Mangrove ecosystem consists of mostly unrelated salt-tolerant trees and shrubs which show similar physiogamy and physiological characteristics and structural adaptations to the habitat (Yanney-Ewusie 1980). The environmental and ecological factors that affect the mangroves are the drying effects of the sun and wind, osmotic imbalance caused by the high salinity of seawater in which it is immersed, and growth in salty, oxygen deficient, and waterlogged soils, the action of tides, exposure to freshwater, destruction effects of storm surges and the diurnal and seasonal fluctuations of temperature. Nature has therefore endowed mangroves with a series of remarkable adaptations which enable them to flourish in an environment characterized by high temperatures, wide fluctuating salinities and shifting of anaerobic substrates. They are adapted to high water stress, exhibit mechanisms that permit water uptake against a gradient and have xerophytic adaptations, extensive and specialized root system (Dawes 1981).

2 Adaptations in Mangroves

According to Warming (1883), the mangroves have adapted to their environment through:

1. Mechanical fixation in loose soil.
2. Respiratory roots and aerating devices.
3. Vivipary.
4. Specialized means of dispersal and
5. Development of xerophytic structures in relation to soil salinity.

Fig. 2.4 Root systems in mangroves. **a** Stilt root of *Rhizophora* sp., **b** Prop root of *Rhizophora* sp., **c** Stilt root of *Rhizophora* hybrid, **d** Planks root of *Xylocarpus*, **e** Knee roots of *Bruguiera* sp., **f** Above ground root of *Lumnitzera* sp., **g** Above ground root of *Xylocarpus moluccensis*, **h** Pneumatophores of *Sonneratia*, **i** Pneumatophores of *Avicennia*

Macnae (1968) has described adaptations of mangroves considering their growth in ill-consolidated mud, specialization of stems and leaves, relationship between root and shoot systems and vivipary.

2.1 Morphological Adaptations

These include various types of aerial roots for proper exchange of gases and support of plant body, leathery shiny glaucous leaves with water storage tissue and viviparous germination in many species.

Mangroves have shallow root systems but they have adapted in a remarkable way to withstand the conditions of the nutrition, absorption of water and oxygen in anaerobic muddy soil and for anchorage on an unstable substratum. A variety of root systems exists in mangroves such as aerial roots, knee roots, stilt roots, tangle roots and pneumatophores or respiratory roots (Fig. 2.4). The aerial roots are highly developed and very extensive, forming intricate tangle making the movement in it very difficult. They help in allowing the atmospheric gases to reach the underground roots which lie in anaerobic soils. The strut and stilt roots in *Rhi-*

zophora species produce lateral roots from the base of the stems which form arch and re-arch before reaching substratum. Prop roots in *Rhizophora* species produce numerous thin unbranched roots at different heights. At ground level, they produce innumerable fibrous roots which help in absorption of water and nutrients. *Avicennia and Sonneratia* possess specialized finger like respiratory roots called pneumatophores with lenticels for passive diffusion of oxygen. Oxygen may pass through non-lenticellular part of the pneumatophores as well. Knee roots found in *Bruguiera, Ceriops, Lumnitzera*, etc., are lateral roots that bend upwards and come above the ground where they make knee-like curve before entering into soil again (Dagar et al. 1991). Lenticels are common on the knee roots and they are also considered to help in gas exchange.

2.2 Physiological Adaptations

Existence of high osmotic and diffusion pressure deficit (DPD) of cell sap, ultrafiltration mechanism of ions and salt-secreting glands are some of the physiological adaptations. Salt tolerance-related physiological adaptations are dealt subsequently in this chapter.

2.3 Anatomical Adaptations

Leaves have thick-walled epidermis with thick layer of cuticle and adequate tissue. Palisade tissue is well developed; water stomata are present in some cases, for example *Aegiceras corniculatum*; Mucillage cells occur in some species such as *Sonneratia, Rhizophora*, etc. The aerial roots on reaching the ground show short elongation zone and almost non-existent secondary growth. Like in aquatic plants, there are no root hairs in true mangroves and the endodermis acts as an effective absorbing layer (Tomlinson 1986). Mangrove woods exhibit unique anatomical features that enable the plants to withstand the high osmotic potential and the transpiration caused by high temperature (Tomlinson 1986). A number of vessels run through the wood and helps in developing high tension in the xylem (Scholander et al. 1964, 1965; Tomlinson 1986).

2.4 Reproductive Strategies

Four methods of reproduction in mangroves have been described by Bhosale and Mulik (1991). These are:

1. Vivipary
2. Cryptovivipary

Fig. 2.5 Vivipary in mangroves (propagules). **a** *Rhizophora stylosa*, **b** *R. mucronata*

3. Normal germination
4. Vegetative propagation.

Among these four means of reproduction, vivipary is the most significant. Vivipary (Macnae 1968; Gill and Tomlinson 1969) means continuous development of the embryo after fertilization while attached to the parent without any intermediate resting or dormant period. Therefore, the term 'propagule' is used for these embryos instead of the term 'seed' (Fig. 2.5). The development of embryo continues during the dispersal by water (Van der Pijl 1972). It is argued that vivipary represents a precondition for increasing salt resistance in the seedling thereby facilitating survival, when detached, in a substrate of high salinity (Dagar et al. 1991). In some mangrove species such as *Aegiceras, Avicennia, Nypa* and *Pellicera*, a more advance state of vivipary known as 'cryptovivipary' is found where the embryo emerges from the seed coat but not the fruit before it abscises (Carey 1934). The advantages of vivipary are obvious for such plants which grow on the fringes of sea. After falling from the tree, the propagules float and remain viable for considerably long period of time. Mangroves have little or no capacity of vegetative propagation but some species such as *Avicennia* and *Excoecaria* have the capacity to coppice and could persist in western India in spite of over-exploitation (Blasco 1977).

3 Salt Stress Regulation Mechanisms

Presence of salt water is not a physical requirement of mangroves (Bowman 1917; Rosevear 1947; Egler 1948). Mangroves are facultative halophytes occurring in tidal areas where fresh water plants, which are intolerant to salt, cannot live. Most mangroves are capable of growing in fresh water (Teas 1979) but mangrove ecosystems are not found in strictly fresh water environment, probably due to the fact that mangroves are not good competitors and the salinity is important in reducing competition from fresh water and terrestrial vascular plants (Kuenzler 1974). Man-

Fig. 2.6 Succulent leaves in *Lumnitzera* and *Excoecaria* species

groves thrive best in muddy coastal plains where adequate fresh water supplies from river discharge with ample nutrients are available.

Each mangrove species is associated with an optimum salinity (Snedaker 1978). Each species occupies the salinity zone to which it is best suited and is best adapted physiologically. There are mainly two mechanisms of salt regulation (Scholander 1968).

3.1 Salt Exclusion

Rhizophora and other members of family Rhizophoraceae have a well-developed mechanism of ultra-filtration in their roots enabling only selective absorption of ions while extracting water from the soil. They may retain a low internal salinity by means of salt-excluding mechanisms in the roots.

3.2 Salt Excretion

Avicennia sp., *Aegiceras* sp., and several other species have salt glands on their leaves which secret salt. *Lumnitzera* and *Conocarpus* have analogous structure to salt glands. Sodium chloride concentration in the xylem sap of these species is about 10 times greater than that in exclusion type. The ions which are excreted by these glands are mostly sodium and chloride. Though salt-excreting species allow more salt into the xylem than the non-salt-excreting species, but still they exclude about 90 % of the salt (Scholander et al. 1962; Azocar et al. 1992). Salt excretion is an active process as evidenced by ATPase activity in the plasmalemma of the excretory cell (Drennan et al. 1992).

Salt accumulation is also another salt-regulatory mechanism found in species of *Lumnitzera* and *Excoecaria* which accumulate salts in leaf vacuoles and become succulent (Fig. 2.6). In some species, salt concentration can also be reduced by

transferring the salts into senescent leaves or by storing them in the bark or the wood (Tomlinson 1986). With increase in water salinity, some species restrict their water use in order to achieve greater tolerance (Ball and Passioura 1993). In addition to these direct regulatory mechanisms, mangroves may also accumulate or synthesize other solutes to regulate and maintain osmotic balance (Werner and Stelzer 1990; Popp et al. 1993). Some species such as *Aegiceras corniculatum*, *Aegialitis annulata* and *Laguncularia racemosa* store mannitol and proline (Polania 1990), *Avicennia marina* stores glycine betaine, asparagines and stychyose (Ashihara et al. 1997) and *Sonneratia alba* synthesizes purine nucleotides that facilitates tolerance to salt load of 100 mM sodium chloride (Akatsu et al. 1996). Scholander et al. (1964) have demonstrated that in order to facilitate water flow from roots to leaves, the water potential at the leaves is held lower (−2.5 to −6.0 MPa) than in the roots (−2.5 MPa). Recent studies also show that mangroves can restrict cytosolic salt contents not only by ultra-filtration (Zheng et al. 1999; Wang et al. 2002; Aziz et al. 2001; Khan et al. 2001), but also by other means such as salt accumulation and ion sequestration (Mimura et al. 2003; Kura-Hotta et al. 2001). Salt-controlling strategies in mangroves are similar to those in glycophytes, but probably mangroves could exclude or sequester salt ions more efficiently (Shan et al. 2008). Many mangrove species (e.g. *Kandelia obovata*, *Avicennia marina* (Zhao et al. 1999; Suarez et al. 2006)) can accumulate inorganic ions and use them as osmolytes to maintain osmotic and water potential. This characteristic confers a survival advantage to these species in a saline environment (Tomlinson 1986). Shan et al. (2008) have shown that while sequestering excessive ions into vacuoles, mangroves could also accumulate organic osmolytes in cytoplasm to get osmotic equilibrium across the tonoplast. Organic osmolytes of mangroves mainly include hydroxyl compounds, free amino acids (especially Proline), polysaccharide (e.g. starch), etc. Oku et al. (2003) studied the relevance of lipid composition to salt tolerance in propagules of *Kandelia candel* and *Bruguiera gymnorhiza* planted with varied salt concentrations. This study result shows that salt stress specifically modulated the terpenoid concentrations in mangroves, whereas phospholipid and fatty acid compositions in both species are not changed with respect to varying salinity.

Salinity increases biosynthesis and accumulation of ABA, which modulates physiological reactions in plant response to salinity (Zhao et al. 1991; Montero et al. 1997; Gomez-Cadenas et al. 1998). It has been documented that ABA induces the expression of antioxidant genes encoding Cu/Zn-superoxide dismutase (Cu/Zn-SOD) (Guan and Scandalios 1998). Calmodulin (CaM), a ubiquitous calcium-binding protein, regulates the activity of a variety of enzymes and proteins that confers salt tolerance (Li et al. 2009). Yang and Poovaiah (2002) demonstrated the role of CaM in regulating H_2O_2 homeostasis, i.e. CaM down-regulated H_2O_2 levels in plants by stimulating the catalytic activity of catalase. Li et al. (2009) recently studied the correlation between ABA, CaM and antioxidant defense in *Bruguiera gymnorhiza* and *Kandelia candel* and found that elevated ABA and CaM concentration under short-term and long-term salt treatment may up-regulate the activity of antioxidant enzymes in the two mangrove species, thus avoiding excess ROS pro-

duction and the subsequent oxidative stress. ABA and CaM likely restricted root-to-shoot salt transport by reducing water flow (Li et al. 2009).

There is experimental evidence that salt stress affects the integrity of cellular membranes, activities of enzymes and the functioning of the plant photosynthetic apparatus (Serrano et al. 1999). An important cause of this damage is the production of reactive oxygen species (ROS; Smirnoff 1993). Oxidative stress generates ROS such as superoxide, hydroxyl and peroxyl radicals and the balance between antioxidation and oxidation is believed to be a critical concept for maintaining a healthy biological system (Jithesh et al. 2006b).

A number of reviews have concentrated on the link between salt stress and antioxidative pathways in plants (Bohnert and Jensen 1996; Dat et al. 2000; Van Breusegem et al. 2001; Arora et al. 2002; Borsani et al. 2003). The plant antioxidative stress pathway comprises two components, the non-enzymatic and the enzymatic components. The non-enzymatic component consists of antioxidants such as tocopherol, carotenoids, ascorbate and glutathione that are free-radical-scavenging molecules (Salin 1987). The enzymatic component consists of enzymes such as superoxide dismutase, catalase, ascorbate peroxidase, monohydroascorbate reductase, dehydroascorbate reductase and glutathione reductase (Salin 1987). Apart from these, an iron-storage protein, ferritin, is also involved in the reactive oxygen-scavenging network (Morel and Barouki 1999; Mittler et al. 2004).

Most of the early studies in mangroves have dealt with the effects of salinity on photosynthesis (Ball and Farquhar 1984) and respiration (Burchett et al. 1989; Fukushima et al. 1997). However, recently, there has been a growing interest in the effect of salinity and its relation to antioxidant enzyme status in mangroves and their associates (Cherian et al. 1999; Takemura et al. 2000; Cherian and Reddy 2003; Parida et al. 2004; Jithesh et al. 2006a). Parida et al. (2004) assessed the activities of some antioxidative enzymes and levels of antioxidants in *Bruguiera parviflora* and suggested that under salinity stress plants are protected against activated oxygen species by the elevated levels of certain antioxidative enzymes, thus avoiding lipid peroxidation during salt exposure and differential changes in the levels of the isoforms due to NaCl treatment may be useful as markers for recognizing salt tolerance in mangroves.

The morphological, physiological and biochemical studies done in the past have not clearly explained the salt-adaptation mechanism and its evolution. Recently, some progresses have been achieved in understanding the mechanism of salt adaptation in mangroves on a molecular level. *Avicennia marina* is one of the well-studied mangroves because of its characters of salt secretion and high salt tolerance. *A. marina* deals with salt stress through accumulating betaine serving as an osmolyte. Hibino et al. (2001) first identified and cloned the *BADH* gene that is involved in betaine synthesis in *A. marina*. *BADH* was up-regulated under salt stress, and this tendency was consistent with the accumulation of betaine in *A. marina*. Two other genes, *AmT1* and *AmT2* (coding for Betaine/Proline transporter) were also isolated from *A. marina* later (Waditee et al. 2002). Jithesh et al. (2006a) reported that in *A. marina* high salinity did not lead to transcriptional change of gene *Sod1*, encoding enzyme Cu/Zn-SOD, but osmotic stress decreased transcript level of this gene and

under oxidative stress, its transcription was transiently up-regulated. *Cat1* was up-regulated by saline or oxidative stress but down-regulated by osmotic stress. *Fer1* was transcriptionally up-regulated by saline or oxidative stress but did not change under osmotic stress.

Aegiceras corniculatum is an another species of high concern. Six hundred EST were obtained from the leaf SSH library of *A. corniculatum* under salt-stress (Fu et al. 2005). *P5CS*, which was related to osmotic regulation, and two aquaprin genes, which participate in water transport (Maurel et al. 2001) were up-regulated in *A. corniculatum* by salt stress (Fu et al. 2005). Expression patterns of these two aquaprins also indicated that *A. corniculatum* could recover from long-term salt stress and adapt to saline environment (Maurel et al. 2001).

Bruguiera gymnorhiza is a well-studied non-secreting true mangrove. Studies on its response to high salinity have been conducted recently at both the gene and genomic levels (Sugihara et al. 2000; Miyama et al. 2006; Miyama et al. 2007; Banzai et al. 2002a; Banzai et al. 2002b; Takemura et al. 2002). For example, in *B. gymnorrhiza* oxygen-evolving enhancer protein 1 (OEE1), the protein was initially isolated and its corresponding gene was also obtained (Sugihara et al. 2000). Currently, there are also ongoing genomic studies of *B. gymonorrhiza*. Miyama et al. (2006) established the first *B. gymonorrhiza* EST library, which collected 14,842 ESTs from leaves and roots after high salinity or hormone treatments. Another non-secreting true mangrove species *Ceriops tagal* has also been of concern recently. More than 5,000 EST clones have been obtained from its root cDNA library and leaf SSH library of *Ceriops tagal* (Liang 2007).

4 Climate Change and Mangroves

Global climate change is considered to have significant adverse effects on mangroves. Global climate change and concomitant effects such as changes in temperature and CO_2, changes in precipitation patterns, storminess, and eustatic sea-level rise as observed over recent decades, are mainly due to anthropogenic activities. Increase in the Greenhouse gas (GHG) concentration in the atmosphere has been the main cause for the observed warming over the last 50 years (Houghton et al. 2001). Following account is based on the IUCN report by Elizabeth and Rodney (2006).

4.1 Temperature

The increase in the Earth's temperature in the past 100 years has been more than 0.6 °C and it is projected to be 2–6 °C by 2100 mostly due to the anthropogenic activities (Houghton et al. 2001). The impact of the projected increases in sea

temperature is not likely to affect mangroves adversely (Field 1995). When mean air temperature rises to 25 °C, most mangroves produce maximal shoot density and when the mean air temperature drops below 15 °C, they stop producing leaves (Hutchings and Saenger 1987). At temperatures above 25 °C, leaf formation rate declines in some species (Saenger and Moverly 1985). Temperatures above 35 °C have led to thermal stress affecting mangrove root structures and establishment of mangrove seedlings (UNESCO 1992). Almost no photosynthesis occurs at leaf temperatures of 38–40 °C (Clough et al. 1982; Andrews et al. 1984). It has also been suggested that mangroves will move polewards with increasing air temperatures (UNEP 1994; Field 1995; Ellison 2005). Migration of some species of mangroves to higher latitudes is limited by temperature. However, extreme cold temperatures are more likely to limit mangrove expansion into higher latitudes (Woodroof and Grindrod 1991; Snedaker 1995).

4.2 CO_2 Concentration

There has been an increase in the atmospheric CO_2 by 90 parts per million by volume (ppmv) during the period from 1980 to 2000 (Houghton et al. 2001). Most atmospheric CO_2 resulting from fossil fuels will be absorbed into the ocean, affecting ocean chemistry. Increased levels of CO_2 are expected to enhance photosynthesis and mangrove growth rates (UNEP 1994). Ball et al. (1997) have shown that increased levels of CO_2 significantly increase photosynthesis and the average growth rates in two Australian mangrove species, *Rhizophora stylosa* and *Rhizophora apiculata*, but only when grown at lower salinity levels.

4.3 Precipitation

It is predicted that the precipitation rates are likely to increase by about 25 % by 2050 in response to global warming. However, at regional scales, this increase will be unevenly distributed with either increases or decreases projected in different areas (Knutson and Tuleya 1999; Walsh and Ryan 2000; Houghton et al. 2001). Changes in precipitation patterns caused by climate change may have a profound effect on both the growth of mangroves and their areal extent (Field 1995; Snedaker 1995). Decreased precipitation results in a decrease in mangrove productivity, growth and seedling survival, and may change species composition favouring more salt-tolerant species (Ellison 2000, 2004). Reduction in precipitation may result in a decrease in mangrove area, decrease in diversity and projected loss of the landward zone to non-vegetated hypersaline flats (Snedaker 1995). Increased precipitation may increase mangrove area, diversity of mangrove zones and mangrove growth rates in some species (Field 1995). Increased precipitation may also allow mangroves to migrate and outcompete salt marsh vegetation (Harty 2004).

4.4 Hurricanes and Storms

According to the International Panel on Climate Change, there have been no reported trends observed in tropical storms and no evidence of changes in the frequency or areas of storm formation, but it is predicted that wind intensities will likely increase by 5–10 % (Houghton et al. 2001). However, the assessment made by Trenberth (2005) indicates that tropical storms will indeed increase in frequency and/or intensity due to climate change, posing an additional threat to mangroves. Large storm impacts have resulted in mass mortality in ten Caribbean mangrove forests in the last 50 years (Jimenez et al. 1985; Armentano et al. 1995). Cahoon et al. (2003) and Ning et al. (2003) have demonstrated mass mangrove mortality due to hurricanes. Roth (1997) suggests that species proportions may shift because they have different rates of regeneration. Storm surges can also flood mangroves and, when combined with sea-level rise, lead to mangrove destruction. Ellison (2000) suggests that flooding caused by increased precipitation, storms or relative sea-level rise may result in decreased productivity, photosynthesis and survival. Due to inundation of lenticels in the aerial roots, the oxygen concentration in the mangroves is likely to decrease, resulting in death (Ellison 2004). It may also decrease the ability of mangrove leaves to conduct water and thereby reduce photosynthesis (Naidoo 1983).

4.5 Effects of Changes in Sea Level

Thermal expansion of the oceans and melting of glacial ice caused by global warming have been primarily responsible for eustatic sea level by 10–20 cm in the last century (Church et al. 2001). Several climate models project an accelerated rate of sea-level rise over coming decades (Church et al. 2001). Tectonic and isostatic adjustments (i.e. ocean basin deformation and land subsidence or emergence) have also influenced the sea-level rise (Kennish 2002). During the twenty-first century, mean sea-level projections range from 0.09 to 0.88 m (Houghton et al. 2001). The greatest climate change challenge that mangrove ecosystems will face is the sea-level rise (Field 1995). Mangroves can adapt to sea-level rise provided it occurs slowly enough (Ellison and Stoddart 1991), if adequate expansion space exists, and if other environmental conditions are met.

4.6 Mangrove Adaptations that help them Survive Sea-Level Rise

Mangroves have adapted special aerial roots, support roots, and buttresses to live in muddy, shifting, and saline conditions. Mangroves may adapt to changes in sea level by growing upwards in place, or by expanding landwards or seawards. Mangroves produce peat from decaying litter fall and root growth and by trapping

sediment in the water. The process of building peat helps mangroves keep up with sea-level rise. Mangroves can expand their range despite sea-level rise if the rate of sediment accretion is sufficient to keep up with sea-level rise. However, their ability to migrate landwards or seawards is also determined by local conditions such as infrastructure (e.g. roads, agricultural fields, dikes, urbanization, seawalls and shipping channels) and topography (e.g. steep slopes). If inland migration or growth cannot occur fast enough to account for the rise in sea level, then mangroves will become progressively smaller with each successive generation and may perish (UNEP 1994).

4.7 Environmental Factors that Affect Mangrove Response to Sea Level

Understanding the impact of sea-level rise on mangrove ecosystems must take into account the factors that affect the ecological balance of that ecosystem such as the substrate type, coastal processes, local tectonics, availability of freshwater and sediment and salinity of soil and groundwater (Belperio 1993; Semeniuk 1994; Blasco et al. 1996). Climatic variability (e.g. changes in rainfall and the frequency and intensity of cyclonic storms) can exacerbate the factors affecting mangrove response to sea level because it can alter the freshwater inflow to mangroves, the sediment and nutrient inputs and the salinity regime. In an analysis of the impacts of sea-level rise on estuaries, Kennish (2002) highlights the importance of local conditions such as the size and shape of the estuary, its orientation to fetch and local currents, the areal distribution of wetlands, the geology of the neighbouring watersheds and land use in upland areas. Tidal range and sediment supply are two critical indicators of mangrove response to sea-level rise. Carbonate settings are often associated with coral atolls and islands, where landwards migration to escape the effects of sea-level rise is not possible and sediments are often limited; thus mangrove communities in carbonate islands are considered extremely vulnerable to sea-level rise (UNEP 1994). Therefore, sea-level rise is expected to decrease the geographic distribution and species diversity of mangroves on small islands with micro-tidal sediment-limited environments (IPCC 1997). Mangroves with access to allochthonous sediments such as riverine mangroves are more likely to survive sea-level rise than those with low external inputs (Woodroffe 1990; Pernetta 1993). It is important to note that although access to sediment is critical for mangroves to survive sea-level rise, too much sediment (e.g. resulting from poor agricultural practices) can bury their pneumatophores and kill mangroves (Ellison and Stoddart 1991).

4.8 Species Response to Sea-Level Rise

Individual mangrove species have varying tolerances of the period, frequency and depth of inundation. Mangrove zones are related to shore profile, soils and salinity,

and changes in these can lead to changes in mangrove species composition. Different species may be able to move into new areas at different speeds making some species capable of accommodating a higher sea-level rise rate than others (Semeniuk 1994).

4.9 Mangroves and Carbon Sequestration

Mangroves are among the most productive primary producers and are important carbon sink. In recent years, they have also been considered very important in relation to carbon sequestration. Mangroves have high carbon sequestration potential through high biomass productivity. Studies have reported high standing biomass, annual litterfall and net productivity of mangrove forests and have shown that these are almost equal to some natural Dipterocarp forests (Whitemore 1984). Jin Eong et al. (1995) have reported that out of the total standing biomass of 114 tera C per hectare, 74 % is in trunk, 15 % in roots and 10.6 % in canopy. Eong (1993) while highlighting production of 18 t dry matter per hectare per year mentioned that if disturbed, mangrove turn out to be CO_2 sources rather than sinks. Mangroves fix 17 tera C per hectare per year compared with 12 tera C per hectare per year by tropical forests.

Mangroves are also one of the nature's best ways for combating global warming because of their high capacity for sequestering carbon. This is a characteristic of mangrove wetlands that now demands our most immediate and undivided attention. One of the greatest contributions that mangroves may have to offer is their great propensity to sequester carbon from the atmosphere and store this in their wetland substrate. According to the February 2007 issue of *National Geographic*, "Mangroves are carbon factories…. Measurements suggest that mangroves may have the highest net productivity of carbon of any natural ecosystem (about a hundred pounds per acre per day)…".

This combined lack of conservation ethics, shortsighted greed and weak law enforcement have allowed massive losses of these coastal wetlands, with one huge and hidden cost arising from the oxidation and release of stored mangrove carbon.

In a study conducted by Dr. Ong of University Sams in Malaysia (2002), it was found that the layers of soil and peat composing the mangrove substrate have a high carbon content of 10 % or more. Each hectare of mangrove sediment might contain nearly 700 t of carbon per meter depth. In building large numbers of shrimp farms or tourist complexes, the resultant clearing of mangroves and subsequent excavation of the mangrove substrate could result in the potential oxidation of 1,400 t of carbon per hectare per year.

Again, according to Dr. Ong, "Assuming that only half of this will become oxidized over a period of 10 years, we are looking at the return of 70 tons of carbon per hectare per year for 10 years, to the atmosphere. This is some 50 times the sequestration rate. This means that by converting a mere 2 % of mangroves, all of the advantages of mangroves as a sink of atmospheric carbon will be lost…".

According to a latest study by the UN's Food and Agriculture Organization (FAO), the current rate of mangrove loss is around 1 % per annum—or around

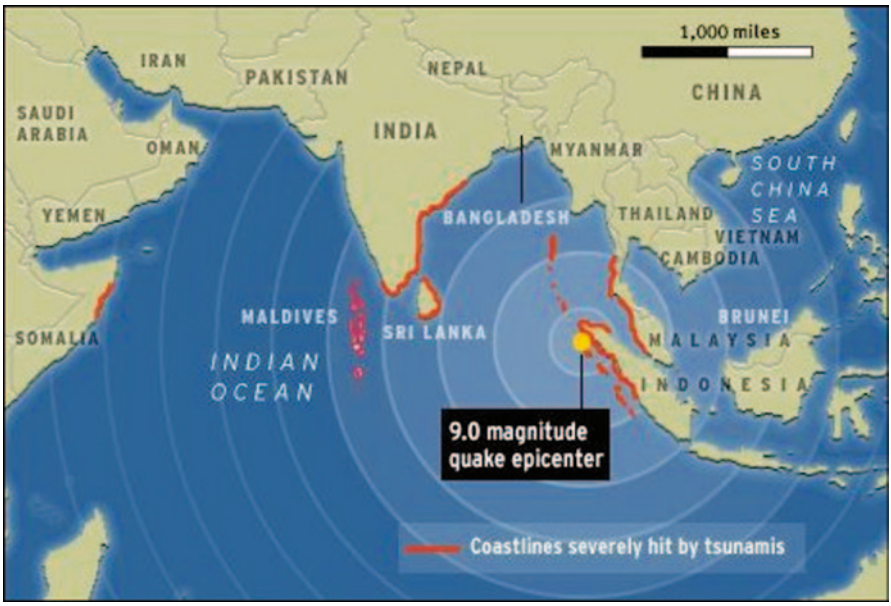

Fig. 2.7 Epicenter of tsunami and location of Andaman and Nicobar Islands. (Source: www.aboriginalastronomy.com)

150,000 ha of new mangrove area loss per year. This translates to around 225,000 t of carbon sequestration potential lost each year with an additional release of approximately 11 million t of carbon from disturbed mangrove soils each year.

5 Impact of Extreme Event of Tsunami on the Mangroves of Andaman and Nicobar Islands (ANI), India—A Case Study

The natural extreme event, i.e. the earthquake of 9.1 on the Richter scale, which struck ANI on December 26, 2004 (Fig. 2.7) and the subsequent tsunami have brought about devastating human tragedy and considerable loss to the ecological resources, including mangroves in these islands. Immediate impact of tsunami on mangroves was not very significant in Andaman group of islands but in Nicobar there had been considerable loss of forest cover, including mangroves as was evident from a few remote sensing-based studies which were carried out immediately after tsunami. These studies were mostly a rapid assessment in nature and without sufficient ground verification. FSI's rapid assessment (2006) showed that there was a loss of 258 ha of forest cover (inclusive of mangrove cover) in Andaman group of islands while the loss was 12,482 ha in Nicobar group of islands. Chatterjee et al. (2008) studied changes in land cover and land use only in South Andaman and reported

Table 2.1 Details of satellite data used for the study

Period	Satellite	Sensor	Path/row	Data period
Pre-tsunami	IRS 1D	LISS-III	115–65; 115–64	January and December 2003
Post-tsunami	IRS-P6	LISS-III	114–65; 114–64	January–February 2005
Post-tsunami	IRS-P6	LISS-III	114–65; 114–64	January–February 2007

that out of the damaged mangroves, only 22 % mangroves were heavily damaged and 15 % were moderately damaged. Ramachandran et al. (2005) studied only four islands of Nicobar group, namely Camorta, Katchal, Trinkat and Nancowry and estimated decrease in mangrove cover ranging from 152 ha in Nancowry to 399 ha in Katchal. Sridhar et al. (2006) studied change in mangrove vegetation of Great Nicobar Islands and reported a decrease of 531 ha. The study carried out by Dam Roy and Krishnan (2005) focused on immediate damage assessment caused by the tsunami. The study carried out by Choudhury et al. (2009) focused on physico-chemical, biochemical and microbial characteristics of soils of mangroves of the Andaman.

All the aforementioned remote sensing-based studies used satellite data of the period immediately after the tsunami happened—mostly of January and February 2005 for comparison with the pre-tsunami data. No study appeared to have been done except for regular biennial assessment by FSI to study the changes that took place subsequently in the mangrove vegetation of these islands. Such studies are considered essential to study long-term effects of tsunami and also to observe whether there is a natural resilience in the degraded mangrove areas. Such studies are also important keeping in view the fact that some geo-morphological changes have been reported in these islands as a consequence of the massive earthquake that preceded the tsunami. Malik et al. (2006) reported that the subsidence of the eastern coast of the areas stretching over the South Andaman Island and all the Nicobar Islands was about 1.2 ± 0.2 m along Car Nicobar and Andaman Islands, and by about 3 m along the southern tip of Great Nicobar Island. Uplifting of the North and Middle Andaman islands occurred, up to a maximum of 3 m in the northern tip of the North Andaman Island. One of the objectives of the present study is to find out changes that may be taking place in mangrove areas gradually as a result of geo-morphological alterations. Therefore, the present study involves studying the satellite data of these islands beyond 2005 and also to observe changes in the ground as well.

For finding out the changes occurring with time in the mangrove cover, satellite data of the Linear Image Self-Scanning Sensor (LISS III) of the Indian Remote Sensing Satellite-1D (IRS-1D) for the pre-tsunami period (2003) and IRS-P6 for the post-tsunami period (2005 and 2007) was used (Table 2.1). The satellite data for the three periods was digitally processed to delineate the mangroves using shortwave infrared (SWIR) band which facilitates the delineation of mangroves with the adjoining forest area. In ANI, mangroves are immediately followed by forest areas and as a result, delineation of mangroves from the adjoining forest areas becomes very difficult using conventional combination of bands, i.e. near infrared (NIR), red and green. Therefore, combination of SWIR with red and green bands has been used for delineation of mangroves after the satellite images were geo-referenced and

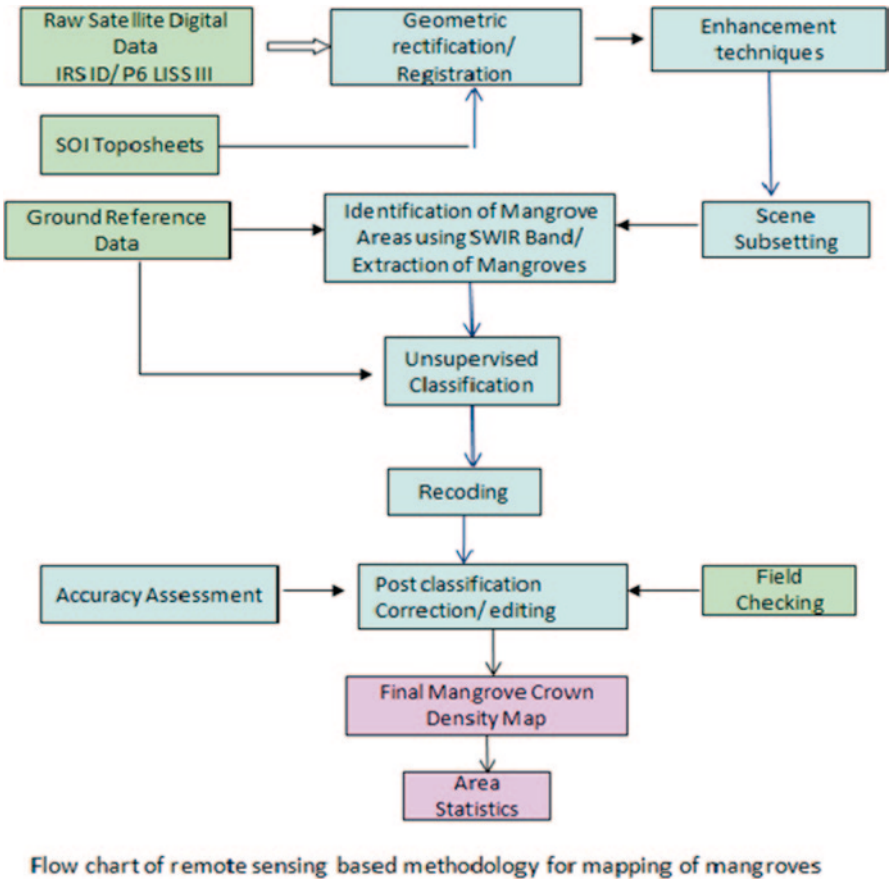

Fig. 2.8 Flow chart of remote sensing-based methodology for mapping of mangroves

radiometric corrections were carried out. Ibrahim et al. (2010) demonstrated that the SWIR is very sensitive to liquid water contents and therefore has the potential as the best index to recognize the mangrove classes. Panigrahy et al. (2009) has also shown the utility of IRS-P6 AWiFS SWIR for crop discrimination and classification. Satellite data was then processed using digital image-processing software and mangroves were classified using unsupervised classification. This was followed by area calculation in delineated mangrove areas. The area figures of the two assessment periods (pre- and post-tsunami) were compared and changes assessed. This was followed by identification and delineation of areas where changes have taken place. The resultant classified data was used for ground verification. The field visits for ground truth verification were made in January–February 2009. For the satellite data of 2004–2005 and 2007, ground verification records of the FSI were consulted. Post-classification, corrections were made after ground truth verification. Figure 2.8 presents the flow chart of the methodology used for the interpretation and the classification of satellite data.

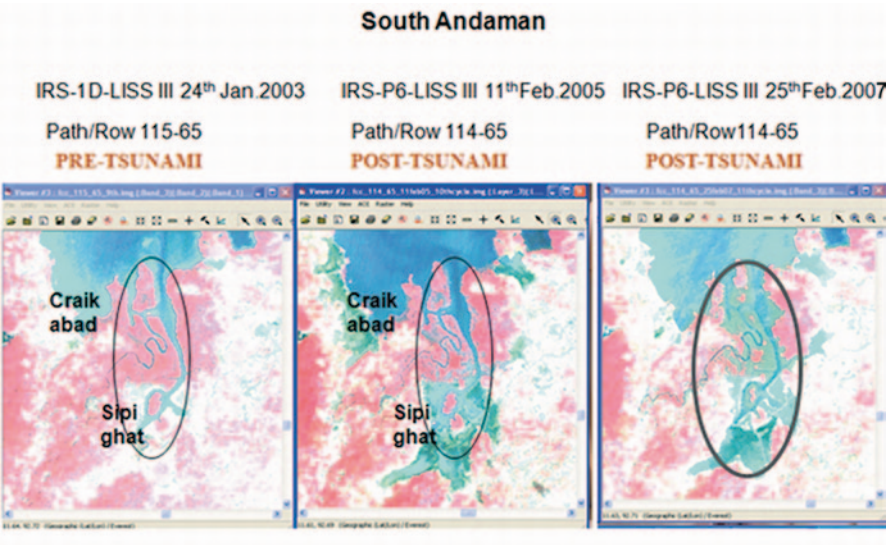

Fig. 2.9 Changes in mangrove cover of South Andaman from 2003 to 2007 (Sippighat area)

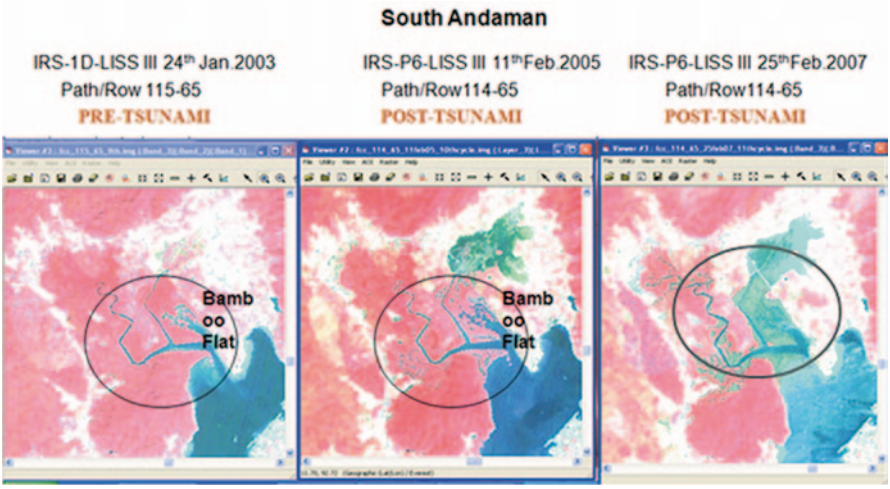

Fig. 2.10 Change in mangrove in part of South Andaman between 2003 and 2005 (Bambooflat area)

The purpose of using satellite data in this study has been to substantiate the field observations of researchers working in ANI and also of the forest staff managing the mangrove areas that continuous degradation is going on in mangroves of North Andaman, certain locations of South Andaman and in Nicobar Islands.

Figures 2.9 and 2.10 show changes in mangroves of two locations of South Andaman Island. Figure 2.9 shows the gradual changes in mangrove area from the year 2003 (pre-tsunami) to 2007 (post-tsunami) in Sippighat area. From the satellite

Fig. 2.11 Field conditions. **a** Sippighat, **b** Bambooflat

images of the area, it is clearly visible that sea water occupied a significant part of land in 2005 after the tsunami due to subduction of land. Dam Roy and Krishnan (2005) and Chatterjee et al. (2008) also had the same observations. The area submerged in water further increased in 2007 as can be seen in the satellite image of 2007. Figure 2.11a shows the field situation in 2009 (subsequent to the period of satellite image). It can be seen that the area is still submerged and there is no improvement. Further deterioration, if any, can be confirmed only after the study of satellite data of the year 2009 which was not available at the time when the study was carried out. Figure 2.10 shows the situation in Bambooflat area of South Andaman. A marginal decrease in mangrove cover but significant increase in water area is noticed by comparing images of 2003 and 2005, but there is a significant change in the land area which is now occupied by the sea water. Considerable loss in mangroves can be noticed between 2005 and 2007. Field situation in 2009 of mangroves of this area is depicted in Fig. 2.11b.

In North Andaman Islands, particularly in Diglipur, impact of these geo-morphological changes on mangrove vegetation has been more pronounced. If satellite images of January 2003 and February 2005 are compared, there does not appear to be any significant change in the mangrove cover immediately after the tsunami (Fig. 2.12), but images of subsequent period (February 2007) show significant degradation in the mangrove area. Dam Roy and Krishnan (2005) also reported that mangrove stands in many locations were exposed. They observed that even during high tide, sea water was not reaching the mangrove plants resulting in the onset of wilting in these plants and their death eventually. Field situation in Shyam Nagar area of North Andaman in 2009 is shown in Fig. 2.13. It shows that mangroves in the area are almost dried up and are dying.

Nicobar Islands have witnessed the maximum damage during the tsunami owing to their proximity to the epicenter of the massive earthquake of December 26, 2004. Satellite data-based results of the present study are given in Table 2.2. There has been a significant decrease in the mangrove cover in all major islands of Nicobar district. Total decrease in mangrove cover in Nicobar district between 2003 and 2005 is 1500 ha. Maximum loss of mangrove cover was noticed in Katchal (567 ha)

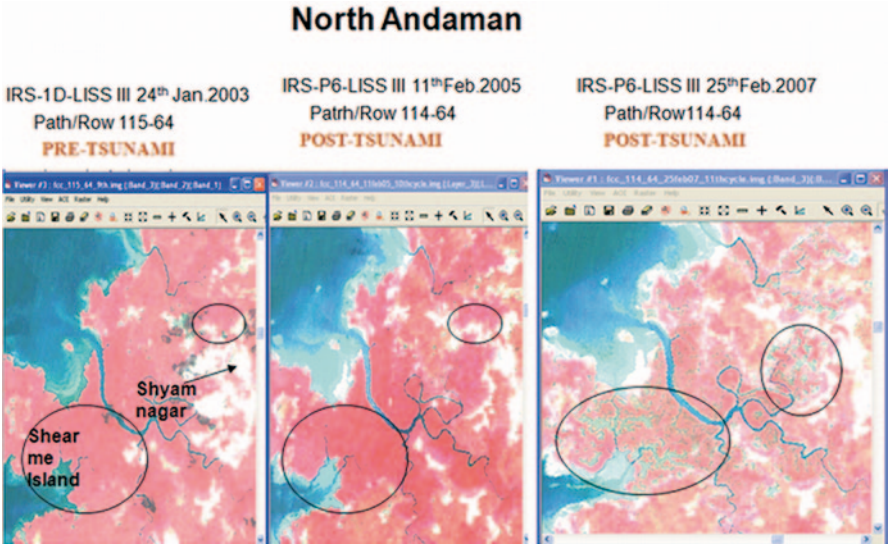

Fig. 2.12 Change in mangrove in North Andaman from 2003 to 2007

Fig. 2.13 Field condition in Shyam Nagar (North Andaman)

followed by Great Nicobar (524 ha), Kamorta and Nancowry (192 ha) and Trinket (117 ha). The loss of mangrove cover continued and it was assessed to be 914 ha between 2005 and 2007. Maximum loss was in Kamorta and Nancowry (581 ha) followed by Trinket (117 ha). In other islands, most of the mangrove area had already been destroyed earlier. In Great Nicobar and Little Nicobar, there are no discernible mangrove left. Total loss of mangrove cover between 2003 and 2007 is estimated at 2414 ha. Now only 3 km^2 (300 ha) of mangrove cover is left in Nicobar compared with 27 km^2 (2700 ha) existing in pre-tsunami period.

Remote sensing-based rapid assessments done by FSI (2006), Ramachandran et al. (2005) and Sridhar et al. (2005) show significant damage in forest and mangrove areas of Nicobar Islands, but these studies are based on comparison of satellite data of the pre-tsunami period with the images of immediate post-tsunami. Rapid

Table 2.2 Loss of mangroves in Nicobar group of islands

Island	2003 (Pre-tsunami)	2005 (Post-tsunami)	2007 (Post-tsunami)	Change from 2003 to 2007
Great Nicobar	610	86 (–524)	ND (–86)	(–610)
Little Nicobar	104	27 (–77)	ND (–27)	(–104)
Car Nicobar	133	90 (–43)	60 (–30)	(–73)
Katchal	588	21 (–567)	18 (–3)	(–570)
Trinket	330	213 (–117)	26 (–187)	(–304)
Kamorta & Nancowry	1000	808 (–192)	227 (–581)	(–773)
Teressa	ND	ND	ND	ND
Bompoka	ND	ND	ND	ND
Chowra	ND	ND	ND	ND
Total	*2745*	*1245 (–1500)*	*331 (–914)*	*(–2414)*

Figures in parentheses show change in mangrove cover per hectare
ND not discernible
(–) indicates loss

Table 2.3 Change in mangrove cover in ANI

Islands	2003 (Pre-tsunami)	2005 (Post-tsunami)	2007 (Post-tsunami)	Change from 2003 to 2007
Andaman	631	623 (–8)	612 (–11)	(–19)
Nicobar	27	12 (–15)	3 (–9)	(–24)
Total	*658*	*635 (–23)*	*615 (–20)*	*(–43)*

Figures in parentheses show change in mangrove cover per km^2
(–) indicates loss

assessment done by FSI describes changes in forest cover which include mangrove cover also. No area figures are separately provided for mangrove cover by FSI in this study. Study by Ramachandran et al. (2005) covers only four islands. A comparison of changes in mangrove swamps shown by them in Table 2.2 shows some variation in the area figures, but both the studies confirm that there was a significant loss in mangrove cover of these islands. The variation in the area figures may be due to the fact that in the present study SWIR band has been used in combination with red and green bands while in the study carried out by Ramachandran et al. (2005) conventional bands were used. In most of the places in ANI, mangrove swamp is immediately followed by dense forest vegetation cover making it difficult to delineate forest vegetation from mangrove forest. Application of SWIR band has been shown to help in better discrimination of crop with more water contents (Ibrahim et al. 2010; Panigrahy et al. 2009). The study carried out by Sridhar et al., (2006) is confined to Great Nicobar Island only and there is not much variation between the figures reported by them and as given in Table 2.2.

Table 2.3 shows the change in mangrove cover in Andaman group of islands and Nicobar group of islands from 2003 (pre-tsunami period) to 2007 (post-tsunami period). Total loss of mangrove cover in ANI had been 23 km^2 between 2003 and

2005 and there was a further loss of 20 km^2 between 2005 and 2007. Total loss of mangrove cover in ANI is assessed to be 43 km^2 between 2003 and 2007. In Andaman group of islands, the decrease has been 8 km^2 between 2003 and 2005 and further loss of 11 km^2 has been assessed between 2005 and 2007 making the total loss to be 19 km^2 between 2003 and 2007.

The Mangrove ecosystem continues to disappear or degrade, especially in the Indian Ocean region due to a variety of reasons mainly anthropogenic in nature. Common causes of mangrove degradation are shrimp culture, wood chip and pulp industry, urban development and human settlements and domestic uses for timber, firewood and fodder and grazing by buffalos, sheep, goats, etc. In ANI, prior to tsunami, degradation occurred only in very small pockets of up to 2,379 m^2 in the preceding 7 years (Ramachandran et al. l998). The mangroves here are less disturbed as compared with those along the peninsular India. The factors responsible for degradation of mangroves are exploitation due to demand for wood and wood products; conversion of mangroves for agricultural and human habitation purposes, exploitation of fisheries, issues of encroachment and tourism. In addition to these, browsing and trampling by wildlife such as deer, which are abundant in the Middle Andaman and livestock, which are often seen in areas close to human inhabitation (Kumar 2000).

It is seen from the results of this study that there has been widespread damage to the mangrove cover due to the massive earthquake and the tsunami that happened on December 26, 2004. However, the earthquake and the tsunami did not have an immediate significant impact on the mangroves of South, Middle and North Andaman Islands as is evident from the pre-tsunami and post-tsunami (2005) satellite data. The remote-sensing data analysis and the field survey done in this study during 2009 show significant degradation in mangroves, particularly in North Andaman Islands. The degradation is mainly due to the decrease in sea level in Diglipur area, as a result of which the mangroves are not getting tidal water and drying up gradually. The situation will deteriorate further with time. In Mayabunder, impact on mangroves is more due to anthropogenic pressures like habitation, construction, garbage throwing, etc. This is resulting in change in species composition in many places. *Avicennia, Nypa, Actrostichum*, etc. are more common near habitation. In Austin creek, mangroves are healthy and do not appear to have been affected by tsunami. Similarly, mangroves in Middle Andaman also, by and large, are not affected by the tsunami. Dam Roy and Krishnan (2005) have also observed the same. In South Andaman, the impact of tsunami is visible in some places, but in many places, mangroves are regenerating. New leaves are also observed in some apparently dying mangroves. Anthropogenic impact is visible in places such as Corbyns cove, Wright Myo, Shoal Bay and Sippighat.

The remote sensing-based results of the study clearly indicate that after effects of tsunami are still continuing in ANI and are likely to continue for some more time. Therefore, as suggested by Dam Roy and Krishnan (2005), there is a need for long-term survey and monitoring of the mangroves in these islands. One of the consequences of climate change caused by human activities is rise in the sea level. In ANI, the sea level has risen in many places and gone down in other places due

to this natural event of tsunami and it is therefore essential to distinguish clearly between the changes caused by human intervention-induced sea-level rise and changes caused by the natural event so as to make proper adjustments in studies or models projecting or predicting consequences of climate change in mangrove vegetation of these islands.

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Chapter 3
Greenhouse Gases Emission from Rice Paddy Ecosystem and their Management

T. B. Dakua, L. Rangan and Sudip Mitra

1 Introduction

The agriculture sector is one of the major sources of Greenhouse gases (GHGs). According to Sharma et al. (2011), agriculture sector contributed 19 % of total CO_2 equivalent emissions in India in 2007 and within the agriculture sector, rice cultivation is a major source of emission of Greenhouse gases in the form of CH_4. At several regional and global conventions, the options for the mitigation of emission of GHGs have been discussed widely. Several studies indicated that methane emission from rice fields can be mitigated through modification of crop cultivation practices like manure and fertilizer management, irrigation management, practice of 'No Tillage', 'Reduced Tillage', adoption of conservation agriculture, crop rotation and various alternative crop management measures, etc. However, in most of the cases, the yield of the crop is often negatively impacted. Improvement of crop varieties has been considered by many scholars as a viable option for reduction of methane emission without impacting the productivity of the crop as methane flux from rice fields is dependent on various cultivar specific properties like properties of root exudates, root porosity and permeability, features of the aerenchyma tissue, stage of the crop growth, methane conductance through the stem, photosynthate allocation efficiency, etc. Modification of such cultivar specific properties that can significantly reduce CH_4 emission, through appropriate crop improvement techniques should be the future research arena for mitigation of GHG emission from rice cultivation.

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2 Emission of Greenhouse Gases (GHGs) from Agriculture

According to the Inter-governmental Panel on Climate Change–Assessment Report 4 (IPCC–AR4 2007), the agriculture sector had contributed 10–12 % of the total anthropogenic GHG emissions in the year 2005. Since 1990, CH_4 and NO_2 emissions from agriculture sector are rising at the alarming rate of 58 Mt CO_2 equivalent/yr (US-EPA 2006a). Agriculture sector's contribution is more significant when emissions from individual sources are considered separately. The agriculture sector contributed 58 % of the total N_2O and 47 % of the total CH_4 emissions in the year 2005 (IPCC–AR4 2007). Emission from enteric fermentation and submerged rice fields constitutes the major source of CH_4 whereas emission from soil constitutes the single largest source worldwide (US-EPA 2006a). Biomass burning and manure management also account for a significant amount of global GHG emission. Net CO_2 emission from agriculture sector is less than 1 % of the global anthropogenic emission (US-EPA 2006b). Terrestrial plants also emit methane, global flux 62–263 Tg/yr, contributing 10–45 % of total global methane emissions (Keppler et al. 2006). Terrestrial plants emit methane through detached leaves as well as whole plant (Keppler et al. 2006; Whiticar and Ednie 2007). Transpiration is the dominant mechanism helping such emission pathway through leaves via xylem. Stiehl-Braun et al. (2011) studied the spatial distribution of methane-oxidizing bacteria (MOB) and proved that methane consumer bacteria can escape the effect of nitrogen (N) fertilization by shifting their zone of activity into deeper soil layers. Nitrogen fertilization and global methane cycling are interdependent and interlinked in both wetland conditions as well as in upland situations. Methanogenic archaea in wetlands is one of the major sources of methane whereas upland soil is a major C sink (Bodelier et al. 2011).

Developing countries contributed around 97 and 92 % of total global emissions from rice production and burning of biomass while developed countries contributed 52 % of total GHG emission from manure management (US-EPA 2006a). South and East Asian nations contributed 82 % of the total CH_4 emissions while countries from Sub-Saharan Africa and Latin America and the Caribbean have contributed about 74 % of total emissions from biomass burning. Yan et al. used the Tier-1 method as described in IPCC (Eggleston et al. 2006) guidelines for estimating global methane emissions and Monte Carlo simulation for estimating the uncertainty range. They have estimated that the total global CH_4 emission in the year 2000 was about 25.4 Tg/yr. They have further calculated that if all of the continuously flooded rice fields were drained at least once during the growing season, a reduction of 4.1 Tg CH_4/yr could be possible (Table 3.1).

Jiang et al. (2000) used The Asian-Pacific Integrated Model for analyzing the long-term Greenhouse gas (GHG) emission scenarios depending on alternative development paths in the developing countries of the Asia-Pacific region. They have taken into account four differeknt scenarios, namely Catch-Up Scenario (Scenario C), Domestic Supply Scenario (Scenario D), Short-cut Scenario (Scenario S) and Regional Equity Scenario (Scenario E). They have estimated that the growth rate of GHG emissions in the Asia-Pacific region is significantly higher than the overall

Table 3.1 Estimated emissions from global rice fields (Tg CH_4/yr)

Country	Irrigated rice	Rain-fed and deep water rice	Total
India	7.41	0	7.41
China	3.99	2.09	6.08
Bangladesh	0.47	1.19	1.66
Indonesia	1.28	0.38	1.65
Vietnam	1.26	0.39	1.65
Myanmar	0.80	0.36	1.17
Thailand	0.18	0.91	1.09
Other Monsoon Asian Countries	2.32	0.67	2.99
Rest of the World	1.2	0.49	1.7
Total	*18.9*	*6.49*	*25.39*

global emission growth rate and under all the scenarios CO_2 emission in year 2100 will be higher than the year 1990 level in the region.

Till date, the actual mechanism of N_2O emissions is not well understood and its emission coefficient calculated by IPCC methodology shows wide variability (Kroeze et al. 2003). The IPCC methodology led to over-estimation of N_2O emissions from legumes (Gregorich et al. 2005).

The CH_4 emissions from rice paddy cultivation under alternate scenarios are more or less stable around 4 Tg/yr. In India, there are diverse cultivation practices in various parts of the country depending upon water availability. Continuously flooded irrigated farming contributes to CH_4 emissions at the rate of 0.0251Gg CH_4/km^2/yr while upland farming contributes very negligible amount of CH_4 emissions (Garg et al. 2001; Fig. 3.1).

The major CH_4 sources in India are livestock farms, paddy fields, coal mining, municipal solid wastes, natural gas exploration and gas flaring and biomass burning etc. In the year 2000, CH_4 (18.63 Tg) and N_2O (0.31 Tg) emissions contributed 27 and 7 %, respectively, to India's CO_2 equivalent Greenhouse gas (GHG) emissions (Garg et al. 2003). The major N_2O emission sources include use of synthetic fertilizers in agricultural fields, indirect emission from atmospheric deposition of NH_3 and NOx, biological N_2-fixation, coal combustion, oil products combustion, crop residue burning and industrial activities, etc. However, emissions from synthetic fertilizer use contributed the maximum percentage (67 %) among all other sources. In the year 2003, the agriculture sector contributed about 65 % of CH_4 emissions and above 90 % of N_2O emissions in India (Garg et al. 2003; Garg et al. 2002).

Parashar et al. (1997) have estimated methane emission from rain-fed low land paddy fields is about 25 t/Km^2, while irrigated rice fields and deep water rice fields contributed on an average 32.46 t/Km^2 and 19 t/Km^2, respectively. The study showed that in 1995, Greater Mumbai (0.51 Tg), Midnapore, WB (0.24 Tg), Bilaspur, MP (0.17 Tg), Burdwan, WB (0.16) and Raipur, MP (0.15 Tg) were the top five districts in terms of methane emission in India. They had also analyzed sectorial emissions in all Indian districts and showed that Midnapore (West Bengal); Cuttack

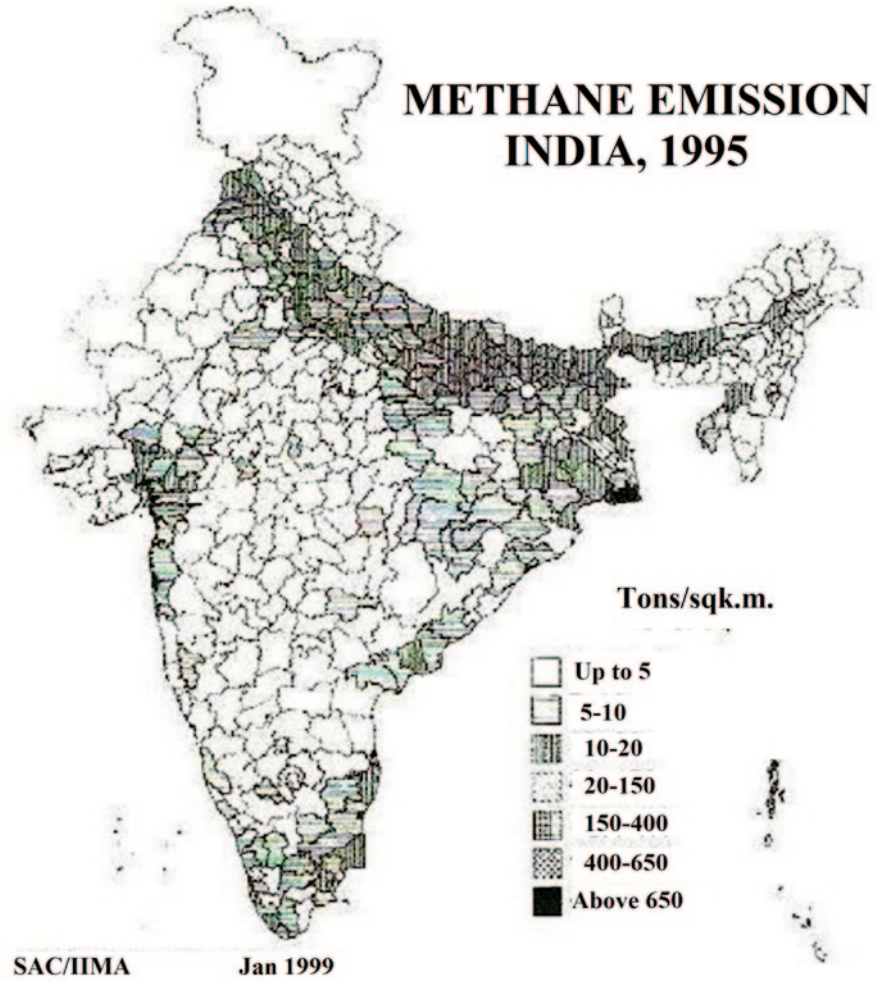

Fig. 3.1 Methane emission in India in 1995. (Source: Garg et al. 2001)

(Orissa), Raipur and Bilaspur (Chattisgarh) were the major methane emitting districts in India.

Garg et al. (2001) have calculated that the total CH_4 emissions in India increased from 17 Tg in 1990 to 18 Tg in 1995. Garg et al. (2001) have analyzed GHG emissions from large point sources (LPS) all over India and showed that in terms of CO_2 equivalent emissions, power plants, steel factories and transport sector together contributed about 47.9 % and agricultural sector, including livestock and synthetic fertilizer sources, contributed about 23.5 % of the total GHG emissions. Garg et al. (2001) have also shown that agriculture-related activities are responsible for about 90 % of Nitrous oxide emissions. The major sources were use of nitrogen fertilizers in agricultural fields (60 % of total NO emissions), biomass burning (10 % of total NO emissions), indirect soil emissions (10 % of total NO emissions) and livestock-related emissions.

3 Methane Emission from Rice Cultivation

Rice paddy field is an important source of methane. Many field and lab based studies have been carried out around the world to explore various aspects of methane production and emission from rice paddy soils. Wassmann et al. (2000a) have studied methane emissions from five different Asian countries (China, India, Indonesia, Philippines, and Thailand) and reported that climate and soil conditions play important roles in regulating methane emission potential from rice fields. They have used automated closed chamber method for estimating methane emissions from the rice fields. Their study revealed that low temperature and subtropical climate limited CH_4 emission in Northern China and northern India whereas tropical stations (Maligaya, Philippines; Beijing and Hangzhou, China) registered higher emission rates (300 kg $CH_4/ha^{-1}/season^{-1}$).

CH_4 emission from rice fields is highly sensitive to existing water regime, local variations in crop management and quality of organic inputs so that in most of the cases their cumulative impact overpowers the impact of soil and climate (Wassmann et al. 2000a). The spatial variations in CH_4 emissions from different rice-growing areas have also previously been reported (Parashar et al. 1996; Yagi et al. 1994).

Wassmann et al. (2000b) showed that distinct period within the season can help to reduce CH_4 emission significantly (20–80 %) in irrigated rice cultivation. Chareonsilp et al. (2000) reported that methane fluxes from deepwater rice fields is lower than that of irrigated rice fields but due to longer seasons and continuous flooding conditions, total emission from deepwater rice fields is quite high, i.e., about 99 kg $CH_4/ha^{-1}/season^{-1}$. Emission of methane from rain-fed rice fields is much lower than that of irrigated rice fields (Setyanto et al. 2000). Garg et al. (2011) estimated that in the year 2008 India's total methane emission was about 20.56 Tg and agriculture sector contributed 23 % of India's total GHGs emission. The study also showed that Uttar Pradesh and Andhra Pradesh were the two highest methane producing states and Mumbai and Anugul (Orissa) districts were the two highest methane producing districts in India.

4 Nitrous Oxide Emission from Agricultural Soils

Flooded rice fields are not the potent source of N_2O emissions because of prevailing anaerobic conditions (Granli and Bockman 1994). Emission of N_2O starts only when the fields are drained and aerobic conditions are created. Use of N-fertilizers increases the rate of N_2O emissions from rice fields (Kumar et al. 2000). Sharma et al. (1995) estimated that N_2O–N emissions from irrigated and upland paddy fields in India are about 0.004–0.21 Tg/yr^{-1} and 0.002–0.01 Tg/yr^{-1}, respectively. However, the emission of CH_4 can itself act as a check on N_2O formation in flooded rice soil (McCarty et al. 1991). Ghosh et al. (2003) reported that in New Delhi, India, total CH_4 emission under upland conditions is in the range of 24.5–37.2 kg/ha^{-1} while N_2O fluxes varied in the range of 0.18–100.5 μg m^{-2} ha^{-1} with CV 69–143 %, and

application of N-fertilizers invariably increased the rate of N_2O emission. Application of nitrification inhibitors like DCD can reduce N_2O emission up to 10–53 % under New Delhi conditions by reducing the availability of NO_3-(Ghosh et al. 2003; Pathak and Nedwell 2001). Kumar et al. (2000) reported that application of DCD with urea and $(NH_4)_2SO_4$ could reduce N_2O–N emission by 11 and 26 % respectively in irrigated transplanted rice grown on Typic Ustochrepts soil in New Delhi, India. Malla et al. (2005) studied the efficacy of five different nitrification inhibitors (neem cake, thiosulphate, coated calcium carbide, neem oil coated urea and DCD) in Indo-Gangetic plains in rice-wheat system and reported that DCD and Ca carbide were more efficient in reducing GWP potential than thiosulphate, neem oil, and neem cake. Bhatia et al. (2010) reported that application of nitrification inhibitors like S-benzylisothiouronium butanoate (SBT–butanoate) and S–benzylisothiouronium furoate (SBT–furoate) could reduce GWP of wheat soil by 8.9–19.5 % under both conventional and no-tillage practice. DCD, one of the most potent nitrification inhibitors, which has been commercially used in Japan and Germany (Bharti et al. 2000) produces non-toxic byproducts upon decomposition (Amberger 1989). The mitigation practices for CH_4 emission and N_2O emission are competitive to each other (Bronson et al. 1997) so a balanced approach should be followed to minimize the Cumulative Radiative–Forcing of both the gases.

Pathak and Nedwell (2001) have shown that application of nitrate (NO_3-N) fertilizers like calcium ammonium nitrate (CAN) in aerobic conditions and ammonium (NH_4-N) fertilizers like ammonium sulphate and coated urea in wetland conditions can significantly reduce N_2O emission. Li et al. (2009) reported that the time of application of nitrification inhibitor, DCD, can increase rice yield as well as reduce the GWP of CH_4 and N_2O emissions from rice fields. They studied the impact of application of DCD at three different stages of crop growth, i.e., Land preparation, tillering, panicle initiation. They have found out that application of DCD at tillering stage had maximum inhibitory effect on N_2O emission (56 % reduction) while application during panicle initiation could reduce N_2O emission efficiently. Application of DCD as basal reduced CH_4 emissions by 35 %.

Soils with high SOM emit more N_2O (Bouwman et al. 2002) and carbon and nitrogen cycles depended on each other in the soil environment (Li et al. 2005a). N_2O is released during both nitrification and de-nitrification. Nitrification inhibitors like Nitrapyrin, DCD and DMPP could be mixed with urea for effectively reducing the N_2O emissions (Pain et al. 1994). No tillage system reduces CH_4 emission from soil as any disturbance in soil environment increases decomposition rate of soil C (West and Post 2002) but effect of no-tillage on N_2O emission is primarily determined by soil and climatic conditions (Marland et al. 2001). Studies indicated that there is an inverse relationship between reduction of CH_4 emission and N_2O emission (Monteny et al. 2006). Zoua et al. (2007) estimated that about 29.0 Gg N_2O–N is emitted during the crop growing period from the rice fields in China which accounts for about 7–11 % of total annual emissions in China. The study also reported that among the different water management systems practiced in China (i.e., continuous flooding (F), flooding-midseason drainage-reflooding (F-D-F) and flooding-midseason drainage-reflooding-moist intermittent irrigation, but without

Table 3.2 GHG emissions from India, Thailand and Philippines. (Source: Gadde et al. 2009)

Country	GHG emission from open field burning (t CO_2 eq/yr.)	Total GHG emission (t CO_2)	% contribution from open field burning
India	556,165	1,218,928,500	0.05
Thailand	425,225	231,546,484	0.18
Philippines	412,803	6,345,154	0.56

water logging (F-D-F-M)), F-D-F and F-D-F-M systems significantly increased the N_2O emissions. A similar result was reported by Wange et al. Their study reported that water management system significantly influences the N_2O emissions from rice fields. The study reported that average N_2O emissions from rice fields under mid-season drainage and continuous flooding treatments were 0.41 kg/N/ha^{-1} and 0.28 kg/N/ha^{-1} respectively. The study showed that N_2O emission gets enhanced mainly in the transition phase of the water management system and 50 % emission reduction under both water management systems can be achieved by integrated application of N fertilizers and rice straw.

5 Emission of GHGs due to Field-Burning of Crop Residue

Burning of crop residue releases GHGs (CO_2, CH_4, and N_2O), other trace gases (CFCs, O_3, CO, Non methane hydrocarbons) and particulate matter into the atmosphere. Sahai et al. (2007) estimated that in the year 2000, about 85,623 Gg of dry wheat residue was generated, of which about 21,406 Gg was openly burnt leading to emission of about 68 ± 51 Gg, 34435 ± 682 Gg CO_2 and 14 ± 9 Gg N_2O. The trace gases released during burning of crop residue also have negative impact on human health and natural environment (Cheng et al. 2000). India's NATCOM (2004) used IPCC methodology and estimated 56, 1 and 40 Gg of CH_4, N_2O and NOx in the year 1994 from in situ burning of wheat residue (Table 3.2).

Gadde et al. (2009) estimated GHG emissions from crop residue burning in three countries namely India, Thailand and Philippines. They reported that 23, 48 and 95 % of the crop residue produced is openly burnt in India, Thailand and Philippines respectively.

6 Modelling GHG Emission from Agriculture

Various models have been used for estimation of various GHGs e.g., CH_4 emission, MERES (Matthews et al. 2000), DNDC (Li et al. 2005b), DayCent (Del Grosso et al. 2009), InfoCROP (Aggarwal et al. 2004) and WNMM (Li et al. 2005b).

The IPCC Tier I is widely used by various scientists for estimating GHG emissions. However, DNDC is one of the very efficient process based biogeochemical models for predicting C sequestration and trace gas emissions from agricultural lands (Li et al. 1992). The DNDC model can predict about N_2O, CO_2, CH_4, crop production, NH_3 volatilization and NO_3^-leaching. Other process-based models include TEM, CENTURY, and ROTHC, etc. The DNDC model has six sub-models, including soil climate, plant growth, decomposition, nitrification, denitrification and fermentation sub-model. Zhang et al. (2011) used DNDC model for quantifying methane emissions from Sanjiang province (North east China) and reported that the region had emitted 0.48–0.58 Tg CH_4-C in 2006. Stephen et al. (2009) used DAYCENT model for estimating GHG emissions from non-rice crops like corn, wheat and soybean. DAYCENT model considers not only N inputs but also other factors like soil texture class, plant N demand, timing of N application, moisture stress, temperature and organic matter decomposition rates for estimating rate of N_2O emission.

7 Mitigation of GHG Emissions from Agriculture

Mitigation of GHGs from agriculture ecosystems without hampering the crop yield is a big challenge. Many research works have been carried out across the world to address this challenge. Many means of mitigation of GHGs from agriculture ecosystems have been identified and tested. Following section will address some of those important mitigation options for GHG management in agriculture ecosystems.

7.1 Through Sequestration of Carbon in Soils

Adoption of agronomic practices like extended crop rotation, cultivation of improved varieties, and use of perennial crops can increase carbon (C) storage significantly in various types of soils (Follett 2001). 'No Tillage' system reduces CH_4 emissions from soil as any disturbance in soil environment increases decomposition rate of soil C (West and Post 2002) but the effect of no-tillage on N_2O emissions is primarily determined by soil and climatic conditions (Marland et al. 2001). Studies have indicated that there is an inverse relationship between reduction of CH_4 emission and N_2O emission (Monteny et al. 2006). In Eastern Canadian soil, crop rotations involving alfalfa had highest amount of carbon stored in the soil (513 kg C/ha/yr) over 20 years. While corn-corn-soybean-soybean rotation had stored the lowest amount, different management practices had significant effect on GHG emissions (Meyer-Aurich et al. 2006). Various tillage practices have very insignificant effect on soil carbon storage in Eastern Canadian soil (Angers et al. 1997; Yang and Kay 2001). Meyer-Aurich et al. (2006) showed that inclusion of alfalfa into crop rotation can mitigate around 2000 kg CO_2 equivalent/ha/yr. Lal (2010) estimated that the global cropland soil can sequester 0.61–2 Pg/yr and soil organic carbon (SOC) concentra-

tion in root zone soil of about 1.1 % is essential for maintaining optimum soil health and agronomic conditions. Soil C sequestration can reduce CO_2 concentration in the atmosphere by locking the C as humus in the soil system for quite a long time. Depletion of SOC depends on climate, soil type and cultural management practices. Adoption of proper management practices can improve the SOC pool as well as increase productivity and enhance soil resilience to adapt to changing climatic scenarios (Lal 2004). Lal et al. (2006) estimated that an increase in SOC by 1 t/ha could increase grain yield by 6.4 million t in Africa and 11.7 million t in Asia. Lal (2011) estimated that increase of 1 t C/ha/yr in the rhizospheric soil can increase foodgrain production by 24–32 million t in the developing countries of the world. The study also quantified the potential of soil C sequestration of the agro-ecosystems of the world to be approximately 1.2–1.3 billion t C per year. The study also showed that if the SOC pool is increased by 10 % over the twenty-first century, it can cause reduction of 110 ppm of atmospheric CO_2 concentration (one billion t of soil C = 0.47 ppm of atmospheric CO_2). Hansen et al. (2008) showed that bio-sequestration can reduce CO_2 concentration by 50 ppm by the year 2150.

7.2 Conservation Agriculture

The concept of Conservation agriculture (CA) was put forward by FAO for addressing the growing concern over sustainable agriculture. Conservation agriculture is a package of management practices that mainly includes reduced tillage, No tillage, direct seeding, soil cover (i.e., cover crops, relay crops, intercrops) to manage soil erosion, improvement of soil health, crop rotation for controlling weeds, etc., (Derpsch 2001). These practices lead to increase in soil organic carbon. No tillage system is better than reduced tillage system as far as accumulation of soil C is concerned (West and Post 2002). Under 'No Tillage' system, SOC gets accumulated in the top soil that creates a vertical stratification of soil C which regulates the soil microbial activity (Dennis et al. 1994; Stockfisch et al. 1999; Moreno et al. 2006). Conservation agriculture helps to improve soil's physical properties like porosity, soil structure, and water holding capacity (Medvedev et al. 2004; Josa et al. 2005). Chivenge et al. (2007) studied the effect of tillage and management practices on SOC dynamics in red clay soil and sandy soil and reported that tillage disturbance is the major factor influencing the C dynamics in agricultural soil. The study also indicated that practice of Conservation agriculture can improve soil C status and maintain long-term sustainability. Ghimire et al. (2011) conducted an experiment in Chitwan Valley of Nepal and reported that 'No Tillage' system is far better than that of conventional tillage system for C sequestration in rice–wheat cropping system. Datta et al. (2011) showed that crop diversification can reduce cumulative methane emission and also reported that rice potato sesame was most suitable cropping system for mitigation of greenhouse gas emissions. The study suggested that methane fluxes from different cropping systems and reported that GWP of rice–rice system is very high whereas rice-potato-sesame system is most profitable in terms of total revenue ($ 1248.21 per ha) as well as C-credit ($38.60 per ha).

7.3 Water Management

Practice of midseason drainage has been followed in China since 1980s and studies showed that it resulted in 40 % CH_4 emission reduction i.e., about 5 Tg CH_4/yr (Li et al. 2005b). However, the effectiveness of water management in reducing CH_4 emissions varied from place to place. Midseason drainage also increased N_2O emissions that offset a part of Greenhouse gas radiative forcing benefit (nearly 32 %) obtained through reduction in methane emission. Maximum Greenhouse gas radiative-forcing benefit can be gained when midseason drainage is applied to soil with low organic content and high clay content (Li et al. 2005b). Husin et al. (1995) studied the influence of various irrigation practices (continuous flooding, intermittent irrigation, and saturated soil conditions) on CH_4 flux from rice fields in Java and Indonesia and proved that the water management treatments significantly influences the average daily methane fluxes. The study showed that CH_4 flux in intermittently irrigated rice fields was 53 % lower than that of continuously flooded fields. Soil Eh status can be maintained easily by altering water management practices. Midseason drainage can increase Soil Eh to the oxidative state (to the level + 450 mV from −160 mV) in just a few days that suppressed the methanogenesis process in the rice soil (Reddy et al. 1989; Patrick and Jugsujinda 1992).

Yagi et al. (1998) studied the impact of water percolation on CH_4. The study suggested that CH_4 emission rate got reduced significantly with an increase in the percolation rates. Yu et al. (2004) reported that under non-flooding (but wet) irrigation system, cumulative global warming potential of rice fields can be reduced up to about 72 %. Nelson et al. (2011) reported that midseason drainage can reduce methane emission effectively as well as promote methane oxidation process which together can reduce Greenhouse gas emissions by 75 million t of CO_2 equivalent. Tyagi et al. (2010) studied the impact of four different types of water management systems (continuous flooding, tillering stage drainage, midseason drainage and multiple-drainage) on CH_4 efflux from rice fields. The study showed that midseason drainage and multiple-drainage are highly effective in reducing methane emissions from rice soil. The study also reported that midseason drainage and multiple-drainage can mitigate GWP of rice soil by 41 and 37 % respectively. Itoh et al. (2011) studied the impact of prolonged midseason drainage on methane flux from Japanese rice fields and reported that seasonal CH_4 emissions and 100-year GWP can be reduced to approximately 69.5 and 72 % respectively by alternative water management without any significant decrease in the grain yield.

7.4 Direct Seeding of Rice

Corton et al. (2000) reported 18 % CH_4 emission reduction by utilizing direct seeded rice practice in Philippines. Wassmann et al. (2004) showed that DSR practice

along with a midseason drainage system practice can reduce CH_4 emissions by 50 %. Ahmad et al. (2009) reported that DSR plus no tillage is a promising option for reducing GWP of rice soil.

7.5 Fertilizer Management

Rath et al. (1999) studied methane emissions for same cultivar 'Gayatri' under rainfed lowland and irrigated condition using different fertilizer management practices (Prilled urea, prilled urea + nimin, Urea super granule and control). They reported that application of nitrification inhibitor, nimin with urea, reduced CH_4 emissions effectively by inhibiting the autotrophic oxidation as earlier reported by Sahrawat and Parmar (1975). Urea super-granule application at the base of the plant is also an efficient option for reducing emission. Bronson et al. (1991) reported that wax coated Calcium carbide can reduce CH_4 emission by releasing acetylene that acts as inhibitor of methanogenesis. Application of muriate of potash reduces active reducing sunstances, Fe^{2+}content and redox potential whereby apart from increasing the grain harvest, it also reduces methane emissions significantly (Babu et al. 2006).

7.6 Silicate Fertilization

Ali et al. (2008) studied the influence of silicate iron slag on rice (*Oryza sativa, cv. Dongjinbyeo*) in Agronomy Farm, Gyeongsang National University, South Korea. Their study showed that silicate fertilization @ 4 Mg/ha could reduce CH_4 emissions by 16–20 % and at the same time increasing the yield by 13–18 %. The growth of the rice plant was enhanced due to increased availability of nutrients. CH_4 emission was limited due to higher concentration of ferric oxides which acted both as oxidizing agent and electron acceptor (Ali et al. 2008). They have reported a strong negative correlation between CH_4 flux and free iron and active iron concentration in soil. Other studies on silicate fertilization indicated that iron oxide suppresses production of organic acid by acting as electron acceptor (Asami and Takai 1970; Watanabe and Kimura 1999). Ali et al. (2009) studied the influence of silicate fertilization on methane production under conventional and no-tillage conditions in Korean paddy fields. Their study showed that methane emission was reduced under conventional and no-tillage conditions by 54 and 36 % with silicate slag application @ 4 Mg/ha^{-1}. Silicate fertilization also reported to improve soil porosity and redox potential, active tillering rate, root volume and leaf photosynthetic rate. Nouchi (1994) and Aulakh et al. (2000) reported that CH_4 emissions get reduced drastically at grain maturation stage due to reduced gas conductivity as well as reduced photosynthetic activity.

7.7 Efficient Management of Manure

Since CH_4 is produced under anaerobic conditions, so by improving organic matter management or incorporating organic matter into soil during off-season drained period, CH_4 emissions can be reduced significantly (Kalra et al. 1996). Debnath et al. (1996) showed that by application of fermented manure like biogas slurry, CH_4 emissions from rice fields can be reduced without hampering the productivity.

8 Crop Improvement for Reducing GHG Emissions

Perhaps one of the most challenging means of mitigation of GHG emissions is the crop improvement. Scientists are pursuing this field of research quite vigorously. Though not much success has been achieved, yet a strong database has certainly been created through worldwide researches on this topic. Many scientists and institutions are working on this aspect for the mitigation of GHG emissions. Like many parts of the world, efforts are on in India also to develop crops with certain characteristics so that the plants emit less GHGs without causing any reduction in the yield.

8.1 Plant Physiology and Molecular Biological Approach

The aerenchyma tissues in the leaf, roots and clum of rice plants act as an efficient channel for gaseous exchange between soil and atmosphere (Raskin and Kende 1985). Satpathy et al. (1998) also reported a negative correlation between oxidase activity of the root tip and CH_4 flux. Higher oxidase activity in the vicinity of the rice plant roots inhibits methanogenesis and increases CH_4 oxidation (Ota 1970). Lueders and Friedrich (2002) reported that addition of electron acceptors stimulates microbial population that is competitive to methanogens by suppressing methanogenic metabolic pathways, thereby reducing methane emission from rice fields. Application of mycorrhiza and methanotrophs can effectively reduce methane emission from rice fields by suppressing methanogen population in rice soil (Lakshmanan et al. 2009).

Rice Cluster I (RC-I) refers to the orders *Methanosarcinales* and *Methanomicrobiales* that carry the *mcr-A* gene coding for methyl coenzyme M reductase (*mcr A*; Grosskopf et al. 1998a). This group of bacteria, abundant in soil in all parts of the world, is responsible for CH_4 emissions through the process of acetoclastic methanogenesis (conversion of acetate to CH_4) or hydrogenotrophic methanogenesis (conversion of H_2O plus CO_2 into CH_4;Conrad et al. 1993). Grosskopf et al. (1998b) and Kudo et al. (1997) studied the *16S rRNA* sequence of Rice Cluster I.

8.2 Genetic Engineering Approach

C_4 crops can assimilate more CO_2 than that of C_3 crops due to specialized C_4 metabolism cycle and can reduce photorespiration by 80 % by increasing bundle sheath CO_2 level significantly (Kajala et al. 2011). In recent times, installation of C4 mechanism into staple food crops like rice, wheat and potato is considered as the futuristic answer to the problem of increasing food insecurity in today's world. International C4 Consortium led by International Rice Research Institute (IRRI) has been trying to install two-cell C4 cycle in rice for achieving higher productivity and higher resource utilization efficiency. This task itself is an enormous challenge to the scientific community, however the conversion is not impossible as all C4 cycle enzymes are found in C3 plants at low level and no new genes are associated with C4 pathway (Sage 2004; Brown et al. 2010). The aim of such a research is to down-regulate the expression of mesophyll cells and change the leaf anatomy i.e., increased vein density and number of M cells in between veins as low as possible. This feature of the C4 rice would help reduce emission of methane as M cells play an important role in determining the methane conductance through the rice plant.

8.3 Temperature Regulation

Various studies have reported a positive correlation between CH_4 flux and soil temperature (Conrad et al. 1989; Sass et al. 1991) but no significant relation is found between methane emissions and amount of light incident on the rice plant (Nouchi et al. 1990). Gas permeability of root epidermal layers and structure of aerenchyma gets adversely affected by aging (Armstrong 1971; Arikado et al. 1990). At maturity, CH_4 emission is reduced due to choking of aerenchyma but increased air temperature during maturation of crop does not play any significant role (Watanabe et al. 1994).

Hosono et al. (1997) reported the effect of temperature on the rate of CH_4 emission. Their study showed that when temperature was increased from 15 to 30 °C, the methane diffusion increased by 2–2.2 times. The study also suggested that air temperature has much less effect on CH_4 conductance than that of rhizosphere soil temperature. The correlation between soil temperature and conductance was reported to be statistically significant ($p < 0.01$). At 28 °C soil temperature, conductance was six times higher than that of at 18 °C.

8.4 Water Management

It has been reported that the degree of water submergence could influence the rate of methane flux from rice plants (Wang et al. 1993). Wang et al. (1997a) have studied

Table 3.3 Variation in CH_4 emissions with various levels of submergence. (Source: Wang et al. 1997b)

Water depth (cm)	% nodes submerged	CH_4 emission (%)
5.5	0	100
12.5	30	77
26.5	67	16
38	100	1

the role of aerenchyma of leaves, nodes and panicles in methane emissions. Emission through the rice plants is controlled by diffusion.

Wang et al. (1997b) reported that under various degree of submergence, the emission rate gradually decreases and it completely stops under complete submergence conditions. The study also proved that CH_4 emission through panicles is far less than that of cracks and porous structure at nodes and increasing submergence reduces CH_4 emissions temporarily until the concentration gradient is readjusted to above water emission sites (Table 3.3).

8.5 Cultivars Development

Das et al. (2008) studied methane emission in traditional cultivar 'Agni' and modern improved cultivar 'Ranjit' under irrigated condition in North Bank Plain Zone of Assam, India. They reported that Agni cultivar emitted more methane gas because of its poor capacity for allocation of photosynthate to the developing grain, which led to increased rhizo-deposition, thus increasing the CH_4 emission whereas 'Ranjit' cultivar emitted less CH_4 it being able to allocate photosynthate efficiently towards panicle and developing grains having smaller root length and smaller leaf area.

Wang et al. (2000) studied three rice cultivars and reported that IR65598 cultivar had higher oxidative activity in the rhizosphere than IR72 and Chiyonishiki. They studied the rate of CH_4 emission in different growth stages. In the tillering stage, all the cultivars showed very low emission rate but at flowering and ripening stage, IR72 and Chiyonishiki had significantly higher emission rate than IR65598. About 60–90 % of methane emitted from rice fields is transported through aerenchyma of the rice plants (Holzapfel-Pschorn and Seiler 1986). Rice plants act as a conduit for CH_4 emissions as well as source of methanogenic substrates. Yunsheng et al. (2008) studied four cultivars (IR65598, IR72, Dular and Koshihikari) under elevated CO_2 concentration in Tsukuba, Japan, and reported that under elevated CO_2 conditions, CH_4 fluxes increased by 10.9–23.8 % and daily CH_4 flux was highest for Dular and lowest for Koshihikari. Mitra et al. (1999) studied six different rice varieties (Pusa 933, Pusa 169, Pusa 1029, Pusa Basmati, Pusa 677 and Pusa 834) in New Delhi and reported that Pusa 933 emitted maximum CH_4 and Pusa 169 variety the minimum. These studies show that use of different cultivars can be a good option for mitigation of CH_4 emission from the rice fields. They also provide clues that through '*on'* and/or '*Off*' the shelf techniques, new plant types could be developed with less methane emitting potentials (Table 3.4).

Table 3.4 Yield and total CH_4 emissions from six rice varieties. (Source: Mitra et al. 1999)

Variety	Total methane emission (Kg/ha)	Yield (t/ha)
Pusa 169	15.63	6.5
Pusa Basmati	26.31	4
Pusa 834	24.02	6.4
Pusa 1019	26.97	4.8–7.1
Pusa 677	16.91	3.2–7.3
Pusa 933	27.24	5.5–7.5

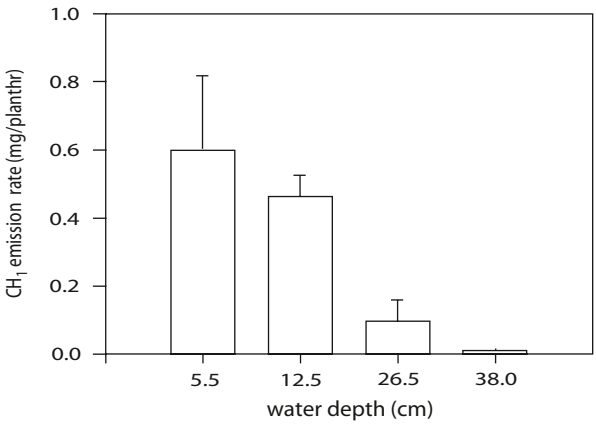

Fig. 3.2 Methane emission from rice culms at different depths of flood-water (mean ± SE, $n = 3$). (Source: Wang et al. 1997b)

The capacity of methane emission varies widely among rice cultivars (Shalini et al. 1997; Sigren et al. 1997; Kesheng and Zhen 1997) but only variation in CH_4 transport capability is insufficient to explain the variability of CH_4 emission potential among different cultivars (Aulakh et al. 2000b).

8.6 Manipulation of Plant Root Properties

Root exudation ability of different cultivars (Wang et al. 1997b; Wassmann and Aulakh 2000), stages of crop growth, gas transport capability, type and amount of aerenchyma (Aulakh et al. 2000a; Butterbach-Bahl et al. 1997) also impart additional variability in CH_4 emissions. Rice plants provide methanogenic substrate through root exudates, help in transport of CH_4 and O_2 through aerenchyma and establishment of an active CH_4 oxidizing-site in the rhizosphere (Wassmann and Aulakh, 2000). Mitra et al. (2005) reported that decomposition of root exudates is one of the causes of CH_4 emission from rice soil. However, the rates of CH_4 production vary with soil types and CH_4 production is positively correlated with degree of aeration in the field. Redox potential of soil is one of the major factors that influence methane production and gas exchange capacity in the rice field (Kludze et al. 1993; Fig. 3.2).

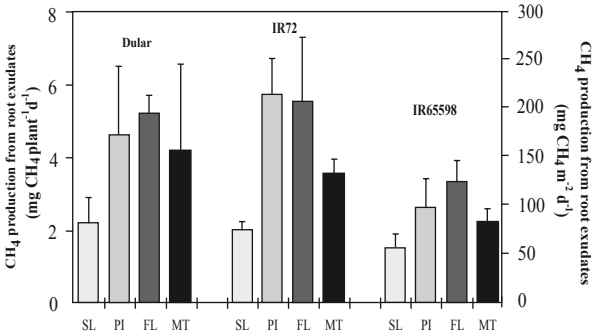

Fig. 3.3 Methane production potential of one-day exudates of Dular, IR72 and IR65598 cultivars at seedling stage (SL), panicle initiation (PI), flowering (FL) and maturity (MT). (Source: Aulakh et al. 2001)

Their study suggested that CH_4 emission was more strongly related to total organic C ($r = 0.920$) than that of organic acids ($r = 0.868$). Rice root exudates act as a substrate for the methanogenic bacteria in anoxic condition. The study also suggested that for cultivation of high-yielding varieties (e.g., IR65598, IR65600) could reduce CH_4 emissions as they produce lowest exudate-induced CH_4 production. Thus, selection of rice cultivars could reduce CH_4 emission in regional and global level.

Various studies have reported about increment of root exudation due to lower membrane permeability and root porosity caused by P deficiency (Ratnayake et al. 1978; Graham et al. 1981; Lipton et al. 1987; Kirk and Du 1997). Low P could stimulate the downward transfer of oxygen and upward transfer of methane due to increased root porosity (Justine and Armstrong 1987; Kludze et al. 1993). P deficiency stimulates a chain of reactions that affect the partitioning of photosynthates and lead to higher root/shoot ratio (Marschner 1996; Kirk and Du 1997). Lu et al. (1999) reported that low P supply to rice plants resulted in significant increase in CH_4 emissions (34–50 micromoles under P deficiency and 10–22 micromoles under ample P supply), increase of root/shoot ratio by factors of 1.4–1.9, better development of root aerenchyma and increase in root exudation by factors of 1.3–1.8.

8.7 Methane Transport Capacity

Aulakh et al. (2000a) have studied methane transport capacity (MTC) of rice plants. They have reported that up to the concentration level of 7500 ppm, methane transport by rice plant increases linearly with increasing CH_4 concentration in the nutrient culture solution surrounding the roots. Their study also reported that MTC of IR72 was lowest at seedling stage (average 8 mg CH_4/plant^{-1}/day^{-1}), then increases gradually until panicle initiation (maximum, 120 mg CH_4/plant^{-1}/day^{-1}) and after that it gets reduced significantly at maturity (Fig. 3.3).

Thus, cultivation of rice varieties having low MTC can reduce methane emissions from rice fields (Butterbach-Bahl et al. 1997). Aulakh et al. (2000b) estimated

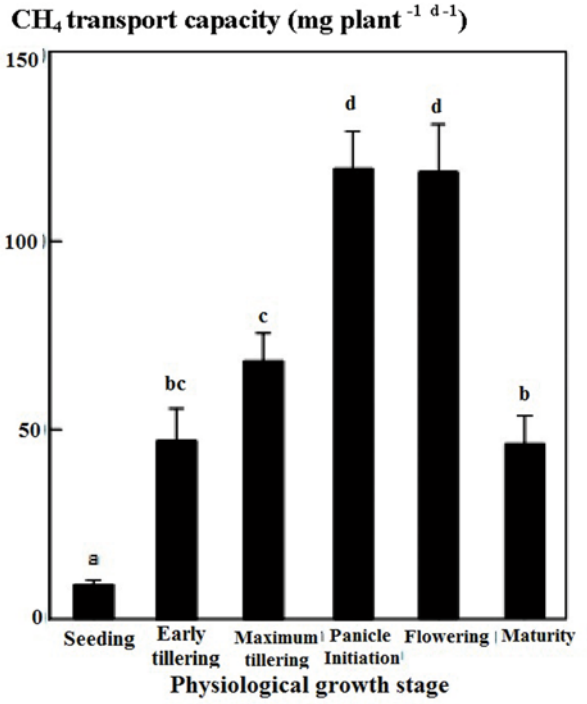

Fig. 3.4 Methane transport capacity of rice plants of cultivar IR72 at seedling, early tillering, maximum tillering, panicle initiation, flowering and maturity. Data shown is means ± SD of three replicate plants each measured in triplicate. Different letters indicate significant differences ($p > 0.05$). (Source: Aulakh et al. 2000a)

the MTC of four high-yielding varieties (IR72 > IR52 > IR64 > PSBRc 20) by using automated system.

Aulakh et al. (2002) studied the MTC of 18 inbred varieties and four hybrids at various growth stages. MTC of different varieties varied from 62 to 445 % of IR72-MTC. The study showed that tiller numbers were linearly co-related to MTC i.e., number of tillers directly determines CH_4 transport. Their study proved that the use of high-yielding cultivars with low MTC (e.g., KDML 105, IR65598 and PR 108) could be a viable option for reducing CH_4 emissions from rice fields (Fig. 3.4).

Nouchi et al. (1994) used modified diffusion model for quantitative estimation of methane transport through the micropores in the leaf sheath and the gaps at the joint of nodal plate and leaf sheath of the rice plants (Nouchi et al. 1990). Methane emission is mainly driven by CH_4 concentration gradient between atmosphere and soil pore water, molecular diffusion (Denier Van der Gon and Breemen 1993) and thermo-osmosis (Schröder et al. 1996).

Yao et al. (2000) reported that CH_4 emissions through rice plants are influenced by many factors like growth stage, rice cultivars, stem inter-cellar volume, length of root bundle and total root volume at matured stage. They studied CH_4 conductance among 11 different rice cultivars and reported that the CH_4 conductance is positively correlated with inter-cellar volume at tillering stage and root volume at the reproduction stage. They have also done regression analysis to prove that in both the stage of growth considered together, CH_4 conductance is significantly correlated

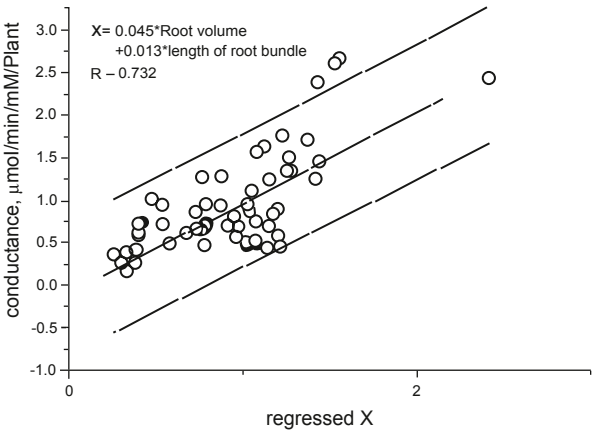

Fig. 3.5 Results of multi-dimensional regression analysis between plant conductance for methane and physical parameters; Regressed value R = 0.793 ($p < 0.01$). (Source: Yao et al. 2000)

with root volume (Fig. 3.5). Jones (1992) reported that the size of the micro-pores, the size of the inter-cellular space and plant conductance are proportional to the size of the rice plant.

9 Conclusion

The agricultural sector contributed 47 % of total CH_4 emissions in the year 2005 (IPCC-AR4 2007) and South and East Asia was the major contributor (82 % of total CH_4 emissions) because of widespread rice cultivation in the region. Many agricultural scientists, who have carried out various studies, recommended various measures for reducing CH_4 emission from the rice fields. CH_4 emission from rice fields is strongly influenced by existing water regime, local crop management practices, cropping rotation and quality of organic inputs used. Practice of no tillage system and cultivation of perennial crops can significantly reduce CH_4 emissions from soil by increasing soil C storage. In upland farming, direct seeded rice cultivation and 'No Tillage' system are two promising options for reducing methane emissions from cultivated rice fields. Use of prilled urea, urea-super-granule, and application of nitrification inhibitor (Nimin) can reduce CH_4 emissions effectively. Management of organic matter, application of organic matter during off-season drained period, application of biogas-slurry to the rice fields are some of the variants of measures to reduce GWP of rice soil. P deficiency in rice soil leads to increase in root exudates amount by lowering the membrane permeability and enhancement of downward transfer of O_2 and upward transport of CH_4, thus management of Phosphorous (P) availability in rice soil would be a viable option for reducing CH_4 emission.

Emission of CH_4 through the rice plants is influenced by various properties of the plant itself, i.e., photosynthate allocation capacity, root volume, oxidase activity in the vicinity of root tip, amount and nature of root exudates, properties of aerenchyma tissue, number and structure of nodes, stages of crop growth and methane

transport capacity (MTC) etc.,. Various cultivars like Ranjit, IR 65598, IR 72, Koshihikari, Pusa 169, IR 65600, KDML 105, PR-108 emit far less CH_4 than that of other traditional varieties due to some variety-specific properties. Improved cultivar 'Ranjit' emits less CH_4 than 'Agni' due its better photosynthate allocation capacity, high-yielding varieties like Pusa 169, Pusa basmati, Pusa 677 emit less methane due to lower root exudation and low MTC.

Thus cultivar improvement in the line of developing new high-yielding varieties having low MTC, lower methane emission through aerenchyma and nodes, low amount of root exudates, can give the breakthrough in agricultural research system for reducing CH_4 emission from rice fields on a regional and global level without hampering the productivity.

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Chapter 4
Remote Sensing Applications to Infer Yield of Tea in a Part of Sri Lanka

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1 Introduction

Crop yields in any location and any species are subject to many dynamic factors of production which are biotic and abiotic. No two agro ecosystems are identical. Here an effort is made to infer the yield of tea in a part of Sri Lanka based on conventional and new techniques of remote sensing applications. Sri Lanka is an agriculture-based country and the sector shares about 12 % of Gross Domestic Product (GDP). Paddy, coconut, tea and rubber plantations are the major crops which cover major portion of the agricultural lands. Rice, which is the staple food, is cultivated in every part of the country during the maha (rabi) season (September to March). However in yala (kharif) season (April to August), it is limited due to unavailability of canal water.

Based on ecological parameters such as rainfall, soil type and topography, Sri Lanka is divided into three major agro-ecological zones namely wet zone, intermediate zone and dry zone (Panabokke 1996). The wet zone is in the southwest quarter of the island where annual rainfall is received during the southwest monsoon period as well as the two inter-monsoon periods, totalling about 3500 mm per year (Samarasinghe 2003). Coconut plantations are confined to low lands in wet and intermediate zones and rubber is primarily in the wet zone.

In Sri Lanka, tea plants are grown as a rain-fed perennial crop which cover approximately 222,000 ha of land. It is the foremost plantation crop which is extensively growing in hill country. However, tea is grown at the altitude range of 0–2,500 m amsl, which fulfils the required minimum rainfall of 1,200 mm per year, but 2,500–3,000 mm per year is considered as optimum (Carr 1972; Squire and

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Callander 1981; Watson 1986). Therefore, tea plantations are distributed in up, mid and low-country regions, belonging to wet and intermediate zones.

Tea production is classified based on its growing regions, such as high, medium and low-grown tea. However, the quality or flavour of each category is unique. Most distinguishable up country tea is characterized by its flavour and aroma while low-grown tea is identical for its strength and colour. In terms of production, low-grown tea dominated over others (173.2 million kg in 2009) followed by high (72.3 million kg) and medium (44.3 million kg) grown tea. Economically, the tea sector is important and contributes about 1 % to the GDP. However, the present average yield of 1,550 kg per hectare is much lower than the average yield of other tea-growing countries like India (1,800 kg/ha), Kenya (2,400 kg/ha) (Annual report 2009).

According to the central bank report (2009) in Sri Lanka, the major reason for the lower productivity is due to senility of the tea bushes. Furthermore, in high-grown areas, around 90 % of tea bushes are more than 100 years old and in low-grown areas, the majority of tea bushes of vegetatively propagated (VP) varieties are older than 30 years. Therefore, main emphasis was laid on replanting programmes to achieve minimum of 3 % replanting per year. However, identification of low-productive fields and suitable land unit for such programme is not straight forward. Therefore, it is vital to have proper scientific basis for the rational decision-making at management level. GIS and remote sensing has proved to be a handy tool to infer land productivity and the suitability.

Remote sensing and GIS technology has advanced over the last few decades and one of the most important applications of it is the database management, to store information for quick access and analysis. GIS data have specific location in space;In other words, those spatial data have been referenced to a co-ordinate system. It is called geographically referenced. It was also possible to store non-spatial or attributes data in such databases. Therefore, GIS is able to store large amounts of different types of data for easy access. Furthermore, the spatial data can be combined with attribute data to generate new information in the decision-making process. Hence, tea plantations where the spatial distribution is massive, use of GIS enhances the ability of rational decision-making process. Turner and Jayakody (1994) emphasised the usefulness of computer-based technology as a tool for planning and managing tea plantations. Because, computer technology is capable of generating required information at its earliest to the higher management levels. However, the success of such a system heavily depends on accuracy of raw data, hence it is essential to have remote sensing with intensive ground-level truth to validate the acquired data. Since 70s, remote sensing technology has proved to be a useful application for evaluating biological activity of vegetation cover. In recent past, spectral data have been widely utilized for crop yield models. Further, it has been recognized as an inexpensive and rapid method for crop monitoring and early warning.

Therefore, this study was designed in a way to investigate the probability of using remote sensing and GIS as management tools for extensive tea plantations in Sri Lanka. However, considering the limitations, the study was limited to Ratnapura

district which is considered as one of the main tea growing districts in the country. Hence, research was conducted with following objectives:

- Application of remote sensing to infer the potential productivity of tea at synoptic and micro level landscapes.
- Potential of using remote sensing as tool for yield prediction and early warning system tea plantations.
- Application of hydrogeomorphology base to study the potential yields of tea plantations.

1.1 Study Area

Sri Lanka lies in the Indian ocean between longitudes 79° 39 and 81° 53′ East and latitudes 5° 54 and 9° 52′ North. It covers a total of 65,609.8 Km^2 area. Geologically, Sri Lanka is located in the South-Asian Tectonic plate and 90 % of country has underlying Precambrian crystalline rocks (Ganashan 1996). The metamorphic basement has been subdivided into three major units, namely the Vijayan Complex in the east, the Highland Complex in the central and the Wanni Complex in the west. Ratnapura, which is the area of interest for this study, is covered by Highland Complex, and is composed of pelitic, mafic and quartzo-feldspathic granulites, abundant charnockitic rocks (Kehelpannala 1997).

Climate condition of the country is basically determined by the geographical location. The mean temperature is 27.5 °C over low lands. However, in higher elevation mountain regions, the mean monthly temperatures vary from 13 to 16 °C. Most parts of the country experience only a small variation in mean monthly temperatures throughout the year. The relative humidity varies generally from about 70 % during the day to about 90–95 % at night.

The two monsoonal periods, the southwest (May-September) and the northeast (December -February) are responsible for major part of the annual precipitation. South western quarter and the central highlands of the country get mostly southwest monsoon rain while north east monsoon produces rain throughout the island. Based on the mean annual rainfall, the country is classified into three major climatic zones: Dry zone (1,250–1,525 mm), Intermediate zone (1,525–2,280 mm) and wet zone (2,280–5,100 mm) (Ganashan 1996).

In the dry zone, the major soil group is the well-drained reddish brown earths. The wet zone is characterized by predominant red yellow podzolic soils with bog, half bog soils, and sandy regosols along the southwest coast. The intermediate zone displays a transition from reddish brown earths to red yellow podsolic soils, with non-calcic brown loam in patches (Panabokke 1996).

Agriculture sector can be divided into two main categories, plantation agriculture and non-plantation sector. About 1.8 million farm families are engaged in non-plantation sector which are characterized by small-scale rice-based farming under rainfed and artificial irrigation. However, plantations involve with largescale cultivation of perennial crops such as tea, rubber, coconut, etc.

1.2 Ceylon Tea

The tea plants (*Camellia sinensis* (L.) O. Kuntze) which belong to the genus camellia probably originated in China. First record of tea dates back to 350 BC in a Chinese dictionary, however, according to Cha Pu, tea drinking started only in the 6th century. The habit of tea drinking later spread to Japan, Portugal, Holland and Europe. In 1834, the British started the cultivation of tea in their colonies. As a substitute crop for the dreaded coffee in Sri Lanka, tea was introduced in 1867 by James Taylor. However, tea was first planted in Royal Botanical garden in Peradeniya way back in 1839. After the experimental plantings, first tea plantation Loolecandera Estate was established in Kandy district (Nathaniel 2008).

One of the oldest tea producing country, Sri Lanka produces predominantly black tea (about 95 %) and is well known as "Ceylon tea". It is ranked among the best tea in the international market. In recent past, Ceylon tea was categorized as world's cleanest tea considering its low pesticide residues, therefore the brand name of Ceylon, guaranteed the quality. According to Ziyad and Zoysa (2008), tea production in the country grew at an average annual rate of about 10 % during last decade and at present the production is 315 million kg. Low-country (0–600 m amsl) tea production is significant and it contributes about 60 % to the total tea production of the country. The rest two regions, mid-country (600–1,200 m amsl) and upcountry (>1,200 m amsl) contribute about 16 and 24 %, respectively. However, 53 % of the estate lands are under old seedling tea and rest 47 % under VP tea. About 90 % of the seedling tea was older than 60 years. Hence, overall productivity of the country remains less compared to other tea producing countries (Anon 2003).

The yield of tea is from the tender young shoots comprising either two or three leaves with a terminal bud. Tea crop is determined by the shoot population density, size of the shoots and the rate of shoot extension. Hence, these factors are widely studied by many scientists to determine the yield (Wjeratne 1996; Odhiambo et al. 1993; Tanton 1992, 1981). However, the number of harvestable shoots per unit area and mean dry weight of single shoot is largely responsible for the tea yield.

Studies have been carried out by several scientists to demonstrate effects of different environmental components on yield. Balasuriya (1999) showed the effect of mean air temperature on shoot development, Wijeratne (1996) exposed the effect of saturation vapour pressure deficit, temperature and soil moisture deficit on shoot population. Apart from environmental factors, crop management practices and environment stresses (biotic and abiotic) affect the agronomic yield or total weight of harvested crop. Thus, yield is a multivariate function and hence, estimation of yield is a puzzling task. However, it is essential for the decision-making process at different levels.

1.3 Primary Production and Yield

Primary production is the process which converts light energy or solar radiation into chemical energy. Basically, green plants are called primary producers because they

are capable of producing primary energy through the process of photosynthesis. However, a fraction of the total production or Gross Primary Production (GPP) is utilized by the primary producer to meet their respiratory demand and balance is Net primary production (NPP), which accumulates as plant biomass (Odum 1960; Whittaker and Likens 1975). NPP is one of the most significant characteristics of ecosystems. However, estimation of NPP in an ecosystem is one of most confusing assignment because it depends on multiple variables such as light, temperature, soil moisture, plant nutrients, plant characteristics, etc. However, in agriculture not only NPP but mostly the agronomic yield, the fraction of NPP which assimilates in harvestable components such as seeds, tubers, leaves, is important.

Though it is simple in concept, in terrestrial ecosystems accurate estimation of NPP is difficult. Measurement based on gas exchange, sampling biomass or carbon flux methods have been devised to measure NPP (Milner and Hughe 1968). However, remote sensing of vegetation indices, such as normalized difference vegetation index (NDVI), promises to provide a means for frequent, non-destructive measurement of NPP at a landscape scale (Tucker and Sellers 1986; Wang 2004).

2 Remote Sensing

With remote sensing technology, vegetation can be inferred through its reflectance signals which are within the visibility and are near-infrared (NIR). In general, green vegetation canopies have a unique feature of reflectance spectra among the green, red, and NIR bands (Fig. 4.1). Green reflectance peak due to chlorophyll, causes peak reflectance in the green band and reflection valleys in the red band. However, leaf tissue gives maximum reflection in NIR band. Hence, this range of reflection spectrum explains the reflection and absorption of green and red bands by the vegetation and the variation can be used as a tool for studying distribution, health and productivity of plants (Buschmann and Nagel 1993). Basically, the shift of the reflection spectrum from the far red towards the near-infrared (red edge) is taken as an indicator of stress or damage to plants (Gates et al. 1965; Chang and Collins 1983). However, the spectral characteristics of a plant canopy largely depend on the composite spectral response of leaves and soil background (Richardson and Wiegand 1977).

As mentioned elsewhere, remote sensing is a widely used tool in studying vegetation. In 1972, Monteith pioneered the concept of calculating NPP based on average photosynthetically active radiation (APAR) and this production efficiency model was tested by using satellite-based and ground data. First global level NPP model was proposed by Hemimann and Keeling in 1989. However, a majority of the satellite-based yield prediction studies are built on correlating yield to various crop parameters like leaf area index, wet biomass, light use efficiency, etc., which are retrieved using various vegetation indices like the Normalized Difference Vegetation Index (NDVI) (Groten 1993).

2.1 Vegetation Indices (VI)

As already mentioned, remote sensing methods provide valuable tools for crop canopy assessment and these tools will provide improved information for agriculture applications. The main advantage of remote sensing is the ability of repeated measurements over the time without damaging the vegetation. Therefore, in remote sensing methods, various VIs are used to generate important information to enhance assessment of different agronomic parameters. However, the purpose of vegetation indices is to enhance the vegetation signal while minimizing the solar irradiance and soil background effects.

The ratio of the near-infrared (NIR)/Red was the first VI which was proposed by Jordan in 1969. He relates this index to Leaf Area Index (LAI) of plants. As mentioned earlier, NDVI is the most commonly used VI which is calculated as the difference between near infrared and red reflection normalized by the sum of the two

$$[\mathrm{NDVI} = (\mathrm{IR} - \mathrm{R})/(\mathrm{IR} + \mathrm{R})].$$

Basically, the wavelength region from 750 to 850 nm is considered as near-infrared (NIR) and 625–675 nm range is considered as red(R) (Sellers 1985; Tucker and Sellers 1986). However, major problem in vegetation studies is reflectance from soil; hence Soil-Adjusted Vegetative Index (SAVI) was described by AR Huete in 1988.

$$\mathrm{SAVI} = (\mathrm{R_{NIR}} - \mathrm{R_{red}})\,(1 + \mathrm{L})/(\mathrm{R_{NIR}} + \mathrm{R_{red}} + \mathrm{L})$$

Adjusted parameter L is typically 0.5. However, this index was further advanced to derive an Enhanced Vegetative Index (EVI).

$$\mathrm{EVI} = 2.5(\mathrm{R_{NIR}} - \mathrm{R_{red}})/(\mathrm{R_{NIR}} + 6\mathrm{R_{red}} - 7.5\mathrm{R_{blue}} + 1)$$

Enhanced vegetative index uses reflectance in blue region of the spectrum. According to Hatfield et al. (2008), this is important to differentiate soil from vegetation at low amount of ground cover. Among the various VIs, assessment of the chlorophyll content of crop canopies has included the Normalized Pigment Chlorophyll Ratio Index described in Merzlyak et al. in 1999 and it was defined as (Red 660 – Blue 460)/(Red 660 + Blue 460). Apart from those indices, Plant Senescence Reflectance Index was proposed as being sensitive to the senescence phase of plant development. Renormalized Difference Vegetative Index (RDVI), Modified Triangular Vegetation Index (MTVI) and Optimized Soil-Adjusted Vegetation Index (OSAVI) are also use to study crop yield.

As mentioned earlier, yield is a multivariate function; factors like different soil types, agricultural inputs and adoption of improved technology affect it, since the spectral reflectance is a manifestation of all important factors affecting the crop. Therefore, stratification of crop area on the basis of crop vigour as reflected

by the spectral data are expected to result in greater efficiency in the crop yield estimation.

2.2 Application of GIS and Remote Sensing in Tea Plantations

Even though remote sensing has been widely used for forecasting the yield of different types of crops, understanding of the spectral characteristics of tea plantations is very important for monitoring the growth of plants and estimating harvested leaf yield. Spectral characteristic of tea estates varies depending on age of tea canopy, type of tea 'jat', and health of tea plantation.

To facilitate convenient height for harvesting, stimulate vegetative growth, maintain healthy frames andoptimize resource utilization. Tea bushes are required to be pruned at regular intervals. The time period between two consecutive prunings is known as pruning cycle. However, the length of the pruning cycle varies according to the location of the estate and type of tea. The pruning cycle of seedling tea can be two, three or four years corresponding to low-country, mid-country and upcountry. Pruning cycle of vegetatively propagated tea is three, four and five years for low-country, mid-country and upcountry, respectively. Generally, most tea plantations show relatively similar spectral characteristics such as higher absorption in red and large reflectance in NIR bands. However, spectral characteristic of tea also depends on its canopy structure, size of the leaves, greenness and maturity of leaves, hence tea plants with different pruning cycles give different spectral signatures (Rajapakse et al. 2002).

According to Rajapakse et al. (2002), the reflectance of clonal and seedling tea depends on tea canopy structure, size of the leaves, greenness and maturity of leaves. However, in general most of clonal and seedling tea types display relatively similar spectral trend. One important factor is canopy age which is calculated from its last pruning and according to Rajapakse (2002) younger plants absorb light between 600 and 625 nm whereas older plants with matured leaves absorb more light at 650 nm. However, the variation depends on tea clones but both younger clonal and seedling tea have low reflectance in NIR (800–1,050 nm) than mature canopy. Therefore, tea plants show different reflectance spectra according to their canopy age and tea clone.

Computer technology has advanced over the last few decades and one of the most important applications of it is the database management, where computers are used to store information for quick access and analysis. GIS data have specific location in space; In other words, those spatial data have been referenced to a coordinate system. It is called geographically referenced. It is also possible to store non-spatial or attribute data in such databases. Therefore, GIS is able to store large amounts of different types of data for easy access. Furthermore, the spatial data can be combined with attribute data to generate new information in the decision-making process. Hence, tea plantations where the spatial distribution is massive, use

of GIS-based information system enhances the ability of rational decision making process. Turner (1994) explained the application and benefits of GIS in tea plantations as a management tool.

3 Study Area

This study was conducted in Ratnapura district of Sri Lanka, which lies between 6040′51.02″ N 80023′42.27″ E and 6 43′ 23.83″ N,80022′10.61″ E latitude and longitude of the upper left and lower right corners respectively. The area primarily belongs to two major agro ecological zones, namely wet and intermediate zones. However, based on rainfall, elevation and soil factors, it is further divided into seven agro ecological sub-zones belonging to low-country and mid-country. On an average, it records 200 wet days and receives more than 3,000 mm of annual rainfall (Annual report 2009). Geologically, area is covered by Precambrian metamorphic rock named as highland complex which is dominated by charnokites and quartzite which supports the tea production (Fig. 4.1).

With respect to total tea area, Ratnapura district has the maximum area under tea cultivation, about 31,116 ha. However, about 75 % of the tea lands in the area belong to small holders (Panabokke et al. 2008). Further, Ratnapura records higher annual tea productivity, 2,159 kg/ha of tea, after Kalutara and Gall districts (Anon, 2003). Basically, tea plantations in the area are of two types, commonly named as seedling and vegetatively propagated tea or clonal tea. Both tea types are harvested throughout the year, however irrespective of the regions, vegetatively propagated tea gives significantly higher yield than seedling tea in the country. Hence, in the recent past many of the seedling tea gardens were diversified to clonal tea and as a result seedling tea cultivation is now confined to only a few estates in the region.

Tea plantations in Ratnapura area are further classified into mono-cropping, and mixed cropping with rubber, coconut and other perennial crops. However, use of coconut and minor export crops dominates over other intercropping types. Even though tea is stated to be a mono-crop, planting of low and high shade trees is a common practice (Fig. 4.2).

Panabokke et al. (2008) classified the tea-growing area into four main productivity zones, based on corporate estate sector tea yield. However, small-scale tea productivity studies, which are essential for the appropriate land use planning are limited. Basically, such information is important when making a decision on new planting, replanting, infilling and crop diversification. Furthermore, it plays a significant role in optimizing resource use efficiency and sustainable tea production. Therefore, formulation and accessibility to productivity information in both macro and micro levels is significant in various levels of decision making. As mentioned elsewhere, remote sensing and GIS are handy in this scenario and this study basically focuses on generating information in micro scale.

The data used in this study were derived from a range of sources, including satellite images, and field collected data and secondary data such as rainfall data, yield

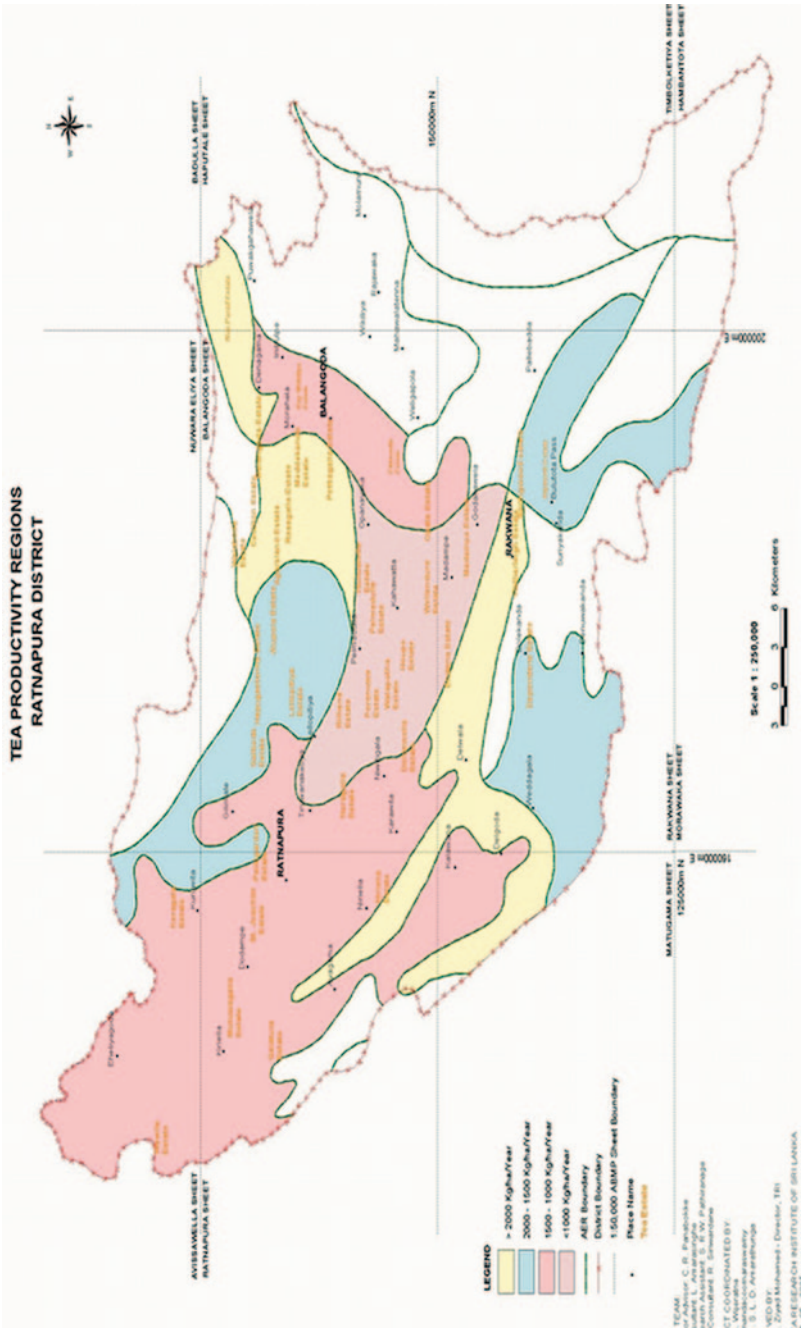

Fig. 4.1 Location of Tea productivity area the study area

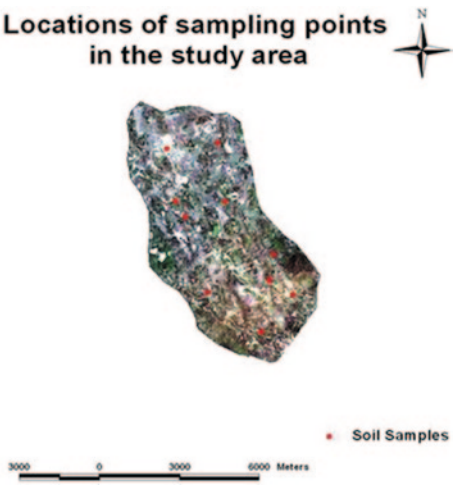

Fig. 4.2 Location of sampling points in the study area

data and cartographic maps. The satellite data set used in this study consists of six images of Landsat TM with the spatial resolution of 30 m. Field data and geo-referenced tea plantation maps which were prepared by Panabokke et al. (2008), were used to identify the existing tea fields in satellite images. Spectral signatures of tea plantations were studied in detail to identify their spatial and temporal variation.

3.1 Sampling and Analysis

Approximately 50 km^2 area was selected as area of interest (AOI) which includes two tea estates, namely St. Joachim and Palm garden. The area was demarcated by including those two estates with small holder's tea gardens. Yield data were obtained from the two estates for a period of past 10 years for the analysis. Soil and groundwater samples were collected from non-agricultural lands within the same area. Soil samples, up to 45 cm depth were taken by excluding top soil and groundwater samples were taken from dug wells. Polypropylene bottles were used to keep acidified (few drops of 2 % HNO_3 was added to water samples while sampling process and those samples were used to metal analysis) and non-acidified water samples, other than soil and groundwater samples, water samples were collected separately from the River "Kaluganga". Properly labelled samples were then brought into the laboratory for further analysis (Fig. 4.3).

3.2 Image Analysis

The imageries for different years were downloaded from www.glovis.usgs.org, WIST (Warehouse Inventory Search Tool) and GLCF (Global Land Cover Facility).

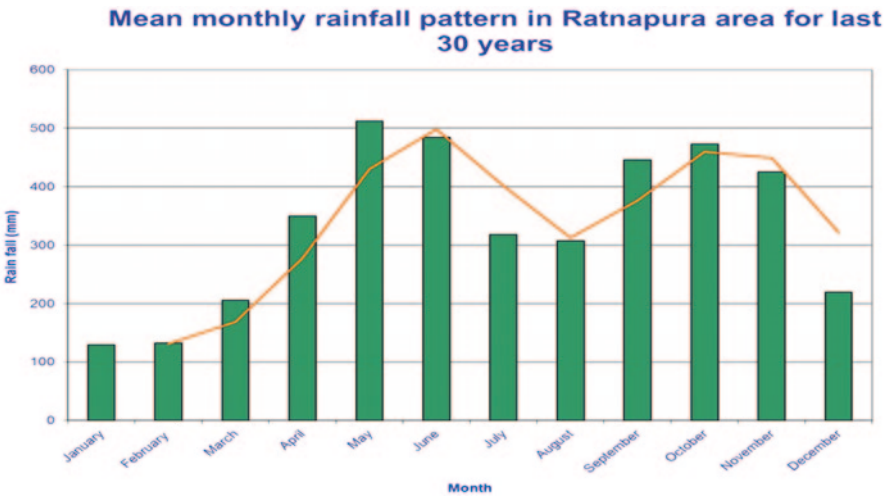

Fig. 4.3 Mean monthly rainfall pattern in Ratnapura for last 30 years. (Source: TRI)

The google earth image provided a good ready reference for the work. Various analyses like NDVI analysis, supervised classification analysis was done on the image using following softwares:

- ArcView GIS 3.2a
- ArcGIS 9.3
- ERDAS IMAGINE 8.5

Statistical analysis of the data for grain size was done using SIGMAPLOT software as an extension of MS EXCEL. Besides all this softwares, handheld Garmin GPS was used to locate the sampling location by acquiring latitudes and longitudes of that place.

4 Result and Discussion

Red yellow podzolic (Rhodudults/ Tropudults) is the major soil group found in Ratnapura area which is characterized by the loam to sandy loam texture at surface and sandy loam clay in subsoil. Furthermore, soil shows higher level of gravel and laterite formation. Generally, all collected soil samples show more than 97 % of sand fraction and none of the sample yielded more than 1.5 % clay or silt. Therefore, soil of study area is classified as sandy soil (Fig. 4.4).

Soil organic matter content in the study area is ranging from 0.5 to 3.7 %. However most part of the study area shows less than 2 % of organic matter. Bringing a virgin soil into agricultural use tends to destroy much of soil organic matter (Tolhurst 1961), hence actual organic matter content in tea fields is expected to be low.

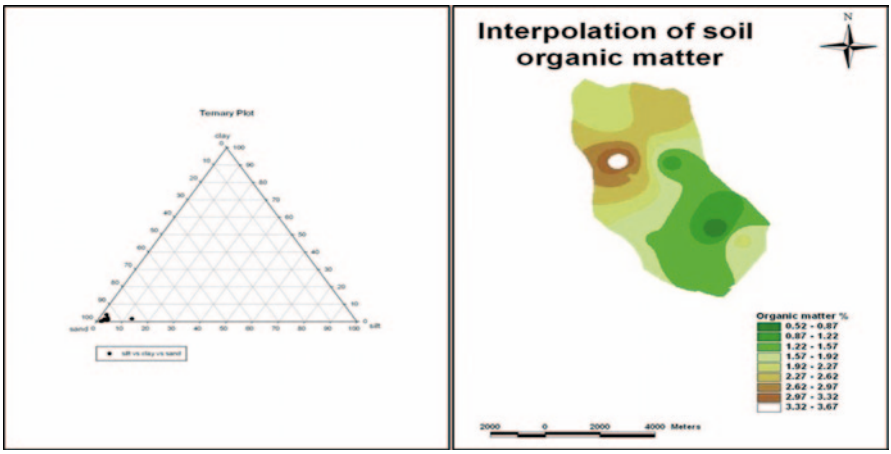

Fig. 4.4 Ternary plot of soil texture and interpolation of soil organic carbon

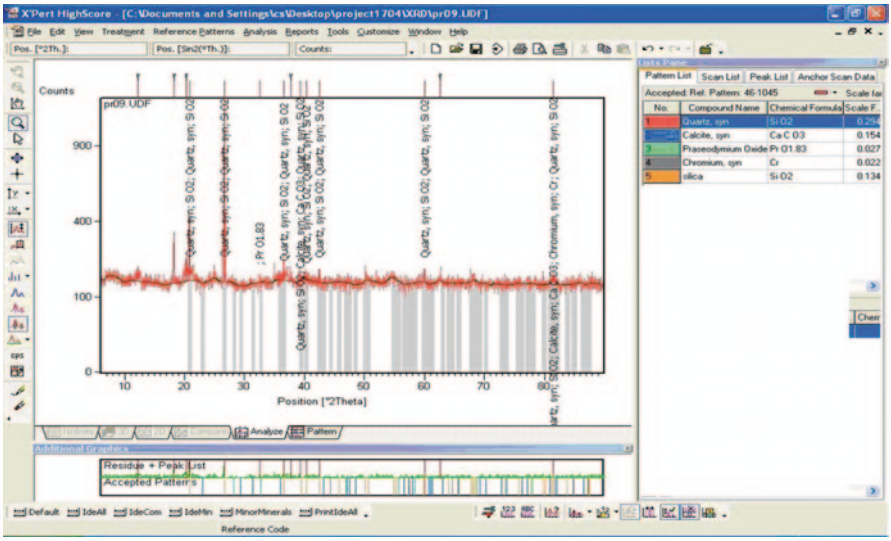

Fig. 4.5 Analysis of soil minerals composition by XRD

The bedrock is characterized by metamorphic rock belonging to highland series which is dominated by charnokites, quartzites, marbles, garnetiferous gneisses and granulites mixed with igneous intrusions (Cooray 1994; Chandrajith 1999). Soil of the study area is dominated by Quartz (SiO_2), Hematite (Fe_2O_3), Calcite ($CaCO_3$), with trace amounts of uranium oxide (UO_2), Bornite (Cu_5FeS_4), Praseodymium oxide ($PrOIB_3$) and Green Cinnabar (Cr_2O_3) minerals (Fig. 4.5).

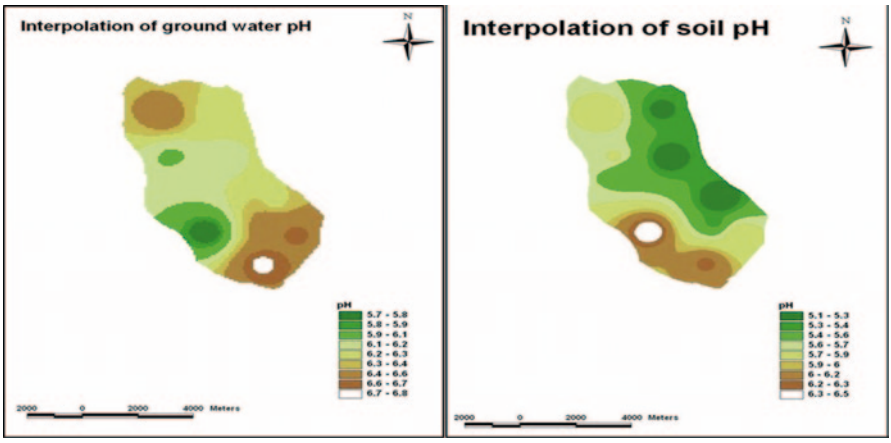

Fig. 4.6 Interpolation of groundwater and soil pH

Basically, Ratnapura belongs to low-country wet zone which receives more than 3,000 mm annual rainfall. It causes extensive leaching of basic cations from the top soil to make it acidic. However, groundwater and soil are slightly acidic in the area where the pH is ranging from 5.7 to 6.8 and 5.1 to 6.5, respectively (Fig. 4.6).

Tea is better grown in slightly acidic soils where growth is optimum at 4.5–5.5 pH range. Considering the fact (optimum pH), major part of the study area which shows soil pH less than 5.7 is therefore suitable for tea plantations. Interpolation of water and soil electric conductivity is given in Fig. 4.6.

Calcium and magnesium are macronutrients which are required in large quantities for better plant growth and productivity, hence, tea yield shows positive correlation to dolomite and kieserite application (Krishnapillai 1991). Sivapalan and Krishnapillai (1988) reported that tea growing soils contain lower Mg concentrations. Furthermore, this is claimed as a possible limiting factor for better yield. Therefore, general recommendation was to add Mg-enriched fertilizer mixtures, particularly when soil pH is 4.2 or low. Mg in the study area is found in the interpolation plate 14. According to this, Mg concentration is less than 4.1 ppm in major part of the study area. Compared to upcountry tea, Mg concentration is higher in low-country tea (Hasselo 1965). Soil Ca concentration in the study area ranges from 8.4 to 28.8 ppm. Typically, Ca concentration in fresh water falls below 10 ppm (Ramesh 1996). The groundwater Ca concentration in the study area is at its lower side, where major part of the area shows less than 4 ppm. However, it was in the range from 0.3 to 11.9 ppm. So, it can be inferred that carbonate rocks like limestone, dolomite, etc., are present in SE part of the study area, precisely in the Palm Garden Estate. Also, anomalies of soil Mg and Ca concentration account for high fertilizer input in those regions.

Soil pH and texture play a significant role in Zinc (Zn) availability. Alkaline and coarse textural soils with low organic matter content are marginal in Zn. The Zn deficiency is basically observed all over the country and the affected plants exhibit

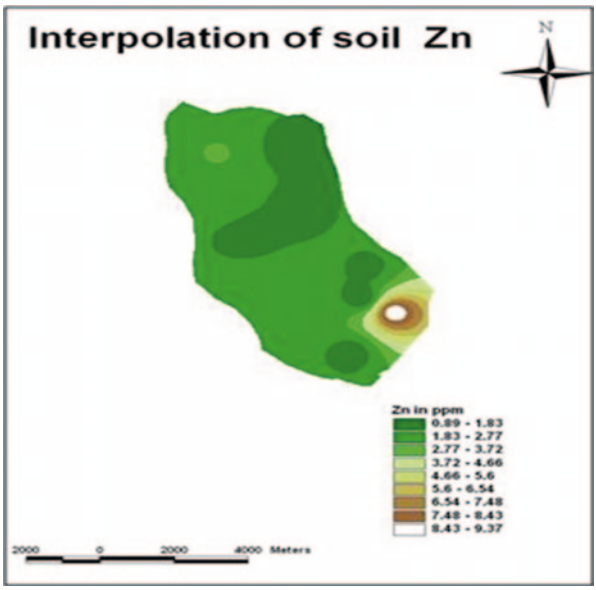

Fig. 4.7 Interpolation of soil Zn

chlorosis, small sickle-shape leaves, clustered at the tips of shoots, etc. However, Tolhurst (1961) reported that deficiency is prominent in low-country. Tolhurst's observation can be justified with Hasselo's (1965) one, according to which Zn concentration in upcountry flush is almost double compared to low-grown tea.

Though Zn level in groundwater is insignificant, its concentration in soil is significant. According to Hettiarachchi and Gupta (2008) and Tolhurst (1961), Zn is identified as promising, yield-responding nutrient. Therefore, application of Zn as a foliar spray was recommended. As seen from the analysis, in most part of the study area concentration of Zn is well above 1 ppm and it is quite sufficient for plant growth (Fig. 4.7).

Lead concentration in ground water is ranging from 0.07 to 0.11 ppm. However, maximum permissible limit for lead in drinking water is 0.05 ppm. Higher level of lead in groundwater might be due to intense gem mining activity in the area. However, further investigations are required for a proper conclusion.

In most part of the study area, manganese concentration in groundwater exceeds the permissible limit of 0.05 ppm. Soil and groundwater manganese concentrations are ranging from 0.01 to 0.3 ppm and 0.66 to 2.78 ppm respectively. Availability of manganese is directly related to soil pH and aeration. In highly alkaline soils, Mn deficiency is common and in highly acidic soils its concentration goes high turning toxic for plants. Considering the tea plant nutrient, symptoms of Manganese toxicity were reported from low-country frequently during the dry weather condition (Hettiarachchi and Gupta 2008). Furthermore, the toxicity is characterized by the leaves chlorosis with pronounced green network of the veins. According to Tolhurst (1973), higher level of N application increases the content of Mn in leaves. Accord-

ing to Hasselo (1965), low-country tea plants contain, on an average, higher amount of Mn as compared to tea plants in upcountry. Therefore, Mn in soil and groundwater is a significant factor which can influence tea productivity.

Nitrogen and phosphorus are the major nutrients absorbed by plants. A significant part of the study area shows soil nitrate concentration above 18 ppm. Nitrate content in groundwater is ranging from 0.7 to 40 ppm in the area. Hence, water is safe for all domestic uses. Concentration of soil nitrate is ranging from 6.03 to 54.24 ppm, however, mostly it is over 18 ppm, and hence it is a favourable factor for plant growth.

Most part of the study area shows less than 119 ppb of soil phosphate. Similarly, it is less abundant in water where it ranges between 0.02 and 873 ppb. However, according to Hasselo (1965) both nitrate and phosphate concentration in low-grown tea is higher than upcountry. Therefore, there is no inherent limitation of those two elements in the area. However, proper nutrient management is required to ensure sustainable yield. Concentration of dissolved silica in groundwater was reported as an indicator to understand the weathering characteristic of basement rock. Therefore, it has been used in various geochemical studies to evaluate the factors affecting chemical weathering rates in a natural environment. However, groundwater mostly shows less than 6.5 ppm dissolved silica. Hence, it indicates the weathering resistance of metamorphic basement rocks. Bicarbonate is a significant anion in groundwater chemistry predominantly present at slightly acidic pH. Bicarbonate ion distribution in the study area is highly correlated with water pH and higher bicarbonate concentration was recorded in areas where groundwater pH is greater than 6.5.

No result was observed for Cu, Ni, Fe and Zn in groundwater at the concentration range of mg/L or ppm. However, soil analysis shows satisfactory result at same concentration ranges. All elements show positive trend toward southward direction where Ratnapura town is located. However, Cu is showing inverse trend of which low concentration was observed in Southern part of the study area. Therefore, higher concentration of elements might be due to anthropogenic influences like mining, which is prominent in SW part near to Kaluganga River.

4.1 Supervised Land Use Classification

Supervised land classification was done in order to identify the areas which show common reflectance characteristics. Based on the reflectance value correlated with the Google earth image, a signature file was prepared for water bodies, paddy fields, vegetation (other than tea and paddy), constructions (built up areas), mature and young tea. Then, land use images were prepared using AOI for years 1992, 2002, and 2005 to identify the changes in land use and land cover over the period (Fig. 4.8).

According to the 1992 AOI image, total extent of paddy is 750.33 ha. However, according to supervised classification paddy extent increased to 770.76 ha in 2002

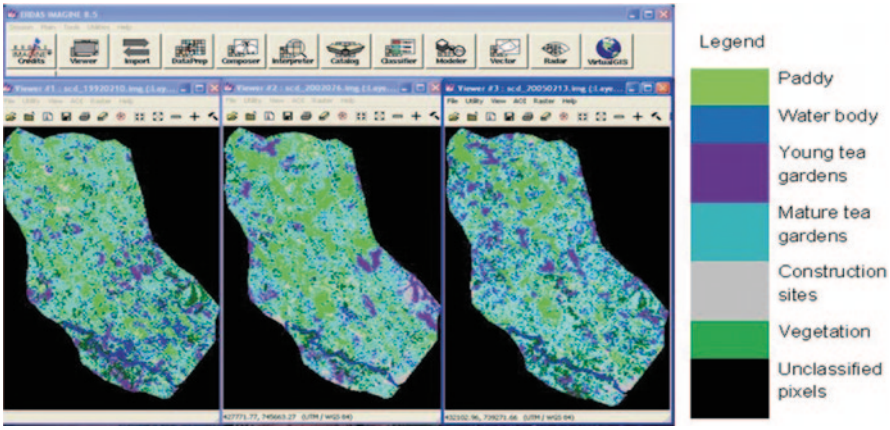

Fig. 4.8 Supervised land classification of satellite images for years 1992, 2002, and 2005

Table 4.1 Land use classifications of 1992, 2002, and 2005 for AOI

	Number of Pixels			Area (ha)		
	1992	2002	2005	1992	2002	2005
Paddy	8,337	8,564	7,700	750.33	770.76	693
Vegetation	5,970	4,269	6,656	537.3	384.21	599.04
Mature tea	22,808	24,103	21,209	2,052.72	2,169.27	1,908.81
Young tea	5,082	4,538	8,709	457.38	408.42	783.81
Construction	4,944	6,825	4,397	444.96	614.25	395.73
Water body	244	1,266	894	21.96	113.94	80.46
Total	*47,385*	*49,565*	*49,565*	*4,264.65*	*4,460.85*	*4,460.85*

and decreased to 693 ha in 2005. Reduction of total pixels of paddy field might be due to land filling, heap up of mining ore or fallowing of paddy fields. However, to reach a proper conclusion, high resolution satellite image like LISS-IV is required. Fluctuation in water bodies over the period might be due to temporal changes of weather. However, an area of 21.96 ha which was recorded in 1992, represents the perennial water bodies and the subsequent fluctuations are due to accumulation of water in seasonal water bodies.

Construction sites, which were characterized by open land with or without man-made structures, were observed. This area for the year 1992 is 444.96 ha. However, it shows significant increase over a period of 10 years and its extent in 2002 was observed to be 614.25 ha. Actual construction site, land clearing for agricultural activities and pruning of existing tea fields can yield similar reflectance. Therefore, the effect can be a short-term influence. However, further studies are required to explain the anomaly in 2005 (Table 4.1).

Total pixel area belonging to tea cultivation is 2,510.1, 2,577.69, and 2,692.62 ha in 1992, 2002, and 2005, respectively. However, according to 2005 census report, total tea extent in this division is 4,726 ha. AOI is only a fraction of Ratnapura division and therefore supervised classification overestimated the tea extent. Similar

Table 4.2 NDVI values for different tea fields representing St. Joachim and Palm garden estates

Sample	19920210	1992313	2001209	2002	2005	20060123	2006303
1	0.232	0.15	0.292	0.403	0.381	0.188	0.312
2	0.288	0.237	0.283	0.385	0.348	0.153	0.303
3	0.267	0.175	0.171	0.175	0.583	0.297	0.6
4	0.333	0.537	0.226	0.443	0.641	0.184	0.597
5	0.333	0.276	0.27	0.343	0.4	0.171	0.528
6	0.314	0.414	0.333	0.267	0.406	0.213	0.541
7	0.218	0.158	0.359	0.113	0.491	0.246	0.291
8	0.461	0.333	0.395	0.314	0.562	0.3	0.231
9	0.472	0.451	0.212	0.37	0.407	0.193	0.38
10	0.307	0.562	0.264	0.3	0.464	0.18	0.218
11	0.377	0.597	0.22	0.429	0.455	0.226	0.338
12	0.355	0.296	0.26	0.388	0.394	0.207	0.534
13	0.295	0.304	0.281	0.237	0.417	0.252	0.48
14	0.358	0.327	0.236	0.365	0.476	0.161	0.592

type of pixel misclassification was observed by Samarasinghe (2003). Therefore, pixel values of rubber, coconut or home gardens also need to be studied intensively in order to classify actual tea estates in synoptic scale.

4.2 NDVI analysis

Normalized difference vegetation index maps for 1992, 2001, 2002, 2005, and 2006 were generated from satellite images. (Plate 34) Representative 7 tea fields from each estate (St. Joachim and Palm garden) were identified and NDVI values were taken (Table 4.2). Annual yield of tea estates was then correlated with NDVI values. The correlation was insignificant with annual yield, hence further studies are necessary with short-term (monthly or weekly) yield data. As it can be broadly seen from NDVI images as to which area is having green cover and which is fallow, it offers a promising tool to evaluate the yield of different seasons. Hence, further refinement of pixel-to-yield correlation is necessary which is better done with high resolution imagery (Fig. 4.9).

5 Conclusion

Soil samples and groundwater samples were collected from the area of interest which is located in a district of low-country wet zone. The following conclusion can be drawn out from the results that were obtained.:

1. Soil is dominated by sand fraction and higher gravel content.
2. Further, quartz, calcite and hematite are the major minerals in the soil samples.

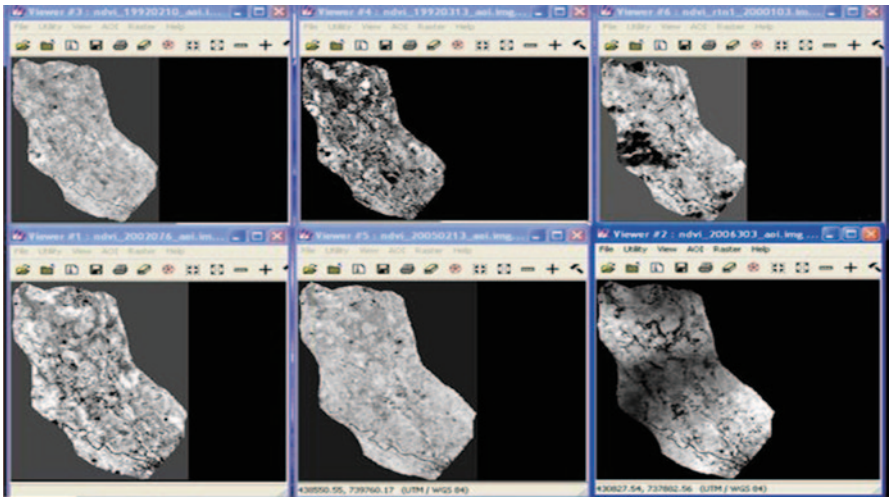

Fig. 4.9 NDVI images for 1991, 1992, 2001, 2002, 2005, and 2006

3. However, concentration of Mn in soil as well as groundwater is significantly higher and has higher potential to influence human and plant health. Furthermore, Hettiarachchi and Gupta (2008) observations of Mn toxicity of tea plantations in the area, especially during the dry weather conditions justify the result.
4. Lead concentration in groundwater is significant. Even though, heavy metals such as Cu, Ni, Fe and Zn in groundwater are not significant.
5. According to supervised classification, reductions of paddy fields were observed over a period of 13 years starting from 1992 to 2005. On the other hand, total vegetation cover has increased. However, pixel overlapping limits the application of remotely sensed data for the tea plantations.
6. Further relationship between annual yield and NDVI was not significant and therefore further studies are required to identify the relationship between reflection characteristic of tea plantation with its yield. Though NDVI is unable to explain the annual yield data, it is important to investigate its potential for shorter timeframes.

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Chapter 5
Polyamines Contribution to the Improvement of Crop Plants Tolerance to Abiotic Stress

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1 Introduction

Polyamines (PAs) are aliphatic biogenic amines present in most Prokaryotes and all Eukaryotic organisms (Takahashi and Kakehi 2010; Fuell et al. 2010). These small molecules are essential for life. At physiological pH, PAs are found as protonated, positively charged molecules containing two (diamine), three (triamine) or four (tetraamine) amine groups, what favors their electrostatic interaction with several macromolecules such as nucleic acids, proteins and lipids (Igarashi and Kashiwagi 2000; Childs et al. 2003). The polycationic nature of PAs is one of the most important properties linking these natural compounds to several cellular and physiological processes, and new connections between PAs and other molecules, revealing new insights into the PA biological role are being continually discovered.

At the cellular level, PAs participate in diverse fundamental processes such as transcription, translation, DNA replication, chromatin condensation, cell signaling, cell division and differentiation, senescence and cell death. In addition, diverse roles in membrane stabilization, ion channel regulation, cation-anion balance, modulation of enzyme activities, and protein modification have also been described (Childs et al. 2003; Shabala et al. 2007; Handa and Mattoo 2010).

In plants, PAs are present from micromolar (~10 μM) to millimolar concentrations (Galston and Sawhney 1990). The most common PAs are spermidine (Spd; $NH_2(CH_2)_3NH(CH_2)_4NH_2$), spermine (Spm; $NH_2(CH_2)_3NH(CH_2)_4NH(CH_2)_3NH_2$) and their obligate precursor putrescine (Put; $NH_2(CH_2)_4NH_2$). Spd is structurally an unsymmetrical molecule that can be aminopropylated at each end, forming either

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Spm or thermospermine (tSpm) (Knott et al. 2007). In plants, PAs distribution differs among tissues and developmental stages, being Put and Spd more abundant than Spm and tSpm (Naka et al. 2010). Also, tSpm seems to be present in all plants, while Spm appears to be restricted to flowering plants (Fuell et al. 2010).

Besides Put, Spd and Spm, less common PAs in plants have been described as cadaverine (Cad), norspermidine, norspermine, homocaldopentamine, homocaldohexamine, 1,3-diaminopropane and 4-aminobutylcadaverine, among others (Kuehn et al. 1990; Fujihara et al. 1995; Kuznetsov et al. 2007).

Although their concentrations in plants are much higher than those of phytohormones, plant PAs are considered as growth regulators, as they play fundamental roles in a wide range of growth, differentiation and morphogenetic processes during the course of plant ontogeny. Roles in embryogenesis, seed germination, rhizogenesis, organogenesis, floral initiation and development, as well as in vascular development, leaf senescence, fruit development and ripening have been described for these molecules (Slocum 1991; Kakkar et al. 2000; Kakkar y Sawhney 2002; Pang et al. 2007). Lately, a great deal of attention has been paid to the protective effect of PAs during plant response to biotic and abiotic stresses (Liu et al. 2007; Gill and Tuteja 2010; Vera-Sirera et al. 2010).

2 Polyamine Biosynthesis

Concentration of intracellular PAs is tightly regulated through their biosynthesis and catabolism, and modulated by cellular transport and conjugation with other organic molecules such as hydroxycinnamic acids and proteins (Bagni and Tassoni 2001; Edreva et al. 2007; Fincato et al. 2011). Polyamines can be found as conjugated forms, e.g., covalently attached to compounds of low molecular weight (typically hydroxycinnamic, p-coumaric, caffeic and ferulic acids) and high molecular weight molecules (proteins or cell wall polymers). Enzymes such as putrescine-cafeoil-CoA transferase are responsible for the formation of hydroxycinnamic acid conjugates (Martin-Tanguy 1997), phenolics compounds that are related to the flowering process and the plant response to pathogen attack (Flores and Martin-Tanguy 1991; Martin-Tanguy 1997). On the other hand, transglutaminases channel the conjugation of polyamines to the γ-carboxamide group of endo-glutamic residues of proteins, especially in the chloroplast, where this activity is stimulated by light (Del Duca et al. 1995; Dondini et al. 2003). In addition, the compartmentalization of enzymes involved in PA metabolism suggests a spatio-specific regulation of these important amines (Borrell et al. 1995; Kamada-Nobusada et al. 2008; Fincato et al. 2011).

The first step in PAs biosynthesis is the diamine Put formation. In plants and some bacteria, this process occurs by decarboxylation of arginine via arginine decarboxylase (ADC; EC 4.1.1.19) in a pathway involving agmatine and N-carbamoylputrescine as intermediates, and the corresponding enzymes agmatine iminohydrolase (EC 3.5.3.12) and *N*-carbamoylputrescine amidohydrolase (EC 3.5.1.53) (Fig. 5.1). The ADC pathway for Put biosynthesis in plants appears to be derived

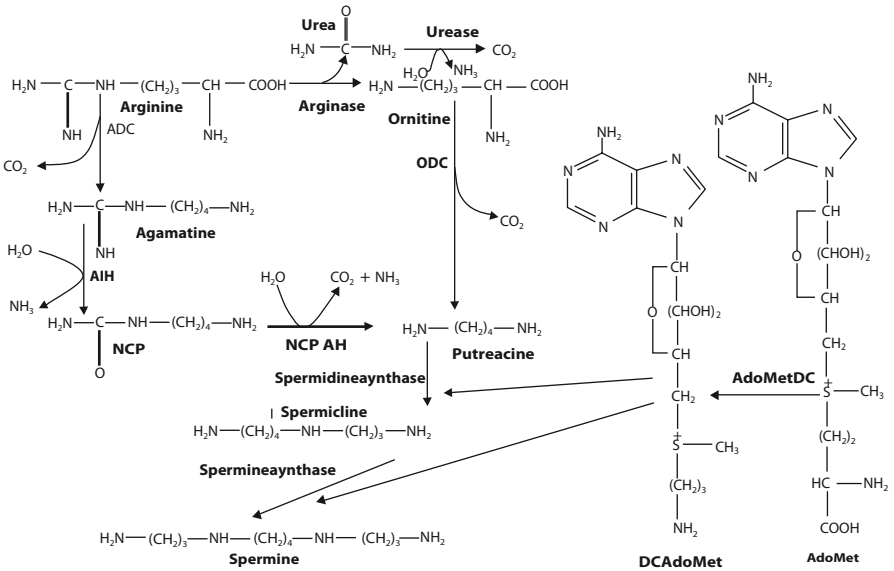

Fig. 5.1 PAs biosynthetic pathways from arginine and ornithine. *ODC* ornithine decarboxylase, *ADC* arginine decarboxylase, *DC AdoMet* decarboxylated AdoMet, *AdoMet DC* AdoMet decarboxylase, *NCP* N- carbamoyl putrescine, *NCP AH* N- carbamoyl putrescine amidohydrolase

from endosymbiotic gene transfer between the cyanobacterium precursor of chloroplasts and the eukaryotic nucleus (Illingworth et al. 2000). In animals, fungi, and also in most plants, Put is synthesized directly from ornithine via the cytosolic ornithine decarboxylase (ODC; EC 4.1.1.17). Evolutionary compartmentalization of Put biosynthesis in chloroplasts is accomplished by ADC signal sequences that import this enzyme into the plastid (Borrell et al. 1995; Illingworth et al. 2000). Both ODC and ADC enzymes use pyridoxal 5′-phosphate as cofactor.

The higher PAs, Spd and Spm are synthesized from Put through the successive activities of Spd synthase (SPDS; EC 2.5.1.16) and Spm synthase (SPMS; EC 2.5.1.22) through the addition of aminopropyl groups. In addition, tSpm is also synthesized from Spd (an asymmetric molecule that allows the formation of two isomers, Spm or tSpm, respectively), through the activity of a thermospermine synthase (tSPMS; Knott et al. 2007). The aminopropyl moiety is derived from methionine, which is first converted into S-adenosylmethionine (SAM) and then decarboxylated via S-adenosylmethionine decarboxylase (SAMDC; EC 4.1.1.50). SAMDC is considered the mayor regulatory enzyme involved in higher PA biosynthesis and plays an essential role in modulating ethylene production in plants, since the precursor of ethylene 1-aminocyclopropane-1-carboxylic acid is also derived from SAM (Bagni and Tassoni 2001). However, Del Duca et al. (1995) and Tassoni et al. (2000) provided data showing the occurrence of a back-conversion pathway: Spd added to *Helianthus tuberosus* chloroplasts and *Arabidopsis* plants,

respectively, was converted to Put, but the enzymes involved in this reaction have not been identified so far.

3 Subcellular Localization and Transport

Polyamines are present in all cell compartments and may be specially detected in actively growing tissues where cell division or elongation takes place. Cytochemical, immunochemical, autoradiographic and subcellular fractions studies suggest that the largest PAs reservoirs in plants are the cell wall and the vacuole (Bagni and Pistocchi 1991; Mariani et al. 1989; Slocum 1991). In addition, PAs have been found in the cytoplasm, nucleus, plasma membrane, mitochondria and chloroplasts. In the latter compartment, PAs are associated with components of the electron transport chain, by both electrostatic and covalent interactions (Bagni and Pistocchi 1991; Kotzabasis et al. 1993; Torrigiani et al. 1986; Votyakova et al. 1999). The information regarding subcellular localizations of enzymes involved in plant polyamine metabolism is scarce. Immunocytochemical and bioinformatic studies indicate that ADC is mainly present in the chloroplast and to a lesser extent in the nucleus (Borrell et al. 1995; Bortolotti et al. 2004; Illingworth et al. 2000). Inhibitor binding and fractionation studies suggest that ODC is located in the cytoplasm and nucleus (Slocum 1991). In plants of *A. thaliana*, ODC activity was observed in plastid membrane (Tassoni et al. 2003). In contrast, Spd synthase and SAMDC activities are generally located in the cytoplasm (Slocum 1991), whereas there is no information on Spm synthase. As mentioned above, Cu^{+2}- and flavin oxidases occur predominantly in the apoplast, although they have also been suggested to have cytoplasmic and vacuolar localization (Cervelli et al. 2004; Cona et al. 2003).

Using cell cultures, protoplasts and petals as models, it has been shown that the transport of aliphatic amines through the plasma membrane of plant cells is bidirectional, saturable, energy dependent and under hormonal control, at least for auxins and cytokinins (Bagni and Pistocchi 1991). It has also been demonstrated that the transport of Put in maize roots is non-competitively inhibited by inorganic cations (Ca^{+2}, Mg^{+2}) and Spm. On the other hand, the existence of at least two transport systems, one for diamines and another for PAs has been put forward (Di Tomaso et al. 1992; Hart et al. 1992). It was also proposed that the interaction between PAs and membranes would arbitrate important cellular events, such as receptor-mediated signal transmission. In *E. coli*, several periplasmic proteins that bind PAs are known such as POTD and PotF, which are part of two transmembrane transport systems (pPT104 and pPT79) and bind Spd and Put, respectively (Sugiyama et al. 1996; Vassylyev et al. 1998). In vascular plants like zucchini (*Cucurbita pepo*) and maize (*Zea mays*), plasma membrane proteins that specifically bind Spd have been identified, purified and analyzed (Tassoni et al. 1996, 2002). Despite being slightly mobile cations, due to their strong interaction with cell wall components, the distant translocation of PAs through xylematic and phloematic conducts has been demonstrated (Antognoni et al. 1998; Bagni and Pistocchi 1991; Caffaro et al. 1994).

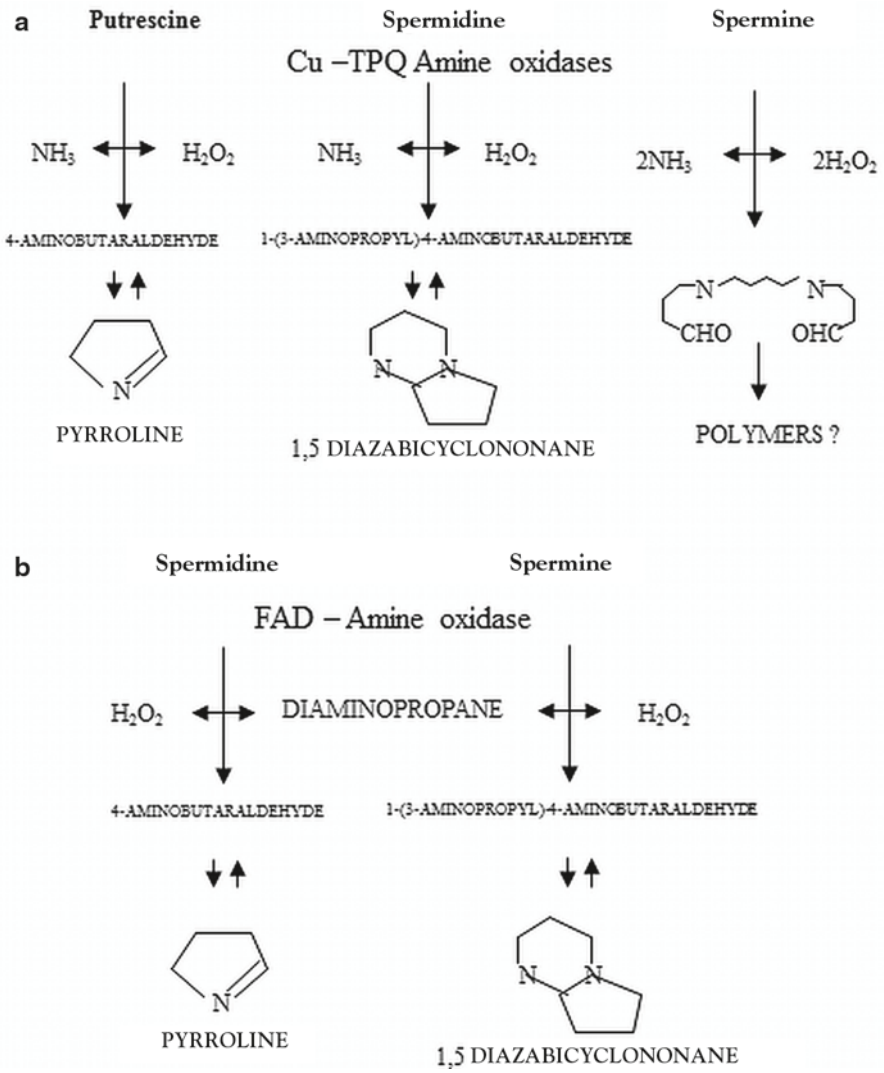

Fig. 5.2 Polyamine catabolism. *DAO* diamine oxidase, *PAO* polyamine oxidase, *1(3-AP)P* 1(3-aminopropyl) pyrroline

4 Polyamine Catabolism

Polyamines may be deaminated by oxidation, which constitutes the main PAs catabolic pathway (Federico and Angelini 1991). Recent data on PA oxidation in plants has led to propose several possible functions that this pathway could fulfill (reviewed by Kusano et al. 2008). In plants, PAs catabolism proceeds via Cu^{+2}-oxidases (Fig. 5.2; diaminoxidase: DAO, EC 1.4.3.6) and flavin-oxidases (polyamine

oxidase: PAO, EC 1.4.3.4), present in the apoplastic and peroxisomal compartments (Medda et al. 1995; Sebela et al. 2001). In the apoplast, Put, Spm, and Spd are oxidized to 1,3-diaminopropane, H_2O_2, and the corresponding aldehyde, while in the peroxisome, Spm is converted to Put, *via* the intermediate Spd (Rea et al. 2004; Cona et al. 2006; Moschou et al. 2008). The Cu^{+2}- amine oxidase oxidizes the primary amine of diamines and polyamines, with the concomitant production of H_2O_2, NH_4^+ and the corresponding aldehyde, while the flavin oxidase oxidizes the secondary amino of Spd and Spm, producing H_2O_2, 1, 3-diaminopropane and the corresponding amino aldehyde. Also, both mono- and dicotyledonous species may catabolize Put to γ-aminobutyric acid (GABA), an important modulator of several physiological processes (Bouchereau et al. 1999).

Aside from their participation in the catabolism and their contribution to cellular homeostasis, amine oxidases take part in important physiological events through their reaction products, mainly H_2O_2. On one hand, they are related to lignin biosynthesis and crosslinking reactions of the cell wall, occurring during xylem maturation and plant cell elongation (Cona et al. 2003; Federico and Angelini 1991; Laurenzi et al. 1999; Laurenzi et al. 2001; Moller and McPherson 1998) as well as to the cell wall strengthening that takes place during the infection by plant pathogens (Cona et al. 2006). On the other hand, they regulate cell PAs level in plants subjected to stress conditions (Bagni and Tassoni 2001; Cona et al. 2006). For example, a steep decline of Spd and Spm observed in rice (*Oryza sativa*) plants, six days after imposition of drought stress was assigned to PAO amino oxidation (Capell et al. 2004). It has also been reported that ethylene promotes DAOs and PAOs activities, which could be related to reductions in PAs contents observed in several crop species (Li et al. 2004).

5 Involvement of PAs on Crop Plant Response to Drought, Salt and Cold Stresses

5.1 Drought Stress

Polyamines are thought to play protective roles during drought stress. These molecules may act as osmolytes and bind non-covalently to the negatively-charged groups of membrane phospholipids, thus contributing to stabilizing membrane conformation (Hanzawa et al. 2000). They also regulate pH alterations due to osmotic stress, by reversing H^+-ATPase and H^+-PPase inactivity. Table 5.1 shows main results obtained in diverse studies on polyamine metabolism in several crop species cultivated under water deficit-related conditions. In barley (*Hordeum vulgare)*, a close relationship between PAs metabolism and leaf turgor was found: when leaf turgor was at or near the control level, Put and Spm accumulated, whereas the levels of both PAs sharply diminished when leaf turgor was lost (Turner and Stewart 1986). Moreover, Flores and Galston (1984) postulated that turgor maintenance is a requirement for PAs accumulation during drought stress.

Table 5.1 Polyamine metabolism in several cultivated species under water deficit-related conditions

Plant species	Observed changes in Polyamine levels	Reference
Hordeum vulgare	Decreased Spd in four cultivars. Put and Spm increased in three cultivars, but decreased in another cultivar	Turner and Stewart 1986
Populus spp.	PAs decreased in sensitive, whereas PAs increased in tolerant	Chen et al. 2002
Hordeum vulgare	Increased ADC transcripts level	Öztürk et al. 2002
Triticum aestivum	Increased Spd and Spm in tolerant genotype. Increased Put in sensitive genotype	Liu et al. 2004
Triticum aestivum	Increased non-conjugated Spd in root	Liu et al. 2005
Oryza sativa	Increased Put, Spd and Spm	Yang et al. 2007
Populus przewalskii	Increased Pas	Lei 2008
Zea mays	Decreased S-adenosylmethionine decarboxylase activity	Zhuang et al. 2008
Theobroma cacao	Increased ADC, SAMDC, SPDS, and SPMS activities in roots. Increased ODC and SAMDC activities in leaves	Bae et al. 2008
Vitis vinifera	Increased PAs	Antolín et al. 2008
Vetiveri a zizanioides	Decreased Put, increased Spd and Spm	Zhou and Yu 2010
Vitis vinifera	Increased PAs in tolerant genotype, whereas Pas decreased in sensitive genotype	Toumi et al. 2010
Oryza sativa	Increased Spd and Spm associated with higher tolerance	Basu et al. 2010
Solanum tuberosum	Increased Spm level, as well as ADC and SAMDC activities	Evers et al. 2010
Capsicum annuum	Increased PAs in leaves. Decreased PAs in root	Sziderics et al. 2010
Vitis vinifera	Increased ADC and SPMS	Liu et al. 2011

Put accumulation and ADC activation occur under unfavorable conditions (Bouchereau et al. 1999). However, it has been often observed that drought stress induces selective accumulation of Put in drought-sensitive plant species, whereas in drought-tolerant ones, PAs metabolism shifts towards Spd and Spm synthesis. When wheat (*Triticum aestivum)* plants were stressed with PEG 6000, increased levels of free-Spd and free-Spm in leaves of a drought-tolerant cultivar were observed, whereas free-Put titer buildup was noticed in a drought-sensitive cultivar of that crop (Liu et al. 2004). Yang et al. (2007) tested whether rice polyamines were involved in drought resistance. Six rice cultivars differing in drought resistance were subjected to well watered and water-stressed treatments during their reproductive period. Water stress increased the activities of ADC, SAMDC, and Spd synthase in the leaves, in consonance with rises observed in leaf Put, Spd and Spm. The augmented contents of free Spd, free Spm, and insoluble-conjugated Put under water stress were significantly correlated with cultivar yield. The authors concluded that to have incremented levels of free Spd/free Spm and insoluble-conjugated Put, as well as early accumulation of free PAs during drought is a desirable physiological trait for rice during its adaptation to this stress. Basu et al. (2010) compared

differential biochemical responses of the salt-sensitive (IR-29), salt-tolerant (Pokkali) and aromatic (Pusa Basmati or PB) rice varieties during polyethylene glycol (PEG)-induced dehydration stress. They found that drought resistant cultivars had higher free Spd and free Spm in the leaves than drought-susceptible ones during the whole period of withholding of water. Moreover, stressed Pokkali rice plants appeared to suffer lesser damage, parallely with the maximum accumulation level of the higher PAs – Spd and Spm. The authors also reported that water stress increased the activities of arginine decarboxylase, SAMDC, and Spd synthase in the leaves, in consonance with rise observed in leaf Put, Spd, and Spm.

The effect of drought stress has also been studied in several non-gramineous species. In 15-day-old chickpea (*Cicer arietinum*) seedlings, fast rise in Spd and Spm levels was observed after exposure to osmotic stress created by incorporating polyethylene glycol – 6000 (PEG) in the growth medium for four days (Nayyar and Chander 2004). Vetiver grass (*Vetiveria zizanioides*) is considered to have future potential as bio-fuel for power generation and cellulosic ethanol (Paul et al. 2008). Zhou and Yu (2010) studied the variations of PAs contents in plants of this species when stressed with 20, 40, and 60 % PEG solutions for six days. Their results showed that under osmotic stress, free and conjugated Put decreased, whereas free and conjugated Spd and Spm amounts increased. Lei (2008) used *Populus przewalskii* as a tree model species to investigate the acclimation and adaptation to drought stress, in particular the ROS damaging effects and their scavenging systems. *P. przewalskii* plants subjected to water withholding showed reduced biomass accumulation, shoot height and basal diameter. Drought stress also increased Put and Spd, while little change was observed in the Spm level (Lei 2008). Cacao (*Theobroma cacao*) plants subjected to 10 days of drought showed augmented Put, Spd and Spm in leaves (Bae et al. 2008). Also, a correlation was found between enhanced expression of TcODC and TcSAMDC with changes in leaf water potential. These expressions were preceded by induction at seven days of TcADC and TcSAMDC in roots. In leaves of this species, TcSPDS and TcSPMS were not responsive to drought, but the expressions of these genes were slightly upregulated in drought-stressed roots. The authors speculated that since PAs are associated with root development, it is possible that the induction of PA biosynthesis genes in roots were involved in shifting the root architecture of cacao plants in response to stress, as it was suggested in the subantartic cruciferous species *Pringlea antiscorbutica* (Hummel et al. 2002). PAs involvement in the development and ripening of fruit emerges from variations observed in their levels in several fruit crops (Serrano et al. 1995; Ponappa and Miller 1996; Geny et al. 1999; Shiozaki et al. 2000). Antolín et al. (2008) investigated how the balances of PAs were affected by drought in grapevine (*Vitis vinifera*). The PAs level was analyzed at distinct stages of berry ripening: onset of veraison, middle veraison and harvest. Their data showed that at the onset of veraison, concentrations of berry PAs were higher in both deficit irrigation treatments than in control grapevines, although those differences disappeared during ripening. Possibly, PAs contribute to increased fruit growth rate (Baigorri et al. 2001; Shiozaki et al. 2000). Also in grapevine, total PAs were significantly lower in the control tolerant and higher in the control sensitive genotype (Toumi et al. 2010), and these titers respec-

tively increased and decreased after drought treatment. Water withholding applied to pepper (*Capsicum annuum* L.) seedlings for one week resulted in elevated levels of Cad and Put in leaves (Sziderics et al. 2010), whereas concentration of PAs was reduced in roots. Authors suggested that PAs might be involved in the stress protection of pepper leaves rather than in the osmotic adaptation to drought, since PAs level was somewhat low. On the other hand, because proline may be synthesized from ornithine via ornithine aminotransferase (Delauney and Verma 1993), thus competing for the substrate (ornithine) with the PA biosynthetic pathway (Theiss et al. 2002), reduced levels of Put, Spd and Spm could be a consequence of a preferential proline synthesis in roots.

Up and downregulation by drought of enzymes involved in PAs metabolism has been studied in several crops. In a largescale study on changes in transcript abundance (Ozturk et al. 2002), drought-induced transcripts of two ADCs were detected in leaves and roots of barley plants subjected to water deficit. By means of a microarray analysis, a decrease in the enzyme SAMDC2 (TM00041253; Tian et al. 2004) was observed in the reproductive organs of maize (*Zea mays* L.), at an early stage of water deficit (Zhuang et al. 2008). With the aim of identifying drought-responsive compounds in potato, Evers et al. (2010) analyzed transcriptomic and targeted metabolites of two potato clones (*Solanum tuberosum* L.) of the Andean cultivar group, Sullu and SS2613. These clones presented different drought-tolerance phenotypes, as exposed to a continuously increasing drought stress in a field trial. Upon drought, genes encoding for PAs biosynthesis ADC and SAMDC enzymes were upregulated in both clones. In grape seedlings grown *in vitro*, ADC and Spm synthase inductions were observed between within one week, after 350 mM of mannitol treatment (Liu et al. 2011).

Addition of exogenous PAs to intact plants has earlier attracted the attention of several researchers as the observed growth promotion effect resembled that of phytohormones (Rastogi and Davies 1991). The binding of Spd and Spm to proteins or nucleic acids protects last compounds from degradation and provides them with a higher conformational stability under stress conditions. The exogenous application of Spd to osmotically-stressed oat (*Avena sativa*) plants stabilized the structure of thylakoid proteins Dl and D2, cytochromes and Rubisco (Tiburcio et al. 1994; Besford et al. 1993). When treated with Spd, water-stressed cucumber (*Cucumis sativus* L.) seedlings showed enhanced guaiacol peroxidase activity and a reduction of SOD and catalase activities compared to untreated, water-stressed ones (Kubis' 2008). The author suggested that PAs are able to moderate the activity of scavenging system enzymes and influence the oxidative stress intensity. Likewise, exogenously applied PAs increased drought tolerance of rice by improving leaf water status, photosynthesis and membrane properties (Farooq et al. 2009). Recently, in vitro *Citrus* plants pre-treated with Spm showed improved tolerance to dehydration stress through less water loss and lower electrolyte leakage (Shi et al. 2010). Pre-treatment with Spm led to higher endogenous PAs content and the activation of antioxidant enzymes. The authors assigned the reduced water loss to increased stomatal closure. On the other hand, they attributed the lower electrolyte leakage to inhibition of lipid peroxidation and biomembranes stabilization due to diminution

of ROS levels. The addition of Spm to the substrate, led to drought-stressed *Pinus strobus* seedlings to sustain higher photosynthesis and lower transpiration rates (Islam et al. 2003).

There is an amount of evidence supporting the influence of PAs on membrane-associated enzymes activities (Srivastava and Rajbabu 1983; Reggiani et al. 1992). Reggiani et al. (1992) reported that PM- H^+-ATPase from rice coleoptiles is activated by PAs. Under osmotic stress, less membrane peroxidation, greater H^+-ATPase activity and reduced senescence were registered in honey brew (*Cucumis melo* L.) supplemented with exogenous Spd or Spm, compared with the corresponding control without PAs (Lester 2000). Treatment with PEG brought about significantly higher increments of noncovalently conjugated-Spd and Spm contents, and H^+-ATPase activity in root plasma membranes of a drought-tolerant than those found in a drought-sensitive wheat cultivar (Liu et al. 2004). In addition, exogenously added Spd alleviated osmotic stress injury in drought-sensitive seedlings, parallely enhancing the root PM-H^+-ATPase activity hugely. Later, it was shown that treatment with methylglyoxyl-bis (guanylhydrazone) (MGBG), an inhibitor of SAMDC, aggravated PEG injury to drought-tolerant seedlings, with a concomitant reduction of the root PM-H+-ATPase activity (Liu et al. 2005). These results pointed to a possible involvement of these PAs in PM-H^+-ATPase activity and water stress tolerance of wheat seedlings.

The over-expression of genes-encoding enzymes that mediate in diverse pathways of PAs anabolism has become a promising approach for obtaining transgenic plants with higher drought stress tolerance. The introduction of a human SAMDC (EC 4.1.1.50) gene under the control of a constitutive promoter (CaMV35S) in tobacco (*Nicotiana tabacum* var. *xanthi*) led to increased conjugated Spd and Put titers and improved drought tolerance (10 % (w/v) PEG, MW 20,000), as well as tolerence to other abiotic and biotic stresses (Waie and Rajam 2003). Sweet potato (*Ipomoea batatas*, cv. Kokei 14) plants transformed with the *Cucurbita ficifolia*-derived Spd synthase gene *FSPD1*, doubled their Spd content and produced higher storage tissue biomass, compared with the wild type (Kasukabe et al. 2006). In addition, transgenic plants were more tolerant to paraquat (a powerful oxidative stress inducer) than the wild-type, suggesting that the observed improved tolerance may be in part due to enhanced oxidative stress tolerance. Previously, Capell et al. (2004) generated transgenic rice plants expressing the *Datura stramonium adc* gene and evaluated their response to drought stress. They observed that wild-type plants responded to the onset of drought stress by increasing endogenous Put levels, but not those of Spd and Spm (the agents that are believed to protect plants under stress). In contrast, transgenic plants expressing *D. stramonium adc* showed improved drought tolerance, in parallel with much higher levels of Put, what led to increased Spd and Spm synthesis.

Prabhavathi and Rajam (2007) introduced in eggplants (*Solanum melongena*) the gene encoding an ADC enzyme under the control of a constitutive promoter of cauliflower mosaic virus CaMV35S. Transgenic seedlings of this crop showed enhanced PAs level due to the augmented ADC activity, and also higher DAO activity. PAs-accumulating transgenic eggplants exhibited an augmented tolerance level

to drought imposed through 7.5 and 10 % PEG (MW 20,000), among other abiotic and biotic stresses. Several lines of a transgenic European pear (*Pyrus communis* L. 'Ballad') overexpressing the gene encoding for the apple Spd synthase (MdSPDS1) were created by *Agrobacterium*-mediated transformation and tested for tolerance to osmotic stress (300 mM mannitol, Wen et al. 2008). The transgenic line having the highest Spd accumulation and expression level of MdSPDS1 (no. 32) showed the strongest tolerance to this stress. On the tenth day after mannitol treatment, a slight decrease in Put, and significant enhancements of Spd (33 %) and Spm titers, and (Spd + Spm)/Put ratio were observed in the transgenic line, compared with the wild type. Later, He et al. (2008) showed that the transgenic line contained superior antioxidant enzyme activities, and less malondiahldehyde and H_2O_2 than the wild type, suggesting that transgenic plants were less stressed. In order to dissect the roles of Put from the higher PAs Spd and Spm, Peremarti et al. (2009) generated transgenic rice plants constitutively expressing an heterologous SAMDC gene from *D. stramonium* so that the levels of higher PAs were increased without affecting Put levels. Although such plants were not drought-tolerant, they returned to the normal phenotype when stress was removed, whereas wild type plants could not recover. Hazarika and Rajam (2011) generated transgenic tomato (*Lycopersicon esculentum* Mill.) plants with the human SAMDC gene, and evaluated the transgenic plants for tolerance to drought, among other biotic and abiotic stresses. Transgenic plants presented higher PAs levels and improved tolerance against drought, with respect to untransformed control plants. In turn, transcription factors may influence PAs-mediated adaptation to a variety of abiotic stresses (Chen et al. 2002). It was shown that overexpression of *CaPF1 (*a *Capsicum annuum* pathogen and freezing tolerance-related protein) in transgenic tissue of eastern white pine (*Pinus strobus* L.), prevented the decrease of PAs and resulted in a dramatic increase in tolerance to drought, freezing, and salt stress (Tang et al. 2007). These authors suggested that CaPF1 may influence, by a so far unknown mechanism on PA biosynthesis, enhancing stress tolerance in pine plants expressing the transgene.

Abscisic acid (ABA) is recognized as a major plant hormone during drought stress, since it inhibits growth and stomatal opening. Upon water deficit, both ABA biosynthesis in roots and its transport to the leaves are enhanced, leading to its accumulation in guard cells. In the stomata, ABA induces the release of water and loss of turgor of guard cells, provoking the closure of the stomata pore (Anderson et al. 1994; Allan et al. 1994). It is known that different *Populus* species and ecotypes may differ in their stomatal responsiveness to ABA (Chen et al. 2002; Yin et al. 2004; Zhang 2009 et al.). Chen et al. (2002) reported that the drought-induced decline of PAs concentrations in a sensitive *Populus* species was accompanied by leaf shedding, whereas the tolerant species maintained higher PAs levels and did not shed its leaves. The same authors showed that in water-stressed intact poplar, xylem ABA reduces PAs contents, and hypothesized that this fact might intensify the sensitivity of the leaf to ethylene, thus accelerating defoliation. Bae et al. (2008) reported that in cacao (*Theobroma cacao*), the induction by ABA (100 mM solution applied to soil) of all five ESTs associated with PA biosynthesis (TcODC, TcADC, TcSAMDC, TcSPMS and TcSPDS) was low, similarly to what has been observed in

rice (Li and Chen 2000), where even a fall in SAMDC1 was registered 12 h after rice plants were treated with ABA. The decline in transcripts was assigned to changes in mRNA stability (Li and Chen 2000). The gene induction patterns triggered by ABA in cacao leaves and roots disagree with those observed in other plant species. ADC2 was highly induced by ABA (50 mM) in *Arabidopsis* (Pérez-Amador et al. 2002). Also in *Arabidopsis*, Alcázar et al. (2010) observed that ADC2, SPMS, and SPDS1 were highly induced by drought and greatly reduced by this stress in ABA insensitive mutants.ABA triggered significant alterations in the PA catabolic pathway of grapevine leaf, but at the same time, it also induced the activity of biosynthetic enzymes ADC, ODC, and SAMDC (mainly in the tolerant genotype), justifying the interplay between PA anabolism and ABA signaling pathways in grapevine (Toumi et al. 2010). This induction (which took place within 1 h post-treatment) resulted in different enzyme induction patterns in the tolerant and sensitive genotypes, with the sensitive genotype responding lately and less profoundly. On the other hand, PAs oxidation concerned PAOs in the tolerant genotype and DAOs in the sensitive genotype. On the basis of this information, the following model was proposed: PA biosynthesis is higher in the tolerant than in sensitive grapevine genotypes; in both genotypes, PAs follow the exodus route and are catabolized in the apoplast by AOs, producing H_2O_2; in the case of high intracellular PA titers/PA anabolism, tolerance is enhanced via induction of additional defensive genes/responses; in the case where PA titers/PA anabolism is low, H_2O_2 enhances the PCD syndrome.

It has been shown that nitric oxide (NO)-treated plants have increased tolerance to drought (Garcia-Mata and Lamattina 2001, 2002). Arasimowicz-Jelonek et al. (2009) demonstrated the occurrence of a functional crosstalk between PAs and NO in cucumber leaves under drought stress. Although exogenous PAs (1.0 mM Spd, Spm, and Put) did not affect NO production in well-watered cucumber seedlings, their treatment with Spm and Spd, prior to water deficit imposition, induced early and higher NO levels (noticed by NO-dependent fluorescence) in leaves of drought-stressed cucumber plants, with respect to the control and Put treated ones.

Recently, Alcázar et al. (2010) discussed advances in the crosstalk between PAs and ABA, integrating them with other abiotic stress-related metabolic routes such as reactive oxygen species (ROS) signaling, generation of NO, modulation of ion channel activities and Ca^{2+} homeostasis.

5.2 Saline Stress

First reports on the induction of plant PAs metabolism over salt stress, as well as its possible alleviating role on plant salt tolerance can be traced back to the eighties (20th Century). Many authors have reported that PAs accumulation is the immediate response observed in different crop plants species after exposure to saline conditions (Erdei et al. 1996; Chattopadhyay et al. 2002; Ghosh et al. 2011). Most significant changes in polyamines level upon salinization appear to be those of Spm, according to data reported in rice (Maiale et al. 2004), maize (Jiménez-Bremont

et al. 2007; Rodríguez et al. 2009) and wheat (Reggiani et al. 1994; El-Shintinawy 2000). Thus, under salinity, the pool of Put would be directed to Spd and finally, to Spm synthesis (Groppa and Benavides 2008). In rice, (Krishnamurthy and Bhagwat 1989; Roy et al. 2005; Roychoudhury et al. 2008) wheat (El-bassiouny and Bekheta 2005) and barley (Liu et al. 2006), the buildup of the Spm level has been described as an indicator of salt tolerance whereas Put accumulation has been associated with salt sensitivity. Roy et al. (2005) clearly demonstrated that deficiencies of salt-sensitive rice cultivars, due to high Na^+ accumulation or salinity stress-induced K^+ loss, could be overcome by exogenously supplied Spd, necessary to Spm synthesis. In general, plants respond to abiotic stress by increasing ADC activity (Bouchereau et al. 1999). Roy and Wu (2001) reported that under salinity, rice plants transformed with a gene encoding an oat ADC increased the PAs level and plant biomass as a consequence of a higher ADC activity. Chattopadhyay et al. (1997) reported that ADC transcripts and activity increased in rice cultivars as early as one hour after the stress treatment was imposed, followed by a sharp decrease after prolonged salt treatment, in the case of salt-sensitive cultivar. Roy and Wu (2002) transformed rice plants with a Tritordeum SAMDC and observed a three-to-four-fold rise in Spd and Spm levels in transformed plants under NaCl-derived stress. Li and Chen (2000) reported that the expression of the *SAMDC1* gene in rice seedlings was dramatically induced by salinity. The transcript levels of *SAMDC1* in two rice varieties differing in salt tolerance were found to be higher in the salt-tolerant than in the salt-sensitive variety. However, authors reported that ADC and SAMDC transcript levels were barely affected by NaCl treatment, although SPDS 2 in maize (Rodríguez-Kessler et al. 2006) and rice (Imai et al. 2004), and SPDS 1 in maize (Jiménez-Bremont et al. 2007) were upregulated by this treatment.

Although the mechanisms that govern PAs metabolism-mediated salt resistance remain unclear, some reports have shed light in the last few years. Mansour and Al-Mutawa (1999) reported that Spd or Spm but not Put alleviates the cellular alterations in wheat roots under saline stress, possibly by plasma membrane protection. Accordingly, Spm and Spd significantly prevented the leakage of electrolytes and amino acids from roots and shoots of rice subjected to salinity (Chattopadhyay et al. 2002). Saline stress-induced elevation of PAs levels may represent an adaptive mechanism in which the uptake of Na^+ and leakage of K^+ from mesophyll cells are reduced (Pang et al. 2007). Pre-treatment with PAs prevented salt-induced K^+ leakage in mature root zone of hydroponically grown maize, apparently by effect on cell membrane transporters in a highly-specific way (Pandolfi et al. 2010). Shabala et al. (2007) showed that PAs treatment substantially reduced the NaCl-induced K^+ efflux from the pea leaf mesophyll, most likely by blocking the non-selective cation channels. Zhao and Qin (2004) reported that exogenous PAs application could maintain tonoplast integrity and function in barley seedlings under saline conditions. Legocka and Kluk (2005) reported higher levels of PAs bound to microsomal membranes in *Lupinus luteus* seedlings in salinity and proposed that PAs most likely stabilized microsomal membrane surfaces, protecting them against NaCl stress damage.

Many authors suggested that PAs act as antioxidants under salinity and other environmentally-adverse conditions, though their precise role as antioxidants is still

a matter of debate (Groppa and Benavides 2008). Under NaCl-induced stress, a higher level of lipid peroxidation was observed in the salt sensitive, relative to the salt-tolerant wheat (El-bassiouny and Bekheta 2005) and rice (Roychoudhury et al. 2008) cultivar, which showed augmented Spd and Spm levels, not observable in the salt sensitive cultivars.

Xing et al. (2007) analyzed the effects of treatments with different NaCl concentrations, with or without aminoguanidine (AG; a specific DAO inhibitor) on endogenous free PAs, GABA accumulation and DAO activity in soybean roots (*Glycine max* (L.) Merr., cultivar Suxie-1). Results showed significant Put, Cad and Spd decreases with increasing salt concentrations, assignable to the promotion of DAO activity by salinity and consequent stimulation of PAs degradation. In parallel, GABA accumulation raised with growing NaCl concentrations, strongly suggesting its origin in PAs degradation.

Reactive oxygen species are necessary in many plant developmental processes (Foreman et al. 2003; Demidchik and Maathuis 2007), particularly in the elongation zone of maize leaves during leaf extension (Rodríguez et al. 2002). In plants of this species, the salt-induced decrease of extracellular ROS contributes to the reduction of leaf elongation (Rodríguez et al. 2004). In turn, the diminution of the extracellular ROS has been attributed to the inhibitory effect of NaCl on the NADPH oxidase complex (Rodríguez et al. 2007). Rodríguez et al. (2009) reported that under saline stress, extracellular ROS registered in the elongation zone of maize leaves are produced principally by PAO, contributing partially to counteract the growth-inhibiting effect caused by salinity. In turn, this same phenomenon was described in soybean hypocotyls grown under NaCl stress (Campestre et al. 2011). In the last species, saline stress increased Spm and Cad level and CuAO activity. Treatment with the CuAO inhibitor showed a significant reduction of ROS in the elongation zone and plants grown in Cad-amended culture medium showed longer hypocotyls in saline condition (relative to the unamended treatment), effect that was abolished by the CuAO inhibitor. Since H_2O_2 functions as a signal molecule activating many of the plant responses deployed to cope with stress, it is believed that its generation from PAs oxidation (along with PA depletion) might orchestrate (at least partially) plant adaptation to these conditions (Moschou et al. 2008; Rodríguez et al. 2009).

5.3 Cold Stress

In temperate climates, plant species have acquired a certain degree of cold tolerance, depending on the genetic background, cold hardness and exposure time (Janská et al. 2010). Plant physiologists use the term freezing to mean temperatures below 0 °C, chilling for temperatures between 0 °C and the minimum temperature necessary for growth, and temperature between that minimum and the optimum is denominated suboptimal temperature for growth. Such a difference in stress terminology is not trivial, since the physiological response of a plant species may be different in each case. Temperate and tropical crop species such as rice, maize and

soybean are exceptionally subjected to freezing periods, more regularly they endure chilling and the most common situation is suboptimal growth temperature. While freezing kills these plant species, chilling and suboptimal temperature constitute an important constraint to productivity. In the last two cases, damage levels depend on the magnitude of temperature diminution and the exposure time. In contrast, wheat, barley and oat crops are normally grown under freezing, chilling and suboptimal growth temperatures, being freezing tolerated to some extent by these species.

There are two different strategies to overcome low temperature stress: avoidance and tolerance. In terms of crop production, avoidance may be determined by the sowing period, growth cycle and agronomic management, but tolerance is a genetic feature, peculiar to each cultivar, which constitutes a major tool for crop production management in areas characterized by low temperatures.

Cultivar response to low temperature stress involves important biochemical and molecular changes. Essentially, plants increase the production of protective compounds that affect cell lipid composition, thus participating in membrane stabilization and maintaining plasma membrane functionality (Janská et al. 2010). Biochemical changes also include the synthesis of cryoprotectant molecules as soluble sugars, (saccharose, raffinose, stachyose, trehalose), sugar alcohols (sorbitol, ribitol, inositol) and low-molecular weight nitrogenous compounds (proline, glycine betaine). Symplastic and apoplastic soluble sugar directly contribute to membrane stabilization (Livingston et al. 2006). Also, compounds such as tripeptidthiol, glutathione, ascorbic acid (vitamin C) and a-tocopherol (vitamin E) are important for their antioxidant activity (Chen and Li 2002). PAs are also involved in the stress response to low temperatures. Changes at the transcriptional and metabolic levels have been reported, mainly in *A. thaliana.* Currently, attempts are being made to manipulate PAs metabolism genes in order to obtain plants tolerant to low temperature stress. Table 5.2 shows variations in PAs levels during the response to cold temperature in several crop species.

In five bean (*Phaseolus sp.*) cultivars differing in chilling response, Guye et al. (1986) found that prior to chilling treatment, PAs levels did not appear to be correlated with chill-tolerance, since levels in non-chilled controls were highest in cultivars of medium chill-sensitivity. These authors also found that the Put levels were increased in tolerant cultivars, whereas no changes were observed in sensitive ones. They concluded that it is the change in Put titer rather than its absolute level what appears to be correlated with chill-tolerance. In two wheat cultivars with slight difference in response to cold tolerance, Nadeau et al. (1987) found a 6–9-fold increased Put level during cold acclimation, whereas a smaller raise was observed in the Spd content and conversely, Spm level decreased. These authors also found an augmentation in Put level of alfalfa (*Medicago sativa).*ADC activity level declined in cold treated plants, related to the untreated control, whereas no major variations were observed in ODC activity levels, suggesting that ADC is the mainly enzyme responsible for the incremented Put levels under the cold-hardening condition. In turn, DAO activity varied in parallel with Put content. In a short term freezing stress experiment, a marked Put and agmatine accumulation was observed in wheat subjected to −2 °C for six hours (Rácz et al. 1996). The buildup of agmatine (which is

Table 5.2 Polyamine modification in several cultivated species under different cold treatment

Treatment	Plant species	Polyamine levels modification	Reference
Cold-hardened-Freezing	*Triticum aestivum*	Increased Put and Spd (Winter) and decreased Put (Spring)	Szalai et al. 2009
Chilling	*Cucumis sativus*	Increased Put, Spd and Spm	Zhang et al. 2009
Chilling	*Zea mays*	Increased Put, decreased Spd and Spm	Gao et al. 2009
Chilling	*Zea mays*	Decreased Put, increased Spd and Spm	Zheng et al. 2009
Chilling	*Solanum tuberosum and Solanum phureja*	Slilght modification, genotype-dependent	Oufir et al. 2008
Chilling	*Cicer arietinum*	Increased Put, Spd and Spm	Nayyar 2005
Chilling	*Oryza sativa*	Slilght modification, genotype-dependent	Pillai and Akiyama 2004
Chilling	*Zea mays*	Increased Put	Németh et al. 2002
Chilling	*Lycopersicum sculentum*	Increased Put.	Kim et al. 2002
Chilling	*Cucumis sativus*	Increased Spd	Shen et al. 2000
Chilling	*Oryza sativa*	Increased Put	Lee et al. 1997
Chilling	*Zea mays*	Increased Put	Szalai et al. 1997
Freezing	*Triticum aestivum*	Increased Put	Rácz et al. 1996
Chilling	*Oryza sativa*	Increased Put	Lee et al. 1995
Cold-hardened	*Triticum aestivum* and *Medicago sativa*	Increased Put	Nadeau et al. 1987
Chilling	*Phaseolus sp.*	Increased Put in tolerant and no change in sensitive cultivars	Guye et al. 1986

an intermediate in Put synthesis and a product of ADC activity) indicated that Put accumulation was mediated by ADC. Again, no major variations were observed in ODC activity levels, reinforcing the idea that ADC is the main enzyme responsible for the increase in plant Put levels during cold hardening. Experiments carried out with chilling (5 °C) temperatures for maize under two different light conditions (darkness and light) by Szalai et al. (1997) found a continuous rise in the Put level, which was more pronounced under the light condition. One day after chilling, Spd also increased in light, whereas it decreased in darkness. After the second day, chilling provoked a 50 % and 80 % fall in the Spd content in light and dark, respectively, compared with the unstressed control. Likewise, experiments performed on winter and spring wheat grown under low and normal light conditions (Szalai et al. 2009) showed that changes in PA contents were markedly light dependent.

Using seedlings of two inbreeded maize lines differing in cold sensitivity (Gao et al. 2009), it was found that Put concentrations escalated after chilling stress in mesocotyl and coleoptile, but the root Put concentration remained unchanged. Inversely, Spd and Spm concentrations decreased after chilling stress in the three mentioned seedling organs. On the other hand, the electrolyte leakage in cold stressed tissues was lower in the tolerant than in the sensitive cultivar, whereas the level

of this parameter was lower in the coleoptile than in the mesocotyl, in both cultivars. Stepwise regression analysis of these data showed that chilling injury in roots was generally correlated with Spd concentration while in the mesocotyl, injury was mainly correlated with Put and Spd concentrations. In the last cultivars, Zheng et al. (2009) found that Put reduced, but Spd and Spm increased during chilling stress (5 °C, 48 h). However, Spd and Spm contents were higher in the tolerant than in the sensitive cultivars. The values of (Spd + Spm)/Put were negatively correlated with malondialdehyde contents, whereas treatment with methylglyoxal-bis-guanyl-hydrazone (MGBG; an inhibitor of SAMDC) resulted in raised malondialdehyde content and reduction of germination percentage and energy, in both maize cultivars.

Working with two rice cultivars differing in their response to chilling condition (5 °C), Lee et al. (1997) observed that levels of Put, Spd and Spm contents, as well as ADC and SAMDC activities, increased under the stress in the tolerant cultivar Tainung 67. However, minor changes occurred in the sensitive cultivar Taichung Native 1. Furthermore, ABA increased, similarly to Put, whereas treatments with inhibitors of Put synthesis or pre-treatment with ABA provoked enhanced sensitivity and improved tolerance to cold stress, respectively. Earlier, a high correlation had been reported between ABA, Put and tolerance levels in 11 rice cultivars (Lee et al. 1995).

On the other hand, Pillai and Akiyama (2004) found that the rice OsSAMDC gene was induced in the tolerant cultivar Yukihikari but not in the sensitive TKM9 one. In agreement with last results, Spd levels increased in shoots of Yukihikari and it was not altered in TKM9, whereas Put and Spm remained unchanged in both cultivars. OsSAMDC was also induced by Ethephon (liquid ethylene) in both cultivars, but this gene was not responsive to salt, drought, submergence, mannitol or ABA.

In cucumber plants, chilling induced a marked Spd rise in a tolerant cultivar, but not in a sensitive cucumber cultivar (Shen et al. 2000). Also, Put built up during the rewarming period in the tolerant cultivar, but there was no change in the sensitive one. In contrast, Zhang et al. (2009) reported increased Put, Spd and Spm in tolerant cucumber plants and a slight Put increase in sensitive ones. Such apparent incongruence might be attributed to the different chilling conditions employed: 3 °C in the first case versus 15/8 °C in the second. Shen et al. (2000) informed that augmentations in Put and Spd were preceded by enhancements in ADC and SAMDC activities. Pre-treatment of sensitive plants with Spd prevented chill-induced increments in leaf H_2O_2 contents and nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase activity, alleviating chilling injury. On the other hand, the application of MGBG to a chilling-treated, tolerant cultivar, prevented Spd rise and enhanced NADPH oxidase activity and chilling injury. A beneficial effect of PAs addition was reported by He et al. (2002). These authors described an improvement of chilling tolerance of the photosynthetic apparatus in cucumber leaves pre-treated with Spd.

In tomato, Put and Spd increased under cold treatment in the wild type, whereas only Put was augmented in the *flacca* mutant, (an ABA-deficient tomato plant). The exogenous application of DFMO (an inhibitor of Put synthesis) intensified the electrolyte leakage, whereas Put addition reversed this phenomenon (Kim et al. 2002). Both ABA and Put had a protective effect against cold stress, but exogenously

Table 5.3 Plants genetically modified for PAs metabolism and their tolerance response to cold stress

Genetic modification	Plant species	Stress response	Reference
S-adenosylmethionine decarboxylase	*Lycopersicon esculentum*	Increased cold tolerance	Hazarika and Rajam 2011
adc1 and adc2 T-DNA mutants	*Arabidopsis thaliana*	Increased cold sensitivity	Cuevas et al. 2008
S-adenosylmethionine decarboxylase	*Nicotiana tabacum L*	Increased cold tolerance	Wi et al. 2006
Spd synthase	*Arabidopsis thaliana*	Increased chilling and freezing tolerance	Kasukabe et al. 2004

applied ABA decreased the endogenous level of Put in the leaves. Authors suggested that ABA is a major regulator in the response to cold stress, although it would not play this role *via* Put.

Chickpea is sensitive to low temperatures. Under (12–15/4–6 °C) cold stress, marked Put, Spd and Spm accumulations were observed at an early flowering stage (Nayyar 2005). Cold stress enhanced electrolyte leakage and declined cellular respiration, while exogenous 10 mM Put, applied during the flowering stage, reverted these effects and increased floral retention, pod set and fertile pods. Also in this crop, Nayyar and Chander (2004) observed that exogenous application of PAs reduced H_2O_2 titer and malondialdehyde content, and raised the antioxidant levels. These effects could be reverted by the ODC inhibitor DFMO. Oufir et al. (2008) observed that Put content rose in two *Solanum tuberosum* genotypes and transiently decreased in a *S. phureja* one, after exposure to 7C/2 °C (day/night), compared with the control condition (21/18 °C). Upon cold, Spd and Spm contents also decreased in *S. phureja*, while in *S. tuberosum,* Spm decreased and Spd levels did not significantly change. In turn, the expression of SAMDC and ADC were upregulated under cold conditions in the three genotypes.

Several plant species that have been genetically modified in their PAs metabolism were studied regarding their tolerance to cold stress treatment (Table 5.3). Kasukabe et al. (2004) reported that the Spd synthase cDNA from *Cucurbita ficifolia* was introduced in *A. thaliana* under the control of the cauliflower mosaic virus 35S promoter. Transgenic plants showed a significant enhanced Spd synthase activity and Spd accumulation in leaves. Under chilling condition (5 °C), leaves of the transgenic plants displayed a remarkable increase in ADC activity and conjugated Spd contents, compared with the wild type. In cDNA microarray assays, it was observed that several genes were more abundantly transcribed in chilling-stressed transgenic *C. ficifolia* than in the corresponding wild type. One of the most remarkable expression features in the last species was the upregulation of genes encoding DREB transcription factors, and stress-protective proteins like rd29A. These results suggested that Spd would be a regulator of stress signaling pathways that are activated in response to chilling stress. Wi et al. (2006) introduced a SAMDC gene from *Dianthus caryophyllus* L. into *N. tabacum* under the control of the cauliflower mosaic virus 35S promoter. Compared with the wild type, these transgenic

plants showed augmented Spd levels whereas they did not present any difference in organ phenotype, compared to the wild type. Transgenic plants displayed reduced sensitivity to chilling (0 °C) injury with respect to wild type plants. Also, overexpressing SAMDC in transgenic plants showed augmented PAs levels, having all transgenic lines higher free Put contents than the wild-type. Hazarika and Rajam (2011) generated transgenic tomato plants that constitutively expressed the human SAMDC gene. These plants showed higher levels of PAs and cold tolerance, compared to untransformed plants. Using *A. thaliana* T-DNA mutants for adc1 and adc2 genes, Cuevas et al. (2008) demonstrated improved sensitivity of these mutants during acclimation response to chilling and freezing. After cold treatment (4 °C for three weeks), mutant plants showed diminished Put accumulation, and reduced expression of 9-cis-epoxycarotenoid dioxygenase (NCED3), a key gene involved in ABA biosynthesis. These authors suggested that Put-controlled ABA levels through modulation of ABA biosynthesis.

In conclusion, low temperatures provoke intense changes in PAs levels. The most common response related with PAs metabolism is the buildup of Put content (Guye et al 1986; Nadeau et al.1987; Lee et al. 1995, 1997; Rácz et al. 1996; Szalai et al. 1997, 2009; Kim et al. 2002; Németh et al. 2002; Oufir et al. 2008), but the magnitude of this response is related to stress severity, as it was shown in *C. sativus* (Shen et al. 2000; Zhang et al. 2009). Furthermore, changes in PAs levels depend on the genetic background of plants exposed to cold treatment (Guye et al. 1986; Pillai and Akiyama 2004; Oufir et al. 2008; Szalai et al. 2009). On the other hand, the relationship between ABA and PAs is not clear: While Lee et al. (1997) observed that ABA levels controlled Put titers in rice under cold stress, Cuevas et al. (2008) showed the opposite in *Arabidopsis* and Kim et al. (2002) reported that ABA and Put act independently from each other in leaves of tomato. It is noteworthy that both rice and tomato are species adapted to warm climate, with minimum temperatures of growth higher than those used in these experiments, whereas *A. thaliana* is a species adapted to cold environment. On this basis, it is possible that the relation between ABA and PAs is dependent either on the stress severity or the interaction between genotype and environment.

6 Conclusion and Future Perspectives

Despite the significant information denoting that PAs are implicated in diverse plant physiological processes, there is still no agreement on whether PAs operate as second messengers or they may be regarded as phytohormones. Crop protection against drought, salt and cold stresses provided by polyamines would presumably rely on their ability to act as antioxidants, stabilizer of nucleic acids, cytosolic pH and biomembranes, and also on their effect as compatible osmolytes. However, the precise mechanism of action of PAs during the plant tolerance response to stress is complex and not fully understood. Neither is the metabolic regulation of the enzymes that synthesize these molecules.

In general, there is a correlation between increased PAs contents and improved tolerance to stress. Several strategies to modify plant PAs levels are currently being developed, including exogenous PAs and addition of specific inhibitors to intact plants, and genetic engineering. Production of higher PAs has been accomplished by the overexpression of *ADC*, *ODC* and *SAMDC* in different crop species and transgenic crop plants with higher tolerance to abiotic stress as compared to non-transgenic plants.

These strategies not only would be a reliable way to develop plants with enhanced tolerance to drought, salt and low temperature stresses, but they also provide useful experimental models to study more indepth PAs metabolism under constraint conditions.

Finally, literature reviewed in this chapter reveals that the bulk of research efforts have turned to the PAs biosynthetic aspects of the PAs-tolerance correlation, while little attention has been paid to the role that PAs catabolism could have played during plant response to this stress. The reinforcement of aspects addressing the role of PAs catabolism on stress tolerance by crops is crucial.

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Chapter 6
Overlapping Horizons of Salicylic Acid under Different Stresses

Mohd Irfan, Shamsul Hayat, Arif Shafi Wani and Aqil Ahmad

1 Introduction

Since the discovery of salicylic alcohol, isolated as a glycosidic derivative first by Johann Buchner in 1928, salicylic acid (SA) has emerged rapidly to establish itself as a class of ubiquitous phytohormone in plant kingdom. It found its role in regulating diverse metabolic and physiological functions in plant tissues, particularly in meristematic and biotically-challenged tissues regulating redox homeostasis. The derivatives of acetyl salicylic acids have been shown to manage plant's internal metabolism at its optimal growth conditions in different normal and stressed regimes of soil salinity (Kaydan et al. 2007; Tari et al. 2004; Szepesi et al. 2005; El Tayeb 2005; Yusuf et al. 2008), water stress (Senaratna et al. 2000; Hamada and Al-Hakimi 2001; Hayat et al. 2008), high temperature stress (Lopez-Delgado et al. 1998; Janda et al. 1999; Larkindale and Huang 2004; He et al. 2005; Chakraborty and Tongden 2005), chilling (Szalai et al. 2002; Tasgin et al. 2003; Korkmaz 2005), and heavy metal stresses (Choudhury and Panda 2004; Panda and Patra 2007; Krantev et al. 2008; Guo et al. 2009; Zhou et al. 2009). SA regulates the internal oxidative state of cell(s) quite differently under two types of microbial invasions, i.e. pathogenesis and symbiosis. In pathogenesis invasion, it synergises reactive (oxygen, nitrogen or free radical) species, simultaneously cross-talking with other active phytoregulators (e.g. nitric oxide, brassinosteroids jasmonic acids and ethylene, etc.), while in symbiosis invasion, it suppresses the over-accumulation of reactive radicals activating detoxification system (antioxidant enzymes and molecules) in co-operation with different plant hormones (viz., auxins, cytokinins and gibberellic acids, etc.). "Resistance induced through a wide range of pathogens (Malamy et al. 1990; Durner et al. 1997), via flash/mild abiotic stress (Ashraf et al. 1999; Azevedo Neto et al. 2005), which may be sometimes taxonomically unrelated (Uknes et al. 1992), facilitates" the accumulation of SA to strengthen resistance at whole plant level.

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2 The Overlapping Regimes of Abiotic and Biotic Challenges in Plants

Salicylic acid has established its worthiness not only in local and systemic pathogenic and symbiotic responses but also surpassed diverse abiotic stresses such as salinity, high temperature, chilling, drought, radiation and heavy metal stresses (Kranteve et al. 2008; Choudhuri and Panda 2004), and sometimes even par-systemically in adjacent plant population (Huang et al. 2005; Heil and Ton 2008, Ortíz-Castro 2009). Therefore, SA appears to manage reactive oxygen species (ROS) signaling at its effective threshold to regulate the balance of free radicals and antioxidation of cell(s) locally, systemically and par-systemically to enable stimulated growth and/or defense. And at the same time, it does not allow cell death, unless under specific conditions where its accumulation is supposed to be regulated externally (Ortíz-Castro 2009).

Converging points of abiotic and biotic signals' crosstalk are still rudimentary (Fujita et al. 2006). Although the role of abiotic stresses in facilitating primary infection has been well recognized (Zahran 1999; Castejón-Muñoz 2008; Babu and Devaraj 2008; Çiçek and Çakırlar 2008), it has not been elaborated in molecular detail. Disease, the outcome of successful pathogenesis, denotes the consistent failure of the host to counter the forced metabolic and redox perturbation of tissue to be invaded. The other causes that favour the microbial ingression include the abiotic factors such as relative humidity, temperature, aeration (Chupp 1946), invader's factors such as virulence and inoculum density of microbe (Navas-Cort'es et al. 2000) and host factors such as genotype and physiological state/age (Millett et al. 2009; Staskawicz et al. 1995, 2001; Basim 1998; Li et al. 2001). The stress-retention time marks the fate of host-microbe relation, and is the manifestation of host resistance capability versus aggressiveness of invader (virulence) under a set of abiotic conditions. If the prevailing abiotic factor favours the invader, a continuous perturbation of redox metabolism triggers instant upsurge and burst of ROS and its organic radical derivatives, favouring subsequent tissue necrosis and eventual plant death (Greenberg and Yao 2004). Therefore, specific mal-physiological symptoms and reduced plant growth become obvious. The bulk diversion of photosynthates and disturbed hormonal distribution could be hypothesized as the reasons for the plant's death (Berger et al. 2007). However, consistent prevailing environment may support host physiology to attenuate microbe disfavouring the inoculum buildup, hence, rendering its ingression to fail. Ingression could be checked though, at different levels of host defense (de Wit 2007) claiming differential loss of tissue size (manifested as intensity of symptoms) denoting eventual combat of host in this tug of war of metabolic management. Apart from genotype, the threshold time of perception and subsequent response of host are crucial determinants to ascertain host defense response. The quick initial defense strategy against microbial invasion includes instant upsurge of free radicals in adjoining cells (Bolwell et al. 2002; Wojtaszek 1997; Lamb and Dixon 1997) at the cost of cellular and microbial death and the rise of neutralizing defense molecules in the vicinity, e.g., phenolics of phenyl propanoid pathway (Bhattacharya et al. 2010; Dixon and Paiva 1995; Baron

and Zambryski 1995; Solecka 1997). The role of phenolic derivatives has been discussed frequently in the studies related to defense strategies of plants (Kurkin 2003; Bednarek et al. 2005; Mandal et al. 2010). Among other things, SA has crucially been discovered in up-regulating the cellular H_2O_2 content and other reactive species, and thus, the induction of hypersensitive response (HR). The level of ROS has extensively been shown to attenuate plant growth physiology at the cost of plant growth and productivity.

An intermediate stage between two extremes of host and invader point of view could be assumed, where forced modulation of host cell metabolism is not as strong to completely overpower its defense response (based on aforementioned triple factors, i.e., abiotic, host and invaders'). Differential push of two during the daily cycle of environment allows the partial resistance/virulence with conditional ingression of invader restricting the growth of intruder within the limits of threshold damage (Berger et al. 2007; Anderson et al. 2010). During the evolution of this semi-compatible system, partial utilization of host metabolites and metabolization of microbial secretions (sometimes toxic products) or cross resistance to abiotic/biotic factors may pave the way to facultative symbiotic relation. After a long gap of growth adjustment, plant system may incorporate these invaders as part of their life cycle at the specific stage of growth or even as a full-fledged integral organelle.

Activation of phenolic compounds under different abiotic stresses and biotic challenges has been reported in several studies (Rivero et al. 2001). It is presumed that the phenolic compounds play a crucial role in signal transduction and defense response under biotic (Holuigue et al. 2007; Malamy et al. 1990; Durner et al. 1997) and abiotic stresses (Dixon and Paiva 1995; Solecka 1997; Helle et al. 1998). SA plays a crucial role, modulating the cellular redox homeostasis in different plant microbe interactive systems, particularly associated with the onset of defense responses up to diversion of stress signaling, executing programmed cell death (PCD; Gut-Rella et al. 1994; Alvarez 2000; Greenberg et al. 2000). Their signaling has recently been implicated in interplant priming of defense responses through mobile signals that travel as volatile intermediates of SA and JA (Heil and Ton 2008). Thus, phenols, particularly SA, regulate plant metabolism under different overlapping environmental and indigenous cues (Fig. 6.1).

3 Activated SA Biosynthesis, Metabolism and Mobilization

The production of H_2O_2 with the interplay of SA and ROS in the local defense reaction is believed to positively feedback the activated biosynthesis of SA via phenylpropanoid pathway (Catinot et al. 2008; Lee et al. 1995; Durner and Klessig 1996; Kawano 2003). Earlier, two routes of biosynthesis of SA from shikimate-derived phenylalanine had been suggested. Phenylalanine is converted to cinnamic acid by phenyl ammonia lyase (PAL), which forms SA either through benzoic acid hydroxylation or through O-coumaric acid de-carboxilation. The benzoic acid and O-coumaric acid may be formed either by the de-carboxilation or 2-hydroxilation

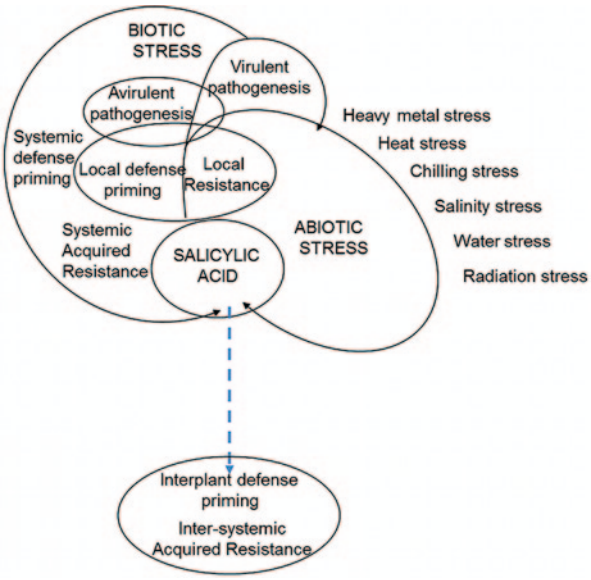

Fig. 6.1 Overlapping horizons of different functions of SA

of trans-cinnamic acid respectively in different species. However, under conditions of inhibition of either of the two pathways in *Arabidopsis*, a third route of biosynthesis of SA via shikimik acid pathway-derived chorismate in chloroplast, is by means of isochorismate synthase (ICS; Hayat et al. 2010). Several conjugated forms of SA have been reported after it is glycosylated, esterified (Balke and Shulz 1987; Popova et al. 1997). The formation of β (beta)-glycosidic SA is catalyzed by the enzyme SA glucosyl transferase (GTase; Balke and Schulz 1987; Yalpani et al. 1992). Other forms identified include 2,3-dihydroxybenzoic acid (2,3-DBHA) and 2,5-dihydroxybenzoic acid (2,5-DBHA; Fig. 6.2).

Volatile MeSA, after infection, in tobacco is produced from SA which on hydrolyzing to SA, can induce defense responses via inter/intra plant signaling (Shualev et al. 1997). It has been found (Chen et al. 1993) that binding of SA to catalase inhibits its activity thereby aiding H_2O_2 production, which activates defense signaling and gene expression (Chen et al. 1993; Durner et al. 1997). H_2O_2, in synergy with ROS, may dispose of the pathogen to death by its direct antibiotic activity providing local acquired resistance (LAR).

Though plants do have prerequisite innate immunity to withstand a biotic challenge, the activation and amplification of inducible form of defense mechanism is more economically augmented during invasion. Mobile signals prepare distal tissues for a possible future attack to induce de novo expression of primary defense mechanism. Recent focus has been on acquisition of primed expression of defense through long distance signaling in plants (Lam et al. 2001). Physical travelling of signals since the work of Ross (1961a, b) on tobacco (TMV infected) and (Green and Ryan 1972) on tomato (insect infestation) till now has well established the concept. However, volatile nature of MeSA and MeJA to prime such responses followed by synergy of vascular transported signal is later suggested by Lam et al. (2001).

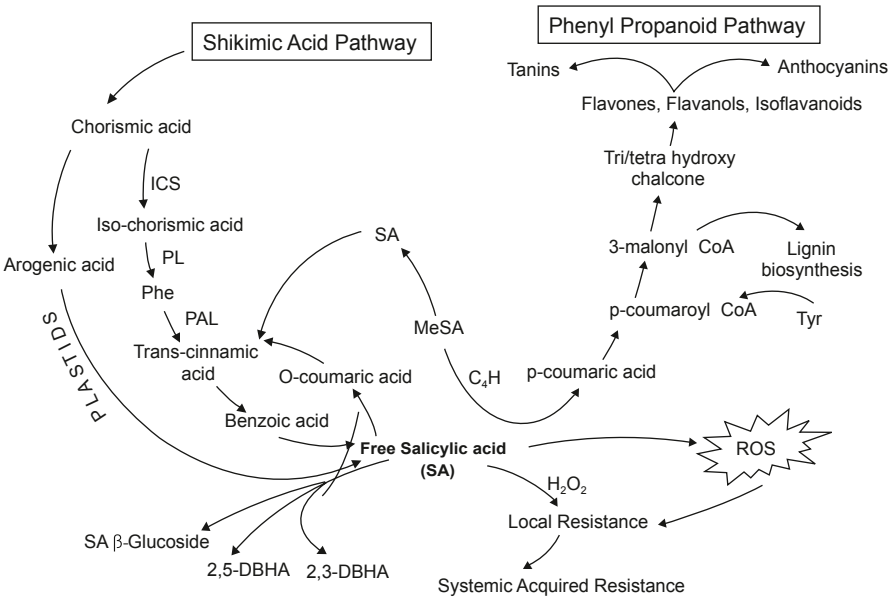

Fig. 6.2 Correlation of SA biosynthesis with phenylpropanoid pathway and elicitation of local and systemic defense (*ICS* isochorismate synthase; *PL* pyruvate iyase; *Pal* phenyl ammonium lyase; *GTase* 3-O-glucosyltransferase; *DBHA* dihydroxybenzoic acids; *C_4H* cinnamate 4-hydroxylase; and *Try* tyrosine)

3.1 Microbial Challenge and SA Upsurge in Tissues

Earlier studies have revealed the major role of SA in counteracting the biotic stress responses in plants. White (1979) first evidenced the involvement of SA in plant defense mechanism. Internal increase in SA levels during pathogenic plant-microbe interaction often facilitates the building-up of the resistance and SAR (Ryals et al. 1996). SA induces resistance against many necrotic or systemic viral, bacterial and fungal pathogens in a variety of plants (Weete 1992; Malamy and Klessig 1992). Durrant and Dong (2004) clearly reviewed the demonstration of SA accumulation and its subsequent role in signaling, by using mutant and transgenic plants. Endogenous SA protects plant from oxidative damage either through aging, abiotic stress or biotic stress (Yang et al. 2004). Bacterial SA degrading enzyme *salicylate dehydroxylase* (*NahG*) when over-expressed in transgenic *Arabidopsis* and tobacco plants, developed inefficient defense response and showed more susceptibility to pathogen infection damage (Gaffney et al. 1993; Delany et al. 1994). Possibly, SA inhibited pathogen growth by repressing the auxin signaling pathways in plants (Wang et al. 2007). Evidence shows that the manipulated host auxin-biosynthesis takes place through interference in normal developmental process (Dharmasiri et al. 2005a, b; Kepinski 2005). Plants probably themselves repress auxin signaling to come up with defense strategy during infection although many pathogens them-

selves produce auxins (Vandeputte et al. 2005; Chung et al. 2003; Ansari and Sridhar 2000; Major et al. 2004) to counter this activity.

3.2 SA Application in Plant Growth and Productivity under Changing Regimes

The applicability of a plant hormone is testified based on an array of physiological responses it manages under normal and stressed conditions which are expressed in plant's optimal growth and yield. Changing climatic conditions led to identification of several phenolic compounds that ensure the resistance of plant to withstand the environmental inhospitable conditions. These phenolics, well known as 'salicylic acids', are also crucial growth regulators under normal plant growth conditions, managing leakage of reactive radicals at their suboptimal level where they can enable ROS signaling and protect it from oxidative damage (Castagne et al. 1999; Yang et al. 2004). SA deficient *NahG* rice contains elevated level of superoxide and H_2O_2, decreased level of SOD and CAT activity, thus increasing susceptibility to oxidative damage caused by the pathogen (Yang et al. 2004). Exogenous applications of SA and its derivatives have shown promising prospects for future agronomical applications, in improving growth and yield of different crop plants such as rice, wheat, oat, maize, soybean, tomato, mustard, gram, carrot and chilli. Furthermore, the dose-dependent exogenous application of SA under environmental or physiological stress conditions viz., high-temperature (Larkindale and Huang 2004; Wen et al. 2008), chilling, water (Hayat et al. 2008; Umebese et al. 2009) and salinity stress (Yusuf et al. 2008) and biotic stress (Esmailzadeh et al. 2008; Chitra et al. 2006; Anand et al. 2008) is well documented, affecting cellular redox homeostasis leading to attenuation of oxidative stress, thereby promoting photosynthesis (Maslenkova et al. 2009), nitrogen metabolism and proline content and growth. The precise internal rise of SA has been evidenced to enable the plant to withstand these harsh conditions expressing defense proteins, optimizing activity of antioxidant system (Krantev et al. 2008; Guo et al. 2009; Yusuf et al. 2008; Hayat et al. 2008), photosynthetic parameters (Fariduddin et al. 2003; Hayat et al. 2008; El Tayeb 2005), enzyme activities, growth attributes and eventual yield of crop plants (Fariduddin et al. 2003; Hayat et al. 2005; Hayat et al. 2007). Therefore, SA finds its extensive applicability in agronomic applications and tissue culture practices (Hayat et al. 2010).

3.3 SA and ROS: A Close Intracellular Interplay

The production of ROS (e.g. superoxide anions) and their secondary derivatives (H_2O_2, OH^-, R*OH etc.) during cellular metabolic processes is a normal phenom-

enon under aerobic conditions (Asada 1999), signaling regulation of plant metabolism internally, and thereby the growth and development of the plant. A drastic change in climatic regimes renders the metabolic machinery to re-setup cell internal molecular profile in charge. This transition costs the leakage of electrons from the electron flow at the cost of reduction of molecular oxygen in chain. These partially-reduced oxygen species' are highly reactive and interact with other molecules to form secondary oxidative free radicals. These free radicals are very toxic if they accumulate beyond a threshold, so they must be detoxified. These highly reactive species interact with different components and biomolecules such as enzymes, transcription factors, inhibitor proteins, nucleic acids and membrane lipids, to disrupt normal metabolic functions. The antioxidation of these species to prevent oxidative injury is achieved by the schematic array of enzyme systems acting in co-operation with low molecular weight thiol buffers, better known as antioxidative molecules. The former one incorporates SOD, APX, GPX, CAT, GRs, while the latter includes ascorbate (AsA), glutathione (GSH; Noctor and Foyer 1998; Asada 1999), proline, polyamines, etc. Restriction of ROS level within a limit to ensure the survival, and relative activity of the antioxidant system is important to balance the redox homeostasis for survival and normal growth (Scebba et al. 1999). This is the reason why stress tolerance is often interpreted in terms of improved efficiency of antioxidative system in plants. In several studies, it was evidenced that endogenous higher level of SA deactivates the activity of antioxidant system, while co-operating with ROS. Furthermore, Kuzniak and Sklodowska (2005) have indicated that tomatoes infected with *B. cineria*, initiate the rise of peroxysomal antioxidant enzyme system activity, which soon declines with eventual development of disease symptoms.

Research in the last few decades has shown that SA interplays with ROS to signal genetically-controlled defense reactions, thus activating related genes followed by progression towards PCD (Overmyer et al. 2003; Durrant and Dong 2004; Fobert and Després 2005; Foyer and Noctor 2005). The concept of crosstalk between SA and ROS in defending stress is considered crucial during local and systemic defense responses (Overmyer et al. 2003; Durrant and Dong 2004). Initial buildup of internal SA favours antioxidative defense system (over-expression and activation), thus altering the activity of regulatory factors through cellular signaling, although on reaching up to a threshold it becomes suppressive to plant growth metabolism (Fariduddin et al. 2003), suggesting oxidation (inactivation) of regulatory factors and saturation of thiol buffers, and thus suppressing the expression and eventual activity of antioxidant enzymes and molecules. This results in instant upsurge of reactive species' and free radicals and subsequent oxidative burst. During the hypersensitive responses, the ROS and SA accumulation at higher levels (Pasqualini et al. 2002) appears to regulate genetic program in favour of cell death propagation (Overmyer et al. 2003). Apoplastic and (Jabs et al. 1996) mitochondrial burst of ROS accelerate PCD in synergy (Maxwell et al. 2002; Dutillent et al. 2003). However, there are several other sites and mechanisms of ROS generation (Rao et al. 1997; Kawano 2003). SA has clearly been shown to inhibit the activity of antioxidant enzymes, namely APX and CAT, leading to over-accumulation of superoxide anion (ROS). SA was shown to block the activity of catalase. This inhi-

bition leads to accumulation of H_2O_2 (Chen et al. 1995). Using the dimethyl urea (a trap for H_2O_2), the effect of SA has been shown to be decreased suggesting that SA acts through H_2O_2 at least partly (Rao et al. 1997). Though the production of H_2O_2 has been suggested to be crucial for the induction of disease resistance, yet H_2O_2 alone is insufficient to trigger PCD (Alvarez et al. 1998; Levine et al. 1994; Orozco-Ca'rdenas et al. 2001; Pellinen et al. 2002). Application of exogenous SA leads to increased cellular H_2O_2 level in plant tissues when applied in suitable dose, and has been found to induce HR signaling and SAR against pathogen (Lamb and Dixon 1997).

3.4 SA Regulated ROS Signaling, the Determinant of Defense Metabolism and Tissue Fate

The role of ROS has also been explained in the regulation plant metabolism, thereby in growth and development of the plant. ROS signals for gene expression (Desikan et al. 2001; Vanderauwera et al. 2005) and to modulate activity of other crucial signaling compounds, e.g. MAP kinase (Rentel et al. 2004). Modification of protein quaternary structure and activity of thiols in cellular pool has also been suggested to have a widespread mechanism affecting the functional proteins regulated by cytoplasmic ROS level (Cooper et al. 2002). Spatio-temporal distribution of ROS, therefore, must be regulated sophistically (Mittler 2006) at the boundary of compartmentalized gradients of organelles. The evolution of antioxidant system enables plants to manage ROS toxicity within the limit to ensure their role as signal transducers (Mittler 2006). Increased level of internal SA binds and inhibits the activity of catalase (Chen et al. 1995) resulting in over-accumulation of H_2O_2. Continuous and sustained accumulation of ROS in the absence of complementary activity of antioxidant system donates free electrons to other electronegative groups secondarily forming reactive nitrogen species and organic free radicals. This initiates an oxidation chain reaction of membrane phospholipids disrupting homeostasis and cellular integrity. The over-phosphorylated defense signaling and change in membrane permeability and cellular pH commits this shift towards death signals. Reactive nitrogen species react with ROS to form lethal peroxynitrile and nitrosonium ions, adding further nitrosative stress (Zhao 2007; del Río 2006; Hayat et al. 2010).

The determining step as to when and where a cell has to commit for cell death is also regulated by the active participation of plant hormones. Once oxidative stress crosses the threshold (a balance between oxidation and induced preventive reduction to prevent minimal required setup for basal metabolic processes), the cell switches over to PCD with simultaneous elicitation (HR) as defense mechanisms in neighbourhood. Different combinations of hormones (SA, JA, NO) and secondary signals (ROS, CaM, H_2O_2, Ca^{2+}, kinases, lipid derivaties) have been found to be involved in the initiation, propagation and containment phase of HR or PCD (Dangl and Jones 2001; Overmyer et al. 2003). SA also mediates the lipid peroxidation,

which plays a key role in initiating defense response. ROS induced peroxidation of membrane lipids and proteins perturbs the homeostasis and induces the decompartmentalization of organelles. Induced ROS level not only have a toxic effect during cell death of oxidizing cellular components, but additionally constitute proximal (H_2O_2) and distal (methylated forms of SA and JA) signals to balance information between metabolism and environment.

3.5 SA Signaling and Regulation of Gene Expression

The induced systemic resistance, effective against multiple pathogen types and root colonizing bacteria, is mediated through R gene which detects specific pathogenic Avr proteins of the pathogen. ISR (Induced Systemic Resistance) is independent of SA and responds to JA and ethylene signaling pathways as indicative of *NahG* mutants. The involvement of *NPR1*-dependent SA signaling with *WRKY* (Chaturvedi and Shah 2007) and *MYB* (Galis and Matsuoka 2007) transcription factors, *SABP-2*, *MRP*-type ABC transporters (Yazaki 2005) and *NPR1-TGA* protein complex in activation of SA responsive elements of PR genes have been recently recognized as components of SA-based gene expression regulation and signal transduction cascade. Interestingly, ISR requires activation of *NPR1* gene product, a crucial mediator of SAR through SA signaling (Wang et al. 2006; Chaturvedi and Shah 1997). This is contrary to JA signaling where plant expresses PR proteins and acquires heightened local as well as systemic resistance against a broad spectrum of pathogens. The expression of *PR1* gene is poorly induced in *NPR1* mutants following SA application or pathogen challenge with the compromised basal resistance and SAR induction (Durrant and Dong 2004). The promoter of *NPR1* contains *WRKY* binding sites (W-box), very common regulatory transcription factors. These are also shown to be involved in the expression of *NPR1* (Yu et al. 2001). DNA binding of *NPR1* to some TGA proteins alters the expression of *PR1* genes (Durrant and Dong 2004; Lebel et al. 1998; Zhang et al. 2003). The cellular redox status also regulates the activity of TGA proteins (Després et al. 2003).

The presence of *NPR1* independent SA signaling was evidenced by comparative studies on *nahG* and *NPR1* mutants where the *nahG* effectively compromised their resistance against pathogen infection, while the *NPR1* mutants did not (Raridan and Delaney 2000; Kachroo et al. 2001; Takahashi et al. 2002). Also *SSI2* mutants constitutively accumulating SA at elevated level, constitutive expression of *PR* genes were demonstrated in *NPR1* mutants (Clarke et al. 2000; Shah et al. 2001) and these double mutants confer resistance against oomycete and bacterial pathogens. This suggests that other pathogen induced signals may be required for the activity of *NPR1*. Li et al. (1999) also reported *Arabidopsis SNI1* mutant which showed SA dependent-*NPR1* independent defense in the absence of *NPR1*. *SNI1* was suggested to be repressor of PR gene expression based on nuclear localization and DNA binding studies (Durrant and Dong 2004). *WHRLY (WHY)* genes encoding DNA binding proteins as the promoter of *PR10a* gene single strand show another example of

SA regulated *NPR1* independent defense mechanism (Desveaux et al. 2002). *NPR1* mutants treated with exogenous SA, induced the sDNA binding of *WHY1* protein of *Arabidopsis thaliana* (Desveaux et al. 2004).

4 Conclusion

Cellular redox status is the imprint of ratio of reduced-to-non-reduced compounds (Kornas et al. 2010) determined through the equilibrium of antioxidant system and ROS (Gill and Tuteja 2010) regulating directly the cell metabolism. The role of SA in controlling the interface of total cellular oxidation state and antioxidation has been evidenced recently through research and literature. SA is not only shown to regulate cell metabolism under abiotic and biotic stress conditions within the systemic and par-systemically, but also during the synergy of two major causes of stress, often met whenever disease epidemiology has been described. Nevertheless, the role of abiotic stress in priming the biotic stress has been often suggested but scarcely explained on the basis of regulatory affairs revealing molecular cause. SA appears to be the potent plant hormone to regulate the defense signaling internally under these conditions and may find its future prospects in modern agronomy to protect the crop at high time of disease incidence to favour enhanced growth and production. The detailed research in this discipline and critical reviews therefore, are awaited to update our understanding of the overlapping roles and regulations of SA.

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Chapter 7
Genotoxic Stress, DNA Repair, and Crop Productivity

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1 Introduction

It is generally acknowledged that plants exposed to adverse environments undergo oxidative stress, resulting in severe injury at the cellular and molecular levels. Environmental pollution, caused by anthropogenic activities, as well as water stress and high temperature conditions associated with rapid climate changes contribute to soil deterioration and thus affect crop productivity (Ahmad et al. 2010). When plants are challenged with oxidative stress, protective responses which include a complex network of integrated molecular and cellular events, are activated. Initial stress perception and transduction of stress signal are key factors leading to modulation of gene expression and finally to the plant response.

Till date, most agronomically relevant genotypes deriving from long-term selection lacks the ability to adapt to environmental fluctuations and suboptimal growth conditions. Such an unfavorable situation is in contrast with the increasing global food demand. According to recent estimates, the world population will rise from 6.8 to 9.1 billion in 2050 and nearly all the expected growth will be localized in developing countries (Alexandratos 2009). For this reason, strong efforts are currently dedicated to understand the multiple aspects of plant stress molecular biology in order to acquire novel insights and provide advanced tools/technologies for improving plant survival and ensure optimal agronomical performance.

In recent years, molecular breeding and genetic engineering have contributed significantly to the basic knowledge of the cellular mechanisms involved in stress response, suggesting new strategies to enhance stress tolerance in plants (Sreeniva-

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sulu et al. 2007). Despite this, there are some aspects of the plant stress response which still need to be explored.

This chapter will focus on the effects of oxidative stress within the nuclear compartment where DNA becomes the main target of the highly toxic reactive oxygen species (ROS). Several abiotic stresses (e.g., water deficit, high salt, UV-light, ionizing radiation, heavy metals, and ozone) can trigger DNA damage by directly acting on the double helix structure or by enhancing the intracellular ROS levels (Tuteja et al. 2009). Oxidative DNA damage requires prompt repair to maintain genome integrity and preserve the fidelity of genetic information. The response of plant cells to genotoxic stress relies on the activity of multiple DNA repair pathways which share some common elements with animal cells, but also own distinctive features, unique to the plant kingdom.

This chapter will cover the recent advances in the field of plant DNA repair, highlighting novel gene functions directly involved in the response mechanism to abiotic stresses, not only at the plant level but also in seeds. As for the molecular mechanisms involved in the regulation of DNA repair, some intriguing findings that link the genotoxic stress response to complex cellular networks, e.g., the small regulatory Ribonucleic acids (RNAs) and the circadian clock, are reported.

2 Genotoxic Stress: Effects Inside and Outside the Nucleus

Differently from animal systems where genotoxic agents are mainly investigated as a major cause of human cancer (Evans et al. 2004), genotoxic stress in plants is essentially considered as a critical factor which impairs fitness and productivity by affecting genome stability (Tuteja et al. 2009; Roldan-Arjona and Ariza 2009).

The most frequent DNA lesions are oxidative base damage, alkylation, deamination, abasic (apurinic and/or apyrimidinic, AP) sites and single-strand breaks (SSBs; Tuteja et al. 2009). Accumulation of 7,8-dihydro-8-oxoguanine (8-oxo-dG), the most common oxidized base lesion formed *in vivo* in animal cells, has been investigated also in plants (Balestrazzi et al. 2009, 2011a) and cell suspension cultures (Balestrazzi et al. 2010) challenged with different abiotic stresses. Other DNA lesions, e.g., UV-photoproducts and bulky chemical adducts, dramatically alter the DNA structure (Tuteja et al. 2009; Roldan-Arjona and Ariza 2009). Perception of genotoxic stress and the activation of signal transduction lead to cell cycle arrest and this allows the regulated expression of DNA repair genes.

Several genotoxic agents can simultaneously cause both DNA damage within the nucleus and oxidative stress outside the nuclear compartment, leading to complex molecular activity which might ultimately result in programmed cell death (PCD). Besides the enhancement of DNA repair, antioxidant mechanisms are activated inside and outside the nuclear compartment. There is evidence to demonstrate that the control of the nuclear thiol-disulfide redox state is dependent on reductant molecules such as glutathione and thioredoxin, as well as on the presence of specific isoforms of antioxidant enzymes. All these nucleus-specific pathways are distinct

from the counterparts located in the cytosol and in other subcellular compartments (Go and Jones 2010). This is the case of the protein 1-Cys peroxiredoxin (1-Cys Prx), activated under oxidative stress conditions in the nucleus of wheat (*Triticum aestivum* L.) seed cells following reduction mediated by the NADPH thioredoxin reductase/thioredoxin h (NTR/Trx h) system (Pulido et al. 2009).

Recently, Vanderauwera et al. (2011) reported that the extranuclear protection of chromosomal DNA in *Arabidopsis thaliana* plants involves the coordinated action of ROS-scavenging pathways localized both in the cytosol and peroxisomes. The antioxidant enzymes catalase (CAT2) and ascorbate peroxidase (APX) were found to play a key role, mediated by a WEE1 kinase-dependent cell-cycle checkpoint, in preserving the plant genome integrity (Vanderauwera et al. 2011).

The emerging picture highlights the complexity of the interactions, involving the nucleus and the other subcellular compartments, which are an integral part of the cell response to genotoxic stress.

3 Genotoxic Adaptation

Genotoxic adaptation represents an interesting aspect of the plant response to adverse environmental conditions. It has been reported that exposure to minimal stress levels causes a limited cellular damage which triggers the so-called "adaptive response" (AR), responsible for increased resistance to high levels of the same stress or other stresses (Dimova et al. 2008). The term "genotoxic adaptation" is used to address the response of cells exposed to low doses of genotoxic agents and the subsequent induction of stress tolerance.

The AR involves activation of signaling pathways and up-regulation of gene expression, which contribute to multiple cell defense mechanisms such as ROS scavenging, DNA repair and production of antioxidant compounds. Thanks to these mechanisms, the "primed" cells can better withstand genotoxic stress. Till date, contrasting findings have been reported concerning the involvement of DNA repair genes in AR. It has been suggested that the up-regulation of DNA repair genes might be essential; however the experimental results have not always supported this hypothesis.

Clastogenic adaptation, defined as the protection of plant cells against chromatid aberrations induced by genotoxic agents, can be obtained by pre-treating cells with non-effective low doses of the same agents in order to elicit a protective response. It has been reported that clastogenic adaptation correlates with removal of O^6-methylguanine from DNA after pre-treatments (Baranczewski et al. 1997). The study carried out by Angelis et al. (2000) in *Vicia faba* root tip nuclei pretreated with alkylating mutagens, demonstrated that adaptation takes place soon after the pre-treatment. The level of AP sites, measured using the comet assay, revealed a significant reduction in the number of these lesions, which were preferentially removed while the majority of DNA damage was not affected. This finding suggests the possible involvement of AP glycosylases, typical components of the base excision repair (BER) pathway, in genotoxic adaptation.

An example of genotoxic adaptation in plants has been studied in the case of aluminum (Al) pollution. At high concentrations (0.5–2.0 mM), Al inhibits root growth inducing DNA damage and cell cycle arrest at G_2 phase (Rounds and Larsenm 2008). Genotoxic adaptation to aluminum has been recently demonstrated by Achary and Panda (Achary and Panda 2010) in root cells of *Allium cepa* L. Low concentrations (1–10 μM) were found to be critical for induction of AR, which conferred protection against genotoxic agents. Similarly, low-dose treatments carried out with cadmium were able to induce genotoxic adaptation in root cells of *Hordeum vulgaris* (Joutchev et al. 2001).

Interestingly, Patra et al. (2005) found that pre-treatments with salicylic acid (SA) could alleviate the effects of oxidative stress in root meristem cells of *Allium cepa* L., since SA enhances the level of hydrogen peroxide, which in turn induces increased antioxidant enzyme activities. They also suggested the presence of two distinct genotoxic signaling pathways, one of them mediated by DNA damage and the other one activated independent of DNA injury.

The recent findings, highlighting the response of different DNA repair genes in relation to abiotic stresses (Balestrazzi et al. 2011a) represent a valuable opportunity for a deeper investigation on the possible links between DNA repair and genotoxic adaptation.

4 DNA Repair in Response to Abiotic Stresses: Current Knowledge and Potential Applications

Environmental stresses, which severely damage the nuclear compartment and thus compromise plant development, are counterbalanced by ROS scavenging and DNA repair mechanisms. However, while the plant antioxidant machinery has been extensively investigated (Gill and Tuteja 2010), research in the field of plant DNA repair is still too limited, despite the relevant link between this aspect of plant physiology and the current agronomical issues (Balestrazzi et al. 2011a). In the present paragraph, attention will be focused on the following environmental abiotic stresses: salinity, drought, air pollution, and heavy metals.

Now a days, excess salt in the soil represents a major cause of soil degradation which limits crop productivity, particularly in the arid and semiarid regions of the world. The molecular mechanisms for salt tolerance, which are not always equally effective in different crop species and varieties, need to be properly dissected, although varieties with salt tolerance have been obtained by traditional breeding as well as using gene-transfer techniques (Lee et al. 2007). High salinity induces osmotic stress accompanied by ROS accumulation and for this reason most researchers have focused their attention on the antioxidant enzymes and genes stimulated under salt stress in tissue- and genotype-specific manner (Mhadhbi et al. 2011).

As for the role of DNA repair pathways in response to salt stress and their possible regulation, both at the enzyme and gene level, information is still scanty. DNA repair genes that are significantly up-regulated under salt stress might represent

interesting tools to improve the plant tolerance and the work carried out on plant DEAD-box helicases has undoubtedly contributed to expand the knowledge in this research field (Vashisht and Tuteja 2006). DEAD-box helicases play an essential role in several basal processes of DNA metabolism, including DNA repair. They are motor proteins that catalyze the ATP-dependent unwinding of duplex DNA/RNA at the damaged area, allowing repair. DEAD-box RNA helicase-encoding genes are up-regulated in response to salinity stress, as reported for the sorghum (*Hordeum vulgaris* L.; Nakamura et al. 2004). Stress-responsive helicases have been characterized also in *Pisum sativum*. The *PDH45* helicase is induced in pea seedlings in response to high salt and the overexpression of the *PDH45* gene in tobacco resulted in salinity tolerance (Sanan-Mishra et al. 2005). A similar response was observed for the *PDH47* gene, induced by cold and salinity stress (Vashisht and Tuteja 2005).

Water deficit is another major abiotic stress, affecting crop growth and reducing sustainable food production. Besides the known general effects of drought stress on vegetative growth, water shortage is detrimental at the reproductive stage in most cereals and strong efforts have been made to improve drought tolerance at this specific stage (Oh et al. 2009). Drought tolerance relies on multiple gene functions, some of them participating in the early response (e.g., signal transduction) and others required during the late response. The latter includes not only genes for water transport and osmotic balance but also antioxidant and DNA repair gene functions (Zhu 2001). As for the contribution of antioxidant cellular mechanisms to drought tolerance, the key role of catalase, superoxide dismutase and peroxidase enzymes has been demonstrated together with the involvement of novel ROS scavenger such as metallothioneins (Seki et al. 2001). Recent studies carried out on the model legume *Medicago truncatula* (barrel medic) have highlighted the novel DNA repair gene functions, participating in the nucleotide- and base-excision repair pathways (NER and BER, respectively), which turned out to be significantly up-regulated in response to water stress (Macovei et al. 2010; Macovei et al. 2011a, b). A detailed description of these results is given in the following paragraph.

Air pollution, resulting from the release of toxic gases and other poisons to the environment, is another abiotic stress responsible for crop yield losses (Seyydnejad et al. 2011). Pollutants include sulfur and nitrogen oxides, carbon monoxide, toxic metals, organic molecules, and radioactive isotopes. As for several other environmental stresses, exposure to air pollutants causes oxidative stress with the consequent activation of ROS scavenging processes. This is another relevant research field in which molecular investigations related to genotoxic effects and protection mechanisms should be supported.

Environmental heavy metal pollution has been dramatically increasing due to extensive mining and industrial activities. Heavy metals, e.g., copper, cadmium, chromium, and lead, released from different anthropogenic sources severely affect ecosystems and crop productivity, posing a risk to the food chain (Peralta-Videa et al. 2009). The genotoxic effects of heavy metals have been widely investigated in animal cells (Garcia-teston et al. 2010). In plants, several deleterious effects induced by metal exposure have been attributed to oxidative stress (Benavides et al. 2005). Intoxication with pollutant metals generates ROS which need to be removed

to avoid oxidative injury. An intriguing aspect of ROS scavenging in plants challenged with heavy metals is related to the small metal-binding cysteine-rich proteins metallothioneins (MTs; Cobbet and Goldsbrough 2002). Besides their role in heavy metal detoxification, MTs have recently been found to act as ROS scavengers and signal molecules outside and inside the nucleus, highlighting their possible interaction with the DNA repair machinery (Wang et al. 2010; Balestrazzi et al. 2011b). Till date, there is only indirect evidence of the putative protective role played by MTs in the nucleus. Balestrazzi et al. (2009) demonstrated that expression of the $PsMT_{A1}$ gene, encoding a metallothionein-like protein from *P. sativum*, confers protection against oxidative stress in the nucleus, reducing the level of oxidative DNA damage. On the other hand, studies on animal cells have reported that MTs are transported into the nucleus where they make zinc available to transcription factors (Cherian and Apostolova 2000). MTs can help overcome defective DNA repair functions as suggested by Yeong et al. (2004) who demonstrated that the overexpression of the *MTIII* gene, encoding a type 3 metallothionein, prevents accumulation of the oxidized base 8-oxoguanine in normal and OGG1(8-oxoguanine DNA glycosylase/lyase)-depleted cells exposed to g-rays.

As for the direct involvement of DNA repair genes in the plant response to heavy metals, evidence has been recently reported in *M. truncatula* by Macovei et al. (2010, 2011). The *NER* and *BER* genes up-regulated in aerial parts and roots of barrel medic plantlets exposed to toxic copper doses, will be described in detail in the following paragraph.

5 Nucleotide Excision Repair Genes Responsive to Abiotic Stresses

Nucleotide excision repair is a versatile DNA repair system involved in the removal of different helix-distorting DNA lesions (Liu et al. 2010) which includes two distinct subpathways, the global genome (GG)-NER which targets lesions at the genome level, comprising the majority of NER activities, and the transcription-coupled (TC)-NER which acts more rapidly and removes lesions from the transcribed strand of active genes (Hanawalt 2002).

The NER pathway is regulated at the transcriptional level by different mechanisms, including circadian regulation (Kang et al. 2009). At the post-translational level, mono- or poly-ubiquitination or SUMOylation of NER components play a key role in the cellular choice between DNA repair and error bypass (Ulrich 2009).

Some genes belonging to TC-NER have recently demonstrated to be significantly up-regulated in response to osmotic and heavy metal stresses (Macovei et al. 2010, 2011; Balestrazzi et al. 2011c). The application of DNA repair enzymes to sites of stalled transcription complexes and the related mechanisms of damage removal is a relevant part of the cellular responses to genotoxic stress.

Transcription Elongation Factor SII (TFIIS), which interacts with RNA polymerase II (RNAPII) allowing the efficient synthesis of long transcripts, participates

in transcription-coupled repair. The proposed TC-NER model in animal cells is essentially derived from the finding that TFIIS is able to assist RNAPII to bypass transcription arrest sites (Gnatt 2002). However, a recent study carried out by MacKinnon-Roy et al. (2011) has demonstrated that the TFIIS function is not a limiting factor for TC-NER since RNA interference directed against TFIIS did not affect the sensitivity of human cell to oxidative stress. In a different report (Jensen and Mullenders 2010), downregulation of human TFIIS by RNA interference significantly delays, but does not suppress, the recovery of UV-inhibited transcription. According to these authors, downregulation of TFIIS causes an increased level of hyperphosphorylated RNAPII, which in turn needs to be degraded.

In plants, there are only a few reports dealing with the TFIIS function (Grasser et al. 2009; Macovei et al. 2011; Balestrazzi et al. 2011c) and the presence of a *TFIIS-like* gene encoding a TFIIS-related protein has been described (Macovei et al. 2011). The *TFIIS-like* gene encodes a product with unknown function, conserved among plant species, and shares some structural features with the canonical TFIIS protein, elongin A (Transcription Elongation Factor SIII), and CRSP70 (Cofactor Required for Sp1 activation). The expression profiles of the *TFIIS* and *TFIIS*-like genes were analyzed in barrel medic plantlets exposed to heavy metal and osmotic stress, respectively. Both genes were significantly up-regulated in aerial parts and roots of plants exposed to toxic copper doses as well as in plants grown in the presence of polyethylene glycol (PEG) as osmotic agent (Macovei et al. 2011).

6 Expression Profiles of Base Excision Repair Genes in Response to Abiotic Stresses

Oxidative DNA damage is associated with the accumulation of the oxidized base 7,8-dihydro-8-oxoguanine (8-oxo-dG), which is highly mutagenic since it frequently mispairs with the incoming dAMP during DNA replication, causing G:C to T:A transversions. Oxidized bases are usually removed through the BER pathway, initiated by DNA glycosylases that hydrolytically cleave the glycosylic bond between the target base and deoxyribose, releasing the damaged base and leaving an AP site that are further processed (Roldan-Arjona and Ariza 2009).

Poly(ADP-Ribose)Polymerase 1 (PARP-1), a nuclear protein which interacts with factors involved in the modulation of chromatin architecture and cell recovery from DNA damage, is among the BER components responsive to stresses (Douchet-Chablaud et al. 2001). Both the *Arabidopsis PARP* genes *AtPARP1* and *AtPARP2* are stress responsive and the preferential accumulation of the *AtPARP2* transcript in response to heavy metal stress suggests for specific roles played in plants by each gene (Douchet-Chablaud et al. 2001).

In plants, 8-oxoguanine DNA glycosylase/lyase (OGG1) and formamidopyrimidine-DNA glycosylase (FPG) play similar roles within the BER pathway involved in the removal of oxidized bases, e.g., 8-oxo-dG and formamidopyrimidine (FAPy) lesions. Macovei et al. (2011b) have investigated the possible roles played in plants

by the barrel medic *MtOGG1* and *MtFPG* genes. The expression profiles of both genes were evaluated in barrel medic plantlets grown *in vitro* under oxidative stress conditions induced by copper and PEG, respectively.

The *Tdp1* gene encoding tyrosyl-DNA phosphodiesterase has been extensively investigated in animal cells, due to the role of this enzyme in the repair of topoisomerase I-DNA covalent lesions (Yang et al. 1996). Macovei et al. (2010) reported for the first time in plants on the *Tdp1* gene family from barrel medic, composed of two members, *MtTdp1*α (alpha) and *MtTdp1*β (beta). The expression profiles of the *MtTdp1* genes were evaluated in plantlets grown *in vitro* using copper and PEG as stress agents. Both *Tdp1* genes were significantly up-regulated in response to heavy metal and osmotic stress, suggesting for a requirement of the *Tdp1* function under stress conditions. From this point of view, the response of *MtTdp1* genes to stress seems to be in agreement with the literature available on the animal *Tdp1* gene (Lu et al. 2004).

In a recent work (Lebedeva et al. 2011), a novel role played by TDP1 has been reported, since the enzyme contributes to the removal of abasic or AP sites, the key BER intermediates (Barnes and Lindhal 2004). The human TDP1 protein can initiate repair of AP-sites within the APE (apurinic/apyrimidinic endonuclease I)-independent BER pathway through the cleavage of the AP site and release of 3′- and 5′ phosphate termini. Due to the multiple functions played by the human TDP1 enzyme in DNA repair, it is possible that the plant *tdp1*α (alpha) and *β* (beta) genes might represent effective tools for biotechnological applications aimed at improving stress tolerance in crops.

7 Environmental Stresses, DNA Repair and Seed Vigor

Despite the relevant effects of environmental stresses on crop productivity, only limited attention has been given to DNA repair in seeds and to its involvement in those mechanisms conferring seed protection against stress. It is generally acknowledged that high crop productivity requires efficient and uniform seed germination and increased seed vigor. The effects of adverse environmental conditions on seed quality can be easily scored using common indicators of viability such as germination speed and percentage (Bewley and Black 1994).

Dry seeds are tolerant to adverse environments; however germination and the first stages of seedling establishment represent highly vulnerable steps of the plant lifecycle (Kranner et al. 2010). Drought, osmotic stress, salt, low temperatures, and heavy metals adversely affect seed germination and in natural environment seeds might frequently be challenged by detrimental combination of stresses (Lee et al. 2010).

The stress response in germinating seeds is regulated at different levels and signaling events play a crucial role. It has been suggested that ROS could be involved in the regulation of cell signaling in dry seeds and within this context distinct roles have been hypothesized for short- and long-lived radicals (El-Maarouf-Bouteau and

Bailly 2008). Short-lived ROS, e.g., OH, react with receptors located in proximity of their production site while long-lived ROS, particularly H_2O_2, can move far from the production site and interact with other cellular targets (Moller and Jensen 2007). ROS signaling in dry seeds might be mediated by short-lived radicals while short- and long-lived radicals might participate in signaling during rehydration (Moller and Jensen 2007). ROS-mediated signals are converted in active antioxidant and repair responses while the failure of protective mechanisms increases oxidative damage, leading to seed deterioration (Kranner et al. 2010). In some cases, the endogenous antioxidant defenses might be enhanced by treatments with exogenous factors such as fungi (*Trichoderma* spp.) which can alleviate abiotic stresses during imbibition and germination (Mastouri et al. 2010).

On the other hand, a complex picture is emerging since novel regulatory mechanisms have been highlighted recently. Indeed, the involvement of miRNA in the control of seed germination under stress conditions has been reported by Kim et al. (2010), who demonstrated that overexpression of *miR402* accelerated seed germination and seedling growth under salt stress conditions while dehydration and cold stress positively affected only seed germination.

Information, although limited, is available currently concerning DNA repair mechanisms in seeds. The role of DNA repair during seed imbibition has been investigated by Macovei et al. (2010) who analyzed the expression profiles of the barrel medic *MtTdp1*α and *MtTdp1*β genes. It is known that water uptake during seed rehydration leads to resumption of respiratory activity and protein synthesis, followed by several cellular and biochemical events, among which DNA repair plays an essential role to guarantee genome stability (Holdsworth et al. 2008). An extension of the G_1 phase, occurring during seed imbibition, allows prereplicative DNA repair, thus preventing the possible deleterious effects derived from a damaged template (Whittle et al. 2001). Although both the *MtTdp1* genes were significantly up-regulated during seed rehydration, a temporal shift in transcript accumulation was observed, since the *MtTdp1α* and *β* mRNAs peaked at 12 and 8 hours, respectively.

The reliability of seed imbibition as a system for the study of novel DNA repair functions was further confirmed in a different study carried out in barrel medic genes (Macovei et al. 2011). The requirement for the *MtTFIIS*-like transcript at 8 hours following seed rehydration was evidenced. The *MtTFIIS* gene was also up-regulated during seed rehydration although a temporal shift in transcript accumulation was observed compared to *MtTFIIS*-like gene (Macovei et al. 2011a). Additional DNA repair functions have been analyzed during seed imbibition. Both the *MtOGG1* and *MtFPG* genes, encoding 8-oxoguanine DNA glycosylase/lyase and formamidopyrimidine-DNA glycosylae, respectively, were up-regulated at 8–12 hours following rehydration, confirming the requirement for the BER pathway during early germination (Macovei et al. 2011b).

Salt stress can affect seed germination through osmotic effects which ultimately lead to uncontrolled ROS accumulation and extensive cellular damage (Bailly 2004). High salt concentration reduces water potential delaying water uptake by seeds. Declines in seed germination under salt stress conditions have been well documented (Jamil et al. 2006) and adaptive strategies such as enforced dormancy,

might be induced to prevent seed germination under stressful environments (Tilki and Dirik 2007).

The expression profiles of several DNA repair genes, namely *MtTdp1a*, *MtTdp1b*, *MtTFIIS-like*, *MtTFIIS*, *MtOGG1* and *MtFPG*, were evaluated in barrel medic seeds imbibed with the osmotic agent polyethylene glycol (Balestrazzi et al. 2011c). This high molecular weight compound cannot cross the cell wall, avoiding cell plasmolysis. When cells are exposed to salt stress, rapid accumulation of ions in the intracellular environment takes place and membrane permeability is compromised. Osmotic stress induced by PEG can be reduced as a consequence of osmoprotectant accumulation (Zhao et al. 2010).

Germination was significantly reduced by PEG which also induced consistent 7,8-dihydro-8-oxoguanine (8-oxo-dG) accumulation. All the genes were responsive to osmotic stress during seed imbibition with the osmotic agent and most of them revealed a delayed and significant up-regulation at 12–24 hours, when the highest levels of DNA oxidative damage were also observed. As expected, there was a temporal shift in water up-take and occurrence of DNA damage in PEG-treated seeds.

Seed germination can be severely affected by heavy metals. It has been reported that cadmium uptake by germinating rice seeds resulted in osmotic effects associated with oxidative damage. A proteomic approach revealed cadmium-induced protein profiles with up-regulated protein profiles related to signal transduction and antioxidant defense (Ahsan et al. 2007). However, no reports dealing with the role played by DNA repair genes in seeds exposed to heavy metals are currently available.

8 Role of Telomerase in DNA Repair and Genotoxic Protection

Telomerase, the enzyme required for telomere synthesis and maintenance, is a critical element for the prevention of aging in animal cell. Telomerase is a ribonucleoprotein composed of a catalytic subunit owing reverse transcription activity (TERT) associated with an RNA subunit (telomerase RNA component, TR). The latter acts as a template for the addition of telomere repeat sequences carried out by TERT (Tomas-Loba et al. 2008).

There is recent evidence to show that telomerase also owns some telomere-independent activities, among which are DNA repair and protection of mitochondrial DNA (mtDNA) against oxidative stress (Majerska et al. 2011). In human fibroblasts, increased oxidative stress accelerates telomere shortening and this is caused by the reversible, stress-induced translocation of telomerase from the nucleus to mitochondria (Haendeler et al. 2009). It has been demonstrated that the human telomerase (hTERT) is localized within the mitochondrial matrix where it binds and protects mtDNA. It seems that both hTERT and hTR participate in the modulation of DNA repair and, consequently, in the cell response to genotoxic stress by stimulating the ATM and Rad3 (ATR)-related sensor kinase (Kedde et al. 2006). Additional

nontelomeric functions such as transcriptional regulation, have been proposed for hTERT whose ectopic expression causes remarkable alterations in gene activity and the recent study by Park et al. (2009) has demonstrated that hTERT is a component of the transcription activator complex of the Wnt/APC/β-catenin pathway.

As for plants, experimental evidence concerning the telomere-independent activities of telomerase is missing. However, it has been reported that tobacco BY-2 cells, exposed to cadmium sulfate and showing chromosome fragmentation, are able to overcome the heavy metal treatment and complete recovery was concomitant with a 2.5-fold increase in telomerase activity, thus suggesting the involvement of this enzyme in DNA repair (Fojtova et al. 2002). In a different work, Riha et al. (2002) investigated DSB repair in *Arabidopsis* plants defective in Ku70, a critical component of the Non Homologous End Joining (NHEJ) pathway localized at the telomere cap, are hypersensitive to gamma irradiation. Differently from animals, in the *Arabidopsis* Ku70 mutant the control of telomere length is altered to increase significantly by the second generation.

Restoration of telomeres at chromosome ends occurs early during seed imbibition, when DNA repair is required to ensure genome integrity and it has been suggested that a level of telomerase might be essential for the control of seed viability during long-term storage (Watson and Riha 2010). Till date, only a few reports are available dealing with the possible role of telomerase in seed aging and for this reason additional studies are required.

9 Modulation of DNA Repair in Response to Genotoxic Stress: Role of small Regulatory RNAs

Recent studies have highlighted the correlation between the ability to withstand environmental stresses and the complex mechanisms of RNA silencing and epigenetic activities occurring in plants (Riuz-Ferrer and Voinnet 2009). Gene expression can be modulated by small regulatory RNAs, a broad class of molecules with regulatory functions which include microRNAs (miRNAs), small interfering RNAs (siRNAs), and transactivating siRNAs (ta-siRNAs; Mallory and Bouche 2008).

The miRNAs represent a highly conserved 18–22 bp RNA family that bind to mRNA, blocking transcription or promoting mRNA degradation, a unique mechanism for the regulation of gene expression. As for the possible involvement of miRNAs in the response to genotoxic stress, most information is currently available from animal systems. The study from Simone et al. (2009) carried out in human fibroblasts suggests that miRNA expression is a common feature of the response to genotoxic agents and the authors hypothesize that DNA damage might modulate miRNA expression by either inducing the transcription of miRNA genes or directly interacting with the processing and maturation machinery of miRNAs.

Yao et al. (2010) showed that *Arabidopsis* mutants impaired in siRNA biogenesis are more sensitive to genotoxic stress. siRNA biogenesis depends on various enzymes, among which are Dicer-like proteins (DCL2, DCL3, and DCL4), ribonucleases that process double-stranded RNA into small RNAs. DCL2 and DCL3

are involved in the generation of viral siRNAs and heterochromatic siRNAs, respectively (Xie et al. 2005) while DCL4 is required for ta-siRNAs biogenesis (Dunoyer et al. 2005). Yao et al. (2010) demonstrated that the *Arabidopsis dcl2* and *dcl3* mutants showed a lower DNA repair ability in response to UV irradiation and increased tolerance to the DNA methylating agent methyl methane sulfonate (MMS) which causes methylation of guanine, a type of lesion generally repaired by the BER pathway. Based on this finding, it ishypothesized that some siRNAs might be deleterious under genotoxic stress conditions and, when depleted, the plant response to adverse environments is enhanced. In contrast, the *dcl4* mutant was more sensitive to MMS treatment and this might be possibly due to a decreased efficiency of the BER mechanisms. Another intriguing aspect of the study from Yao et al. (2010) was the observation that the *dcl2* mutant was more tolerant to MMS than the wild type.

10 Role of Circadian Proteins in the Response to Genotoxic Stress

The involvement of circadian proteins as modulators of the cell response to genotoxic stress has been reviewed in animals by Antoch and Kondratov (2009). The circadian system (clock), extensively investigated also in plants, results from a complex network of transcription/translation feedback loops, comprising positive and negative components. The positive regulators are transcription factors which control the rhythmic expression of several genes while other proteins act as negative regulators and inhibit the transactivation events mediated by the positive components. The clock proteins are strictly regulated both at transcriptional and posttranslational levels, the latter being responsible for their stability, nuclear/cytoplasm distribution and functional activity (Gallego and Virshup 2007). The multistep regulation ensures high plasticity and fast adaptation in response to environmental changes, and within this context, links between circadian proteins and some key regulators of the cell response to oxidative stress have been highlighted. Almost all the information comes from studies carried out in mice with mutations of circadian proteins. The circadian proteins PER1 and TIM interact with the two critical components of the stress-related cell cycle checkpoints, the ATM and ATR kinases and this represents a novel mechanism of circadian modulation of the response to genotoxic stress (Antoch and Kondratov 2009). On the other hand, genotoxic agents (e.g., ionizing and UV radiations, MMS) can affect circadian parameters and the resulting effect is a shift in the phase of oscillation in expression of circadian genes. In some cases, both advances and delay in the phase of oscillation have been reported while only advances have been reported in response to gamma radiation (Antoch and Kondratov 2009). This might be due to the presence of different signaling pathways connecting the clock proteins and the DNA damage response (Antoch and Kondratov 2009). The response of the circadian system to genotoxic stress is likely to be evolutionary conservation as demonstrated in *Neurospora* (Pregueiro et al. 2006).

11 Future Perspectives

Plants, as other living beings, are continuously exposed to a wide range of abiotic stresses and, due to their sessile lifestyle, they have evolved highly sensitive stress responses. Abiotic stresses are critical constraints to crop productivity which has to maintain stable yields, associated with good quality and high nutritional value in order to address the increasing food demand. Drought, salinity, and land desertification are increasing on a global scale together with enormous economical losses. The impact of genotoxic stress, resulting from adverse environmental conditions, on the plant defense machinery represents a key issue, which has not been investigated properly so far.

Studies on the effects of genotoxic stress on the global gene expression have been carried out in the model plant *Arabidopsis*, highlighting the involvement of different gene categories, e.g., defense-related, DNA-repair, cell cycle-regulating and signal-transduction genes. Some of these functions, particularly those related to the plant antioxidant response, have been deeply investigated taking advantage of genetically modified systems designed for the overexpression and/or silencing of the target genes. In other cases, the defective mutants turned out to be a valuable tool in eliciting more information.

The role of DNA repair genes as essential components of the plant response to genotoxic stress has been recognized, but the direct link between these specific functions and the protective mechanisms activated under adverse environmental conditions, needs to be better defined. There is general awareness that the study of DNA repair genes might be informative for the genetic improvement of crops and for the design of new plant breeding strategies.

The physiology of DNA repair deserves more attention at the level of stress perception, by defining the molecular determinants (receptors, signal molecules, transcription regulators). The current knowledge on DNA repair pathways should be expanded, assessing the roles played in plants by newly identified genes. The functional characterization of DNA repair genes by transgenic approaches is also desirable for a better understanding of the plant response to genotoxic stress. Despite the availability of innovative and high throughput technologies such as DNA microarray and protein profiling, till date only a limited number of DNA repair genes has been clearly demonstrated to be stress-responsive.

Information derived from model organisms will be translated in practical applications directed toward agronomically relevant species and varieties, as it would be in the case of the model legume *Medicago truncatula* and the valuable *Medicago sativa* accessions. The urgency for fulfilling all these specific goals is quite evident.

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Chapter 8
In Vitro Haploid Production—A Fast and Reliable Approach for Crop Improvement

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1 Introduction

1.1 Overview of Haploidy

Haploids have attracted great interest of plant physiologists, embryologists, geneticists and breeders ever since the discovery of the first natural haploid embryos and plants in *Datura stramonium* in 1922 by Blakeslee et al. Subsequently, haploidy has been reported in many species, but at low and variable frequencies and was regarded as a special biological phenomenon. The low frequency of spontaneously arising haploid plants severely limited the utilization of haploids for crop improvement and genetic studies. The remarkable discovery that haploid embryos and plants can be produced through in vitro culture of anthers of *Datura* (Guha and Maheshwari 1964, 1966) brought renewed interest to haploidy. This method of androgenic haploid production was quickly attempted in many species to hasten the breeding programme in several economically-important plants.

The life cycle of angiosperms (higher plants) is characterized by alternating generations of sporophytes and gametophytes. The gametophytic phase arises when the diploid cells undergo meiosis (reduction division) to form male and female gametes. This phase is shortlived as fertilization of the egg re-establishes the diploid sporophytic phase. The sporophytic phase characterized by diploid (2n) chromosome number is the product of fertilization of male and female gametes, containing the haploid (n) set of chromosomes from each parent (Forster et al. 2007). Therefore, haploid is a generalized term for plants that contain the gametic chromosome number (n). This is in contrast to diploid plants, which contain two sets (2n) of chromosomes. Haploids are sexually sterile and, therefore, doubling of the chromosomes is

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required to produce fertile plants which are called doubled haploids or homozygous diploids.

Spontaneous production of haploids usually occurs through the process of parthenogenesis (embryo development from an unfertilized egg). However, occasionally, they bear the characters of male parent only, suggesting their origin through 'ovule androgenesis' (embryo development inside the ovule by the activity of the male nucleus alone where elimination or inactivation of egg nucleus occurs before fertilization) (Bhojwani and Razdan 1996). Although in vivo occurrence of haploids has been reported in several species, the frequency is very low (Pochard and Dumas de Vaulx 1971; Lacadena 1974). Several methods have been employed for producing haploid plants. In 1970, Kasha and Kao reported haploid production in barley following wide hybridization and the subsequent preferential elimination of the chromosomes of wild species during early embryogenesis. This provided a system that produced a large number of haploids across most genotypes (Choo et al. 1985). However, this program has been restricted to only a few crops so far. In vitro haploid plants can be obtained by triggering the male or female gametic cells to undergo sporophytic development. In vitro androgenesis (anther-microspore culture) is one of the most preferred techniques for obtaining haploids, but in vitro gynogenesis (unfertilized ovary–ovule culture) can prove to be a complementary technique in species where anther culture is inaccessible or less productive. It means that not only the microspore, but also the megaspore of angiosperms can be triggered in vitro to undergo sporophytic development. However, production of haploids via gynogenesis is more tedious, less efficient in comparison to androgenesis. In addition to the above techniques, *in situ* parthenogenesis (pollen irradiation and chemical treatment) can be employed for generation of haploid plants (Kurtar and Balkaya 2010).

Haploid and diploid lines play a vital role in genomics and have been used for the purpose of physical mapping, genetic mapping and also for integration of genetic and physical mapping. Additionally, haploid and doubled haploid plants are adapted for mutagenesis and genetic transformation experiments, presenting the advantage of immediate production of homozygous lines. It is also expected that, in the near future, haploid and doubled haploid plants will play an increasingly important role in whole genome sequencing projects, where homozygosity is of particular advantage.

1.2 History of Haploids

Dorothy Bergner in 1921 was the first to describe the natural occurrence of sporophytic haploids in the weed species *Datura stramonium* and this was reported by Blakeslee et al. in 1922. This report was followed by similar reports in several other crop species. The initial attempts to use haploidy in breeding were started by Chase in 1952 who selected the low frequency of parthenogenetic haploids in maize and then applied chromosome doubling treatments to produce inbred lines (Kasha and Maluszynski 2003).

Ever since the discovery of the first haploid in *Datura stramonium* in 1921, several researchers attempted to induce the unfertilized egg cell or other cells of the embryo sac to undergo parthenogenesis via in vivo means. In vivo induction of haploidy is carried out by the application of various physical, chemical or biological stimulants (Yang and Zhou 1982). This was followed by repeated attempts by various scientists to improve the frequency of parthenogenesis. While ovule and ovary culture suffered setbacks, Guha and Maheshwari (1964) made a breakthrough in anther culture (Yang and Zhou 1982). Pioneering work by Guha and Maheshwari in 1964 and 1966 opened a new vista for haploid breeding through in vitro means. It was a major advancement in haploid breeding of higher plants in which development of numerous pollen plantlets through in vitro anther culture of *Datura innoxia* was achieved. They wanted to study normal breeding development, but they found development of embryos in their cultured anthers (Maheshwari et al. 1980, 1982). These embryos developed into plants with a haploid chromosome number. However, the frequency of haploid production was significantly low. These studies led to further experiments to improve the frequency of haploids through in vitro anther culture. Plant regeneration via anther culture has been reported in more than 250 plants (Maluszynski et al. 2003a).

Consequently, attention shifted mainly to anther culture and culture of female tissues was neglected for about a decade. However, other researchers continued to work in the field of in vitro gynogenesis and in 1971, Uchimiya et al. observed the division of haploid cells in callus tissues obtained from cultured unpollinated ovaries of *Zea mays* and ovules of *Solanum melongena*. But this came into limelight only in 1976 when San Noeum reported development of haploid plantlets in unfertilized ovary cultures of *Hordeum vulgare*. Subsequently, Zhu and Wu (1979) obtained haploid plants from cultured unfertilized ovaries of *Triticum aestivum* and *Nicotiana tabacum.* This was followed by development of haploid plantlets via unfertilized ovary culture in several economically-important plant species such as *Zea mays* (Tang et al. 2006), *Psoralea corylifolia* (Chand and Sahrawat 2007), *Cucurbita pepo* (Shalaby 2007), *Guizotia abyssinica* (Bhat and Murthy 2007), *Cocos nucifera* (Perera et al. 2007), *Morus alba* (Thomas et al. 1999), etc.

1.3 Current Status

There are a number of excellent reviews on the production of haploids and doubled haploids so far, including those of Andersen (2005), Dunwell (2010), Germanà (1997, 2006, 2007, 2009), Kasha (1974), Magoon and Khanna (1963), Maluzynski et al. (2003a, 2003b), Palmer et al. (2005), Touraev et al. (2009), Zhang et al. (1990) and Xu et al. (2007). The doubled haploid techniques have been well established in a range of economically-important crop species, including major cereals and cabbage (Wedzony et al. 2009). Regeneration via anther culture has been reported in more than 250 species belonging to Solanaceae, Cruciferae and Gramineae families (Dunwell 1986; Germanà 2011; Hu and Yang 1986), while there are limited reports

on many legumes and woody plants as they are rather recalcitrant (Sangwan-Norreel et al. 1986; Bajaj 1990; Raghavan 1990; Wenzel et al. 1995; Germanà 2006, 2009, 2011). Recent advances in anther culture have been reported by Dunwell (2010), Wedzony et al. (2009), Pratap et al. (2009), Srivastava and Chaturvedi (2008); and Touraev et al. (2009). Unfertilized ovary–ovule culture has been applied to species including sugar beet, tulip, cucumber, sweet potato, onion, squash, gerbera, rice, maize, niger and tea (Chen et al. 2011). Wide hybridization method for production of haploids is routinely used in wheat and other cereal breeding programmes. The general method followed involves a phase of embryo rescue in vitro, usually followed by chromosome doubling with colchicine (Maluszynski et al. 2003a).

1.4 Efficiency of Haploid Production

Homozygous lines are of utmost importance in breeding programmes. Wide hybridization and anther/microspore culture are two of the most preferred techniques for doubled haploid production in crop plants. There is not much difference in the cost of haploid production or in the time and amount of labour required to produce haploids via these means. Currently, gynogenesis is the least favoured technique because of the low efficiency of production of haploids, but the value of doubled haploids in species that do not respond to other methods of haploid production makes this method worthwhile (Maluszynski et al. 2003a; Touraev et al. 2001). The limitation of wide hybridization is that it is restricted to the cereals where the chromosome elimination system appears to operate. Each crop has different requirements and, thus, there is need for extensive research to develop an efficient system. Wide hybridization has the advantage of being quite effective across genotypes and produces little or no induced variation from cultured embryos. However, anther/microspore culture scores higher over distant hybridization due to the fact that anthers harbour large numbers of haploid microspores per anther. In barley and wheat, where both systems have been comparatively well developed, the yield of green plants from isolated microspore culture can be up to 100 times higher than from wide hybridization in the most responsive genotypes (Kasha and Maluszynski 2003).

Anther culture is feasible in most species but it generally takes plenty of time to develop a competent system in some crops. Moreover, a good aseptic technique is a necessity even though the available methods are simple and reproducible (Maluszynski et al. 2003a). In general, haploid plants are regenerated in vitro from the microspores contained in the anther and require chromosome doubling treatments using chemicals such as colchicine, pronamide, trifluralin, oryzalin and amiprophos methyl (APM) (Wan et al. 1991). A few species such as barley regenerate a large number of doubled haploids as a result of induced chromosome doubling during early division of the microspores (Kasha 2005). Although the application of anther culture is widespread, the presence of the sporophytic anther wall serves as

a hindrance towards complete access of the microspores. There is a need to ensure direct embryogenesis to eliminate an intermediary callus phase that can promote gametoclonal variation among regenerants. The large number of successful reports on haploid production through anther culture stands as a witness of the immense benefits that this system offers. However, the presence of extraneous tissue in the form of anther wall makes this system complex for genetic studies where precision is inevitable.

As haploid plants in crop species obtained from microspore cultures through embryogenesis rather than from callus, the problem of extensive culture induced variability found in earlier reports on anther culture can be significantly done away with. With improvement of frequencies from microspores, the genotypic competence has also been reduced so that the system can be used in breeding programs. The feature of a high frequency of spontaneous chromosome doubling in some crops which results in completely fertile doubled haploid plants without subsequent doubling treatments, is another advantage of microspore culture. In breeding, the instant production of true breeding lines in diploid or allopolyploid species saves a number of generations in the breeding program. High density cultures of synchronized microspores can be set up containing more than thousand embryos per ml of culture media. Although a newer technique in comparison to anther culture, the potential of microspore culture has been realized quite early and efficient procedures have been successfully developed to produce doubled haploids in microspore cultures of tobacco, rapeseed, pepper, wheat, barley, rice, etc (Maluszynski et al. 2003a; Touraev et al. 2001).

2 Methodologies of In Vitro Haploid Production

2.1 Androgenesis

In androgenesis, immature pollen grains are induced to follow the sporophytic mode of development with the application of various physical and chemical stimuli. There are two methods for in vitro production of androgenic haploids, viz anther culture and pollen culture.

2.1.1 Anther Culture

Anther culture imparts an easy and one step protocol for haploid plant production (Chaturvedi et al. 2003).There is no single specific condition or protocol for inducing in vitro androgenesis in all plants since different species and even different cultivars within a species show diverse requirements. The following methods were adopted by Chaturvedi et al. (2003) to initiate in vitro androgenesis in neem plants (*Azadirachta indica* A. Juss.):

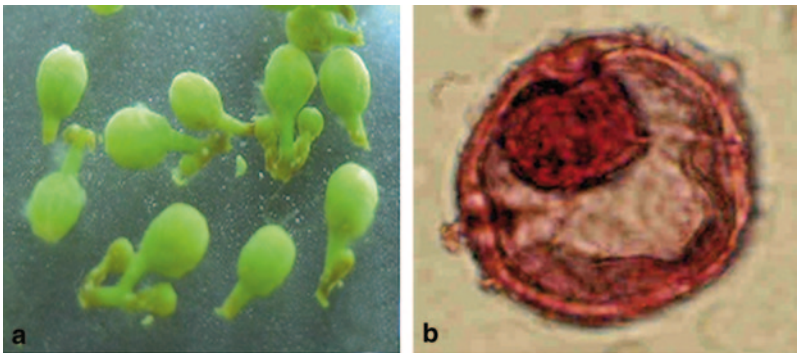

Fig. 8.1 **a** Neem flower buds of 2 mm size bearing correct stage of microspores (6X) **b** An uninucleate microspore of neem stained with acetocarmine (2300X)

Plant Material

For anther culture, 2 mm size flower buds (Fig. 8.1a) were collected early in the morning during the flowering season (April-May). The stage of microspore development was determined by acetocarmine squash preparations. Anthers containing early-to-late uninucleate stage of microspores (Fig. 8.1b) were cultured in the laboratory.

Establishment of Aseptic Cultures

The flower buds, collected from a field, were rinsed with sterile distilled water (SDW) several times, followed by surface sterilization in a glass vial with 0.1 % mercuric chloride solution ($HgCl_2$) for 7 min. Finally, the buds were washed three to four times with SDW. The buds were dissected out with the aid of a binocular microscope using pre-sterilized Petriplates, forceps and fine needles. Damaged anthers, if any, were discarded. Twenty anthers, from two buds, were cultured in 55 × 15 mm pre-sterilized, disposable Petriplates containing 10 ml of MS (Murashige and Skoog 1962) medium. The Petriplates were sealed with parafilm and subjected to specific treatment conditions.

Culture Media Preparation

A number of media compositions containing different concentrations of auxins and cytokinins were used for inducing in vitro androgenesis in neem. MS media, devoid of any growth regulator, did not induce any morphogenic response in anther cultures. For successful induction of callus from anthers, a combination of auxins and a cytokinin was found to be necessary in most of the cultures.

Analytical grade chemicals were used for preparation of stock solution and cultured media. Stock solution for MS/N_6 media preparation can be summarized as follows:

- Macronutrient – 10X; Micronutrient – 20X; Iron stock – 20X; Vitamins – 20X
- Growth regulator is used at a concentration of 1×10^{-3} M

All constituents were stored at 4 ºC for further use. Myo-inositol and sucrose were added freshly at the time of media preparation. The pH of the medium was adjusted at 5.8 with 1N HCl or 1N NaOH. The media were solidified with 0.8 % agar and autoclaved for 15 min at 1.06 kg cm^{-2} and 121 ºC, before pouring in 60 mm size Petriplates.

Culture Conditions

The anthers on each media were subjected to an array of pre-treatment conditions, like cold and heat shock for various duration under both dark and light incubation. Light and dark incubation at 25 ºC temperature served as a control in all the experiments. All the cultures were maintained continuously at 25 ± 2 ºC and 50–60 % relative humidity under 16/8 h (light/dark) photoperiod with diffuse light (1,000–2,000 lx) provided by cool daylight fluorescent tubes (Philips TL40 W). Anther cultures were initially kept continuously in the dark, but after eight weeks, the calli that had developed from these cultures were transferred to multiplication medium and maintained in light.

Twentyfour cultures were raised for each treatment, and each experiment was repeated at least three times. The cultures were observed periodically and the morphological changes were recorded at weekly intervals.

2.1.2 Pollen/Microspore Culture

Anther culture is beset with a number of problems. One of the major problems of anther culture is the concomitant callusing of anther wall along with the pollen, as a result of which the final tissue derived may not be of purely gametophytic origin. Moreover, the plants arising from an anther would constitute a heterogenous population. It has been observed in some species that anther cultures show asynchronous pollen development, the older grains may suppress the androgenic potential of younger grains by releasing toxic substances (Bhojwani and Razdan 1996). Kameya and Hinata (1970) reported for the first time callus formation in isolated pollen cultures of *Brassica oleracea* and the hybrid *B. oleracea* X *B. alboglabra* following which successful pollen-derived androgenic plants have been produced in many crop plants. There are numerous advantages of microspore culture over anther culture for haploid plant production. Unlike anther culture, isolated microspore culture allows haploid plant regeneration directly from microspores, assuring pure gametophytic origin and, thus, avoiding mixing of proliferating anther walls. A homogeneous prep-

aration of pollen at the developmental stage, most suitable for androgenesis, can be obtained by gradient centrifugation. Isolated pollen can be modified genetically by mutagenesis or genetic engineering before the culture and a new genotype can be selected at an early stage of development. Some of the important factors that affect induction of androgenesis in cultured pollen are the composition of the culture medium, pre-treatment and plating density. In most of the cereals, pollen culture involves pre-culture of the anthers for a few days or co-culture of pollen with a nurse tissue. Treatment of pollen-derived embryos and pollen derived callus to recover complete plantlets is similar to that of anther culture. However, the nutritional requirements of isolated pollen in culture are more complex than those of cultured anthers.

2.2 Gynogenesis

As mentioned earlier, in vitro gynogenesis is used as a complementary technique in species where anther/pollen culture is inaccessible or unsuccessful. Young flowers, ovaries or ovules have been used as explants to produce gynogenic haploids where the plants develop from unfertilized cells of female gametophyte (embryo sac) either through direct embryogenesis or via callusing. The following techniques are generally used for production of haploids via in vitro gynogenesis.

2.2.1 In situ Parthenogenesis Induced by Irradiated Pollen Followed by In Vitro Embryo Culture

Parthenogenesis induced in vivo by irradiated pollen followed by in vitro culture of embryos can be an alternative method of obtaining haploids in fruit crops. Gynogenesis by *in situ* pollination with irradiated pollen has been successfully used for *Malus domestica* (L.) Borkh (Zhang and Lespinasse 1991), *Pyrus communis* L. (Bouvier et al. 1993), *Actinidia deliciosa* (A. Chev) (Pandey et al. 1990; Chalak and Legave 1997). This method is based on in vitro culture of immature seeds or embryos, obtained as a result of pollination by irradiated pollen with gamma rays from cobalt 60. The method is useful in those species in which in vitro anther culture has not been successful. Irradiation does not hinder pollen germination but prevents pollen fertilization, thus stimulating the development of haploid embryos from ovules. The success of this technique is dependent on the choice of radiation dose, the developmental stage of the embryos at the time of culture, culture conditions and media requirements.

2.2.2 Ovary Slice Culture

Ovary slice culture technique involves culture of transverse sections of unpollinated ovaries on culture media. The various steps of ovary slice culture of tea are sum-

marized below. The following methods were used to induce in vitro gynogenesis in tea plants (Hazarika and Chaturvedi; unpublished data).

Plant Material

For ovary slice culture in tea, unopened and unpollinated mature flower buds (6–10 mm) size were collected early in the morning. Some of the buds were fixed in FAA (5:5:90 v/v/v Formaldehyde: Acetic acid: 70 % Ethanol) for 48 h and then stored in 70 % alcohol. Later, the appropriate developmental stage of the embryo sac was determined by histological analysis.

Establishment of Aseptic Cultures

The mature unpollinated tea flower buds were surface sterilized with 0.1 % $HgCl_2$ for seven minutes, followed by rinsing with sterile distilled water at least thrice. Carefully dissected transverse sections of ovaries were cultured on Murashige and Skoog's (MS) media supplemented with varying concentrations of auxins and cytokinins. Six ovary slices containing unpollinated ovules were cultured in 60 × 15 mm pre-sterilized disposable Petriplates containing 10 ml MS medium. The sealed Petriplates were subjected to various regimes of temperature and light treatments.

Culture Media Preparation

Murashige and Skoog (1962) basal medium was used throughout the studies to raise ovary slice cultures. MS basal medium was supplemented with a range of growth regulators, viz., 2, 4-Dichlorophenoxyacetic acid (2, 4-D), α-Naphthalene acetic acid (NAA), N_6- Furfuryladenine (Kinetin), 6-Benzylaminopurine (BAP) and Thidiazuron (TDZ) either individually or in combinations. The media contained 3 % sucrose and was gelled with 0.8 % Agar.

Culture Conditions

The ovary slice cultures from each media were subjected to an array of pre-treatments, like cold (4 °C) and heat shock (33 °C), for various durations under both light and dark incubation. The light and dark incubation at 25 °C served as a control in all the experiments. After the application of treatments, the cultures were maintained continuously at 25 ± 2 °C condition with 50–60 % relative humidity under 16/8 h (light/dark) photoperiod irradiance (1000–2000 lx) provided by cool daylight fluorescent tubes. The cultures were observed periodically and the morphological changes were recorded at weekly intervals.

2.2.3 Ovule Culture

Embryos are difficult to excise. To prevent damaging the embryos during the excision process, they are sometimes cultured while still inside the ovule. This technique is referred to as ovule culture or in ovulo embryo culture (Reed 2005). The unfertilized ovary is surface sterilized and the ovules are taken and placed into the culture. Excision of ovule, followed by culture on specific media may be either extremely easy to accomplish, as in case of large-seeded species in which only a single ovule is present, or time-consuming and intricate, as in small-seeded polyovulate species. Two types of ovule support systems have been developed. The filter paper support system involves culturing of the ovules on top of filter paper placed over liquid medium, whereas the vermiculite support technique demands placing the ovules on a sterile vermiculite/liquid media mixture (vermiculite support) with the micropylar side down. Unpollinated ovule culture has been used for haploid production in sugar beets and onions. Since there is usually only one egg cell per ovule, ovule culture has much less potential than microspore culture (Kasha and Maluszynski 2003). In *Nicotiana rustica* cv Rustica ovules with placenta were isolated from flower buds and were cultured on N_6 medium supplemented with growth regulators (Katoh and Iwai 1993).

2.3 Wide Hybridization

Haploids can also be induced by a process of selective chromosome elimination that follows certain interspecific pollinations. This phenomenon was discovered first in barley with crossing between *Hordeum vulgare* and *H. bulbosum* (Kasha and Kao 1970) and is now used routinely in wheat and other cereal breeding programmes; haploids were induced in these species following pollination with maize pollen. High frequency gynogenic haploids of *Triticum aestivum* have been raised by crossing them with *H. bulbosum* followed by embryo culture (Barclay 1975; Zenketler and straub 1979; Inagaki 1990). The process involves a phase of embryo rescue in vitro, usually followed by chromosome doubling with colchicine. The term "embryo rescue" refers to an vitro techniques the purpose of which is to promote the development of an immature or weak embryo into a viable plant (Reed 2005). Embryo rescue has been widely used to raise plants from hybridization in which failure of endosperm development causes embryo abortion. In embryo rescue procedures, the artificial nutrient medium serves as a substitute for the endosperm, thereby allowing the embryo to continue its development. Embryo rescue techniques are among the oldest and most successful in vitro procedures. One of the primary uses of embryo rescue has been to produce interspecific and intergeneric hybrids. While interspecific incompatibility can occur for a wide variety of reasons, one common cause is embryo abortion. The production of small, shrunken seed following wide hybridization is indicative of a cross in which fertilization occurs but seed development is disrupted. Embryo rescue procedures have been very successful in overcoming

this barrier among wide hybridizations in a wide range of plant species (Collins and Grosser 1984). In addition, embryo rescue has been used to recover maternal haploids that have developed as a result of chromosome elimination following interspecific hybridization.

3 Factors Affecting Haploid Production

There are numerous endogeneous and exogeneous factors that affect in vitro haploid production. These factors can be genetic, physiological, physical and chemical may also interact amongst each other to divert the microspores/egg cells to enter into a new developmental pathway. Some of the crucial factors affecting haploid production in plants are discussed below:

3.1 Genotype of the Donor Plant

The genotype of the donor plants has great influence on anther culture response and has been known since the early days of development of plants from pollen grains (Nitsch and Nitsch 1969; Maheshwari et al. 1980). However, it is only in recent times that the genetic factors affecting androgenesis have been studied more intensively. Many crop species are quite recalcitrant in their in vitro response which is governed by specific genes on the chromosomes (Datta 2005). Certain regions of the chromosomes appear to be associated with the formation of embryo like structures (Wan et al. 1992). As has been mentioned by Zhou in 1996, additive gene effects explain most of the variations observed across diverse genotypes, but cytoplasmic influences and non additive gene effects also play important roles in determining in vitro regeneration ability of anther-derived cultures. In earlier studies, significant difference in callus formation using varieties or crosses was observed. In some species, only a few genotypes have responded of the many that were tested. In fact, genetic factors contribute in a major way to the differences in the number of haploid plants produced (Custodio et al. 2005; Sopory and Munshi 1996).

As in anther culture, a difference in response also exists among donor cultivars in ovary and ovule culture. It was reported by Zhu et al. (1981) that the percentage of ovaries producing gynogenic calli in four wheat cultivars varied from 1.3 to 10.9 %. In *Nicotiana tabacum*, two cultivars had an induction frequency as high as 75 % and 80 %, but in another species, *N. rustica*, it was only 8 % (Wu and Chen 1982). Similar genotypic competence for gynogenesis has been reported in a number of crop species (Keller 1990; Lux et al. 1990; Tosca et al. 1999; Kobayashi et al. 1993; Sibi et al. 2001; Alan et al. 2004). It has been observed that genotypic competence exists even between different cultivar types within the same plant species, as has been reported in *Guizotia abyssinica* (Bhat and Murthy 2007).

3.2 Physiological Status of the Donor Plant

The physiological conditions of the donor plant, i.e., the environmental conditions and age of the donor plant, directly affect both in vitro androgenesis and in vitro gynogenesis in almost all plant species. A correlation between plant age and anther response has also been demonstrated by various scientists. Generally, the first flush of flowers yields more responsive anthers than those that are born later. Similar is the case with ovary culture. The frequency of androgenesis is usually higher in anthers harvested at the beginning of the flowering period and showed a gradual decline in relation to plant age (Bhojwani and Razdan 1996). However, it has been reported in *Brassica napus* and *B. rapa* that pollen from older, sickly looking plants yielded a greater number of embryos than those from young and healthy plants. Varying temperature and light conditions during the growth of donor plants also affect anther response. In anther culture of grape, the induction frequency of embyoids, derived from spring flowers, was higher than that derived from summer flowers (Zou and Li 1981). The microscopical observations showed that some varieties of rubber trees often have a lot of degenerated and sterile microspores in their anthers, in early spring or hot summer, due to the influence of unfavourable climatic conditions. As a result, no pollen embryoids were obtained from such anthers, but only somatic calli were obtained (Chen et al. 1982).

3.3 Stage of Explants Material at the Time of Inoculation

3.3.1 Stage of Microspores

The stage of microspores at the time of inoculation is one of the most critical factors for induction of androgenesis. Detailed cytological studies conducted on poplar, rubber (Chen 1986) and apple (Zhang et al. 1990) have shown that androgenic callus and embryos were mainly induced through a deviation of the first pollen mitosis to produce two undifferentiated nuclei. Besides affecting the overall response, the stage of microspore at culture also has a direct effect on the ploidy of plants produced in anther culture (Sunderland and Dunwell 1977). About 80 % of the embryos obtained from binucleate microspores of *Datura innoxia,* a highly androgenic species, were non haploids (Sunderland et al. 1974). In a vast majority of species where success has been achieved, anthers were cultured when microspores were at the uninucleate stage of microsporogenesis (Chaturvedi et al. 2003; Pedroso and Pais 1994; Sopory and Munshi 1996).

3.3.2 Stage of the Embryo Sac

It has been reported that the effect of ovule development on gynogenesis is profound as it harbours the embryo sac comprising the egg cell. The stage of embryo sac is an important determining factor for in vitro gynogenesis in various plant

species. However, it is difficult to know the stage of embryo sac at the time of inoculation. Several authors prefer to describe the inoculation stage according to the developmental stage of the flower bud or stage of pollen development. However, this could not be possible in several species, where male and female gametophytes do not mature simultaneously, a phenomenon known as protandry, the maturation of anthers before carpels (e.g., onion, leek, sunflower, sugar beet, carrot,) and the opposite protogyny (e.g., pearl millet). In such cases, the stage of embryo-sac at culture can be determined by histological preparations of ovary/ovules that are at identical stage with that of cultured ovary/ovules (Srivastava et al. 2009).

The unpollinated ovules collected two or three days before anthesis were non responsive on culture media as has been reported in Niger whereas unpollinated ovules collected one day before anthesis were most responsive with about 5 to 13.3 % embryogenesis recorded (Bhat and Murthy 2007). Although a wide range of embryo sac stages are responsive to gynogenic development, in most cases, nearly mature embryo-sac stage gave better results. This is quite contrary to anther culture in which mature pollen is nonresponsive to androgenesis. In barley and rice, unfertilized ovary cultures with late staged mature embryo sacs gave good results (San Noeum 1976, 1979; Wang and Kuang 1981) while others reported success with ovary cultures containing uninucleate to mature embryo sacs (Zhou and Yang 1981; Yang and Zhou 1982; Kuo 1982; Huang et al. 1982).

3.4 Culture Media

3.4.1 Basic Media

The constituents of the basal medium and combinations of growth regulators serve as an important factor in eliciting successful androgenesis and gynogenesis. However, it is difficult to suggest one single culture medium with a particular growth regulator for all the systems. Regeneration of androgenic and gynogenic plants may occur directly via embryogenesis or via callus formation from pollen/egg cell, followed by organogenesis. In the later stages of plant development, the media constituents may vary according to culture conditions and requirement by the plant itself.

Most species exhibit androgenesis on a complete nutrient medium (mineral salts, vitamins and sucrose) with or without growth regulators. Most commonly used basal media for anther culture are N_6 (Chu 1978) medium, MS (Murashige and Skoog 1962) medium with slight modifications, Nitsch and Nitsch (1969) medium, and B5 medium (Gamborg et al. 1968). Half strength MS medium is suitable for Solanaceae and N_6 medium has been used for cereals (Chu 1978).

Most early work in 1950s used Nitsch medium for unpollinated ovule and ovary culture; however since the 1970s, Miller (1963), MS or N_6 media have been used in successful experiments. In *Gerbera*, MS seems better than the Knop's and Heller medium (Cagnet-Sitbon 1980). An increase in the content of B group vitamins and glycine in H medium has been reported to have promoted induction-frequency in tobacco ovary culture (Wu and Chen 1982).

3.4.2 Growth Regulators

The requirement of growth regulators and culture medium in terms of kind and concentration may vary with each and every plant system. Generally, there is an agreement that the source and amount of total nitrogen as well as combination of a cytokinin and an auxin is necessary for pollen embryogenesis and pollen callusing in several woody plants (Chaturvedi et al. 2003; Chen 1986; Nair et al. 1983). It has been reported in most members of Solanaceae, that addition of an auxin to the induction medium is not a pre-requisite for anther response, but the addition of auxins and cytokinins alone or in combination is crucial for microspore-derived embryo induction in majority of the plants, especially the recalcitrant ones (Maheshwari et al. 1982). The type and concentration of auxins seem to determine the pathway of microspore development (Ball et al. 1993), with 2, 4-D inducing callus formation, and indole-3-acetic acid (IAA) and Napthaleneacetic acid (NAA) promoting direct embryogenesis (Armstrong et al. 1987; Liang et al. 1987). Gibberellins and abscisic acid have been occasionally added to the media.

Growth regulators, especially, auxins are widely used for induction of gynogenesis and their optimum concentrations have been reported to vary considerably from species to species (San Noeum and Gelebart 1986). In sunflower, gynogenesis occurs only when 2,4-D or NAA is added to the medium (Gelebart and San Noeum 1987). As observed in many species, a combination of auxin and cytokinin was also reported to be useful for gynogenesis in allium species (Alan et al. 2003) and mulberry (Thomas et al. 1999). In mulberry, gynogenic haploids are also produced on BA or Kinetin medium (Lakshmi Sita and Ravindran (1991). These researchers observed gynogenesis in *ab initio* ovary cultures of mulberry. Thomas et al. (1999) obtained maximum gynogenic response when excised ovaries from inflorescence segments of mulberry were cultured on MS supplemented with BAP (8.5 μM) + 2,4-D (4.5 μM), followed by transferring them to MS + 2,4-D (4.5 μM) + Glycine (6660 μM) + Proline (1738 μM).

3.4.3 Growth Additives

The supplement of other substances, such as free amino acids (glutamine, proline, glycine), biotin, myo-inositol, casein hydrolysate, coconut water, silver nitrate (ethylene antagonist) and polyvinylpyrrolidone has been reported to enhance gynogenic response (Reinert and Bajaj 1977; Powell 1990; Achar 2002). Moreover, the addition of exogenous aliphatic polyamines (PAs) to the culture medium has been found to increase the number of pollen-derived embryos in potato (Tiainen 1992), in some Indian wheat cultivars (Rajyalakshmi et al. 1995), cucumber (Ashok kumar et al. 2004 and Chiancone et al. 2006). Polyamines such as putrescine, cadaverine, spermidine and spermine are low molecular mass polycations which are involved in in vitro organogenesis and embryogenesis (Bagni and Tassoni 2001; Kumar et al. 1997). Similarly, the use of additives has also been reported in unfertilized ovary cultures. Thomas et al. (1999) obtained maximum gynogenic response in mulberry

when excised unfertilized ovaries from inflorescence segments were transferred from MS + BAP (8.5 μM) + 2,4-D (4.5 μM) to MS + 2,4-D (4.5 μM) + Glycine (6660 μM) + Proline (1738 μM).

3.4.4 Carbon Source

Sucrose has generally been used as the major carbohydrate source in the culture medium. Sucrose concentration in induction medium has a major effect on osmosis, and the development of embryos is apparently influenced by osmosis (Wakizuka and Nakajima 1975). The effect of sucrose on anther culture has been investigated in a number of species. The necessity of sucrose for successful androgenesis was first demonstrated by Nitsch in 1969 for tobacco and later by Sunderland in 1974 for *Datura innoxia*. Generally, sucrose is supplied at 2–3 % concentration. However, increase in its concentration can lead to beneficial morphogenic potential (Agarwal et al. 2006; Sopory and Munshi 1996) by suppressing the proliferation of anther wall (somatic tissues) (Ouyang et al. 1973). For potato, 6 % sucrose proved distinctly superior than 2–4 % sucrose in terms of the number of anthers forming pollen embryos (Sopory et al. 1978). High sucrose levels (6–17 %) are required in Gramineae and Brassicaceae families in which mature pollen is shed in the tricellular condition (Dunwell and Thurling 1985), whereas for those in which mature pollen is bicellular (e.g., Solanaceae), lower level of sucrose, such as 2–5 %, is usually beneficial (Dunwell 2010). All *Brassica* species require 12–13 % sucrose for androgenesis in anther and pollen cultures. According to (Dunwell and Thurling 1985), high sucrose concentration favours better survival of pollen grains, thus improving the frequency of androgenesis in *Brassica napus* (Last and Bretell 1990). Wedzony et al. (2009) have reported the beneficial effects of maltose on androgenesis in anther cultures of wheat, triticale, rye and rice.

The type and concentration of sucrose used in the medium for inducing in vitro gynogenesis varies from species to species. High sucrose concentration (8–10 %) in the culture medium has been shown to be helpful in some species, like sweet potato (Kobayashi et al. 1993) and onion (Campion et al. 1992), whereas in summer squash, 9 % sucrose is detrimental for production of any embryos (Shalaby 2007). Sucrose concentration used in unfertilized ovary and ovule culture was 3 to 10 % in barley, 4 to 8 % in wheat, 3 to 6 % in rice, 2 % in tobacco and 3 to 6 % in *Gerbera* (Yang and Zhou 1982).

3.5 Culture Conditions

Pre-treatments, such as chilling, temperature shock, high humidity, water stress, anaerobic treatment, centrifugation, sucrose, nitrogen starvation, ethanol, gamma radiation, microtubule disruptive agents, electrostimulation, high medium pH, heavy

metal treatments are particularly popular approaches in anther and microspore cultures (Shariatpanahi et al. 2006).

Temperature treatment is considered to be the most effective treatment to induce pollen embryogenesis and it may be applied to excised flower buds or whole inflorescences before culture or cultured anthers or pollen grain, prior to their transfer to standard culture room conditions, in order to divert the gametophytic pathway to sporophytic mode of development. The optimum temperature and duration of pretreatments vary with the genotype. Cold pre-treatment (4 °C for 2–3 days) is employed routinely in the anther culture of many crops and its effect is also genotype-dependant (Osolnik et al. 1993; Powell 1988). In *Brassica* species, a short-duration high temperature pre-treatment at 30–35 °C to cultured anthers before shifting them to 25 °C is required to efficiently switch the developmental pathway.

Light does not seem to be necessary for the induction of androgenesis. For pollen culture of *Datura innoxia* (Sangwan-Norreel 1977), *Nicotiana tabacum* (Sunderland and Roberts 1977) and *Annona squamosa* (Nair et al. 1983), an initial incubation of cultures in dark, followed by diffuse light was found to be suitable. Isolated pollen cultures are more sensitive to light than anther cultures (Nitsch 1977). In *Brassica juncea* (Sharma and Bhojwani 1989) and *Hordeum vulgare (*Xu 1990) species, light is detrimental even for anther cultures. Likewise, neem callusing from microspores requires continuous dark incubation of anthers (Chaturvedi et al. 2003).

A beneficial role of cold treatment on gynogenesis has been reported in some species whilst in others, no significant effects on gynogenesis were observed. Pretreating the capitula of sunflower at 4 °C for 24–48 h before culture, significantly increases the induction frequency (Yan et al. 1987). Cai et al. (1988) observed a promotory effect of cold treatment to young panicles of rice at 7 °C for one day prior to ovary culture in gynogenesis. In *Cucumis melo,* pollination of pistils with irradiated pollen is essential to obtain ovules capable of forming gynogenic haploids (Katoh et al. 1993).

3.6 Other Miscellaneous Factors

3.6.1 Anther Wall Factor

One of the important research subject in anther culture for woody plants is to avoid the over-proliferation of callus from anther wall tissues and to achieve a high yield of pollen embyoids and pollen calli. In anther culture of most woody plants, both pollen calli or embryoids and somatic calli from anther wall tissues grow simultaneously. The development of callus from somatic tissues of anther can be avoided by culture of isolated microspores. However, there are not many successful reports on microspore culture in woody plants (Chaturvedi et al. 2003). Pelletier and Ilami (1972) had introduced the concept of “wall factor”, according to which the somatic tissues of anther play an important role in the induction of sporophytic divisions in pollen. In anther culture of rubber, 47 % of the microspores in close contact with

the surrounding somatic cells could develop into multicellular masses as compared to only 5 % of microspores away from the wall (Chen 1986). Anther wall callusing is regarded as a pre-requisite for the formation of androgenic haploids (Chaturvedi et al. 2003; Chen et al. 1982; Chen 1986).

3.6.2 Microspore Culture Density

The culture density is a critical factor in isolated pollen culture. Huang et al. (1990) made a detailed study on the effect of culture density on embryogenesis in pollen cultures of *Brassica napus*. According to this report, the minimum density required for embryogenesis is 3000 pollen/ml of the culture medium but highest embryo yield was obtained at 10,000 to 40,000 pollen/ml.This high plating density is crucial only for the initial couple of days. Dilution of the density from 30,000–40,000 to 1,000 pollen after two days of culture did not reduce the embryogenic frequency. Arnison et al. (1990) reported the effect of culture density in anther cultures of *B. oleracea*. The frequency of pollen embryogenesis was enhanced if the anther culture density was increased from three anthers per 4 ml to 12–24 anthers per ml of the medium. Cardy in 1986 reported that in *B. napus*, the response was better when anthers were cultured at a density of two anthers per ml.

3.6.3 Effect of Female Flower Position

Position of female flowers on the plant stem affected induction of embryos from ovule cultures of *Cucurbita pepo* L (Shalaby 2007). One of the possible explanations for enhancing responses of tissue culture could be attributed to indigenous hormonal level (Johansson 1986).

4 Haploid Production in Economically Important Dicotyledonous Species

4.1 Azadirachta indica A. Juss (Family: Meliaceae)

Azadirachta indica A. Juss, commonly referred to as neem, is an adaptable, tropical, evergreen tree of the family Meliaceae. It thrives best in hot, dry climates where shade temperatures often reach 50 °C and annual rainfall ranges from 400 to 1,200 mm. The neem plant is a native of South and Southeast Asia and grows well in tropical and subtropical areas of the world. The plant is well known for its numerous medicinal, agrochemical and economic uses, which can be attributed to the presence of azadirachtin, a highly oxidized limonoid (triterpenoid) found prominently in the seed kernels. It possesses insect repellent, antifeedant, larvicidal,

growth inhibiting properties against a wide range of pests and thus, has been well accepted as an eco-friendly, biodegradable biopesticide. Today, due to the remarkable properties shown by azadirachtin and other related triterpenoids, the tree has attained universal significance. Almost each and every part of this tree, particularly the leaves, bark and seeds, has manifold applications. Besides being a popular avenue tree, with a large crown, the wood of this tree is used as timber for house building, furniture and other domestic and agricultural tools.

Androgenesis is a very useful technique for resolving the problem of self-incompatibility, heterozygosity and long gestation period in this tree species. However, limited attempt has been made for the overall improvement of this valuable tree through in vitro haploid production. Gautam et al. (1993) made the initial attempts to produce haploids of neem for which they cultured anthers at the uninucleate stage of microspores and observed formation of multicellular pollen. However, all the plants regenerated from anther callus were diploids. Chaturvedi et al. (2003), for the first time, generated androgenic haploids of neem by anther culture, at early-to-late uninucleate stage of pollen. Callusing from anthers was induced on MS basal medium (with 9 % sucrose) supplemented with 2, 4-D (1 μM), NAA (1 μM), and BAP (5 μM). The calli multiplied best on MS medium (with 3 % sucrose) supplemented with 2, 4-D (1 μM), and Kinetin (10 μM). MS supplemented with BAP (5 μM) was optimum for regeneration from younger calli (75 % cultures differentiated shoots), but older calli showed the best regeneration at 7.5 μM BAP. As per histological investigations, in four-week-old cultures the anther-wall cells had started dividing, while the microspores appeared unchanged. However, in eight-week-old cultures, it was observed that the entire anther locules were filled with microcalli. Calli maintained on MS medium containing 2, 4-D exhibited good regeneration potentiality, but those calli that were maintained on MS medium containing 2, 4-D (1 μM) and Kinetin (10 μM), retained the regeneration potential for a longer period. Elongation of shoots was achieved at a lower concentration of BAP at 0.5 μM. These shoots were multiplied by forced axillary branching and were rooted through in vitro on ¼ strength of MS medium supplemented with IBA (0.5 μM). The plants were subsequently established in soil. Of the plants that regenerated from anther callus, 60 % were haploids (2n = x = 12), 20 % were diploids (2n = 2x = 24) and 20 % were aneuploids (2n = 2x–2 = 22). Srivastava and Chaturvedi (2011) reported a new improved method of haploid production from androgenic cultures of *Azadirachta indica* A. Juss. In this investigation, the best callus induction response was obtained in the induction medium with 12 % sucrose concentration on MS + 2,4-D (1 μM) + NAA (1 μM) + BAP (5 μM). Maximum shoot regeneration frequency obtained was 98.5 % with an average of 8.5 shoot-buds/ explant on MS + BAP (2.2 μM) + NAA (0.05 μM), as against 75 % with cultures forming an average of 4.5 shoot-buds/ explants on MS + BAP (5 μM) in the earlier report of anther culture of neem by Chaturvedi et al. (2003). Cytological analysis of the calli and regenerants confirmed their haploid status with the chromosome number as 2n = x = 12.

To date, there is only one single report on in vitro ovary culture of neem by Srivastava et al. (2009). However, the regenerated plants were diploid in nature. Un-

fertilized ovaries obtained from closed flower buds of an adult 54-year-old neem tree were used as explants. Maximum shoot regeneration (78 %) was observed when calli, induced from ovaries of 4 mm size flower buds and proliferating on MS + 2,4-D (0.5 μM), were transferred to MS containing BAP (5 μM). Histological analysis revealed that 4 mm sized flower buds corresponded to a 2-nucleate stage of the embryo sac. The best medium for inducing calli from unfertilized ovaries was MS medium with 9 % sucrose, 2,4-D(1 μM) + BAP(5 μM). The shoots were multiplied by forced axillary branching on MS medium supplemented with BAP (1 μM) and Casein hydrolysate (250 mg/l). The shoots were rooted on ¼ strength MS medium supplemented with IBA (0.5 μM) at a frequency of 79 %. The plants were subsequently hardened with transplantation rate of 81.8 %. Although all the regenerated plants were diploid, an efficient protocol for ovary culture of neem was established which can be used as a platform to produce gynogenic haploids of neem in future.

4.2 Camellia spp (Family: Theaceae)

Camellia sinensis (L.) O. Kuntze commonly referred to as tea, belonging to the family Theaceae, is an evergreen, perennial, cross-pollinating plant. It grows naturally as tall as 15 m; however, it is usually trimmed to below 2 m when cultivated for its leaves (Mondal and Parathi 2003). The cultivated taxa of tea consists of three main natural hybrids. They are *Camellia sinensis* (L.) O. Kuntze or China type, *Camellia assamica* (Masters) or Assam type and *Camellia assamica* sub spp *lasiocalyx* (Planchon ex Watt.) or Cambod or Southern type. Tea is the oldest non-alcoholic caffeine containing beverage in the world. The medicinal properties of tea are also widely acclaimed. It is an important socio-economic crop that plays a significant role in the foreign exchange of developing countries like India. Polyphenols account for about 25–35 % of the total dry weight of freshly plucked tea leaves of which two-thirds is contributed by catechins alone (Saravanan et al. 2005). Research progress on tissue culture of tea has been rather slow for the last 20 years because tea is less amenable to these techniques, due to the high level of polyphenols, the presence of systemic bacterial contamination and its recalcitrant nature in tissue culture medium (Dood 1994).

The initial attempts to produce haploids through anther culture of tea were pioneered by Katsuo (1969) and Okano and Fuchinone (1970). They obtained roots from anther-derived callus. Later, Hoken Toi (1981) also achieved similar response on medium supplemented with NAA (9.67 μM) and Kinetin (10.1 μM) (Mondal et al. 2004). However, it was Chen and Liao in 1982, who produced complete plantlets from tea anthers of cultivar Fuyun No-7 out of nine different tea cultivars on which they worked. The plantlets were obtained when the anthers were cultured on N_6 medium supplemented with Kinetin (9.3 μM), 2,4-D (2.26 μM), L-Glutamine (800 mg/l), and Serine (100 mg/l), followed by sub culturing on N_6

medium supplemented with Zeatin (9.12 µM), adenine (148 µM) and Lactoalbumin hydrolysate (10 mg/l). On this medium, the calli continued to proliferate either into shiny mass or shoots. These shoots were subsequently rooted on medium containing IAA (0.57 µM). While three out of four plants were haploids, the rest were aneuploids with a chromosome number 2n = 18. Later, in (1992, Saha and Bhattacharya reported formation of globular structures in tea which failed to differentiate further on MS medium (with 7 % sucrose). This was supplemented with NAA (0.53 µM), 2, 4-D (0.45 µM), Kinetin (0.46 µM), and Glutamine (400 mg/l). However, the differentiation of true pollen embryos and regeneration of haploid plants were described by Raina and Iyer in (1992 and Shimokado et al. (1986). Pedroso and Pais (1994) tested 17 different media combinations based on MS and N_6 with various concentrations of carbon source, growth regulators and amino acids, such as Serine and Glutamine for *C. japonica*. The embryogenic calli from microspores were obtained when isolated microspores were cultured on 2, 4-D (4.53 µM), and Kinetin (0.46 µM) and subsequently on MS medium supplemented with BAP (2.22 µM). However, further growth ceased at maturation stage. Seran et al. (1999) reported the highest response in terms of micro calli formation in a Sri Lankan tea clone TRI-2043 (78–98 %) out of five different clones selected for study. On ½ MS + 2, 4-D (9.06 µM) + Kinetin (4.65 µM) + IAA (5.71 µM) under dark condition, 98 % anther response was achieved. Determination of ploidy levels in the callus cells showed that the frequency of haploid cells was greater (68 %) in comparison to diploid cells (6 %). However, plantlets could not be regenerated. Mishra and Chaturvedi (unpublished) has obtained for the first time microspore embryogenesis in anther cultures of TV clones of tea (TV19 and TV21) via an intervening callus phase. Within three weeks of culture, the anthers were swollen and after six weeks longitudinal dehiscence occurred along the anther wall (Fig. 8.2a), followed by emergence of transparent white callus from within (Fig. 8.2b). In both TV19 and TV21, calli were obtained when anthers were subjected to dark incubation at 25 °C. After successful induction of callus, embryogenesis in these calli and embryo germination occurred when cultures were transferred to light conditions (Fig. 8.2c, d).

So far, there have been no reports on in vitro gynogenesis in tea. Hazarika and Chaturvedi (unpublished data) have obtained for the first time, successful callus induction from unpollinated ovules of tea when thin sections of ovary cultures were subjected to various regimes of temperature and light treatments. Within a week of the culture, the ovules swelled to almost double their original size (Fig. 8.3a) and callusing was observed. In a few cultures, white, friable callus tissue emerged from within the cultured ovules (Fig. 8.3b) whereas in others profuse callusing from the entire sections occurred. The nature of callus obtained from within the ovules was friable and it was white in colour. Histological study of the ovary slice sections at the time of culture showed the presence of a mature egg cell within the embryo sac of the unpollinated ovules. Following successful callus induction, formation of dark green nodulated structures occurred on various media compositions. Histological investigations revealed the formation of tracheids in the callus tissue.

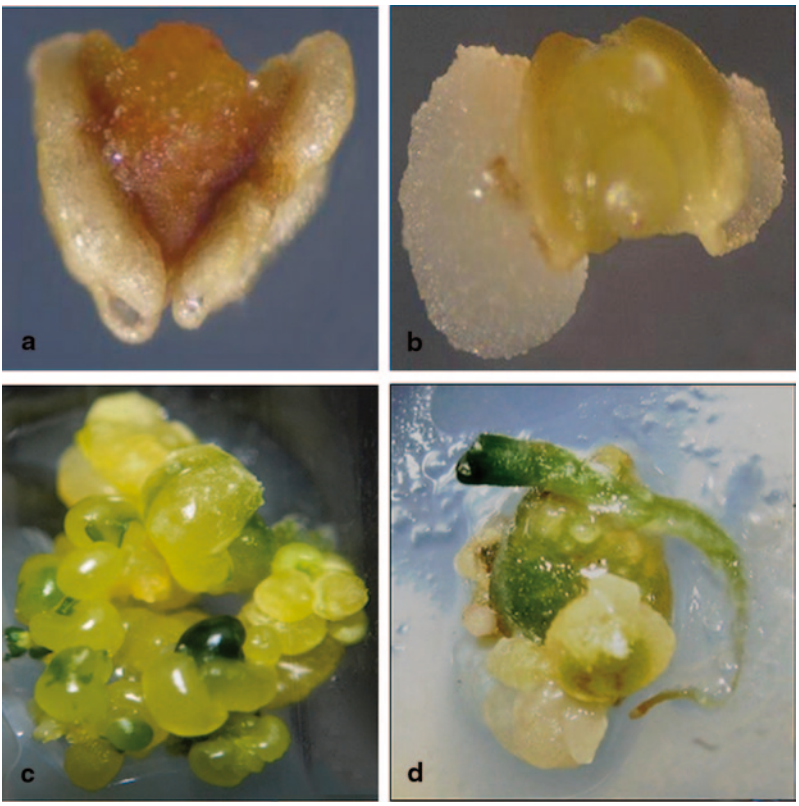

Fig. 8.2 **a** Six-week-old anther culture of tea, showing longitudinal rupturing of anther walls (220X). **b** 10-week-old anther culture of tea. Note the emergence of calli from inside the anther walls (90X). **c** Embryogenesis occurring in anther-derived callus of tea, after eight weeks of culture. Embryos at various stages of development can be seen in the Fig. (4.5X). **d** A germinated embryo of tea (6X)

4.3 Coffea spp (Family: Rubiaceae)

The coffee plant is a woody perennial, evergreen, dicotyledon that grows relatively high, therefore, more accurately described as a coffee tree. Coffee is a brewed drink prepared from roasted seeds, called coffee beans, of the coffee plant. While there are several different coffee species, the two main cultivated species are, *Coffea arabica,* known as Arabica coffee accounting for 75–80 % of the world's production and *Coffea canephora*, known as Robusta coffee, accounting for about 20 % of the total production. Genetic improvement of coffee, being an important commercial crop, is essential for improving the production and quality of coffee. Development of haploids via anther culture facilitates the production of homozygous plants in one generation and opens the way for new breeding strategies.

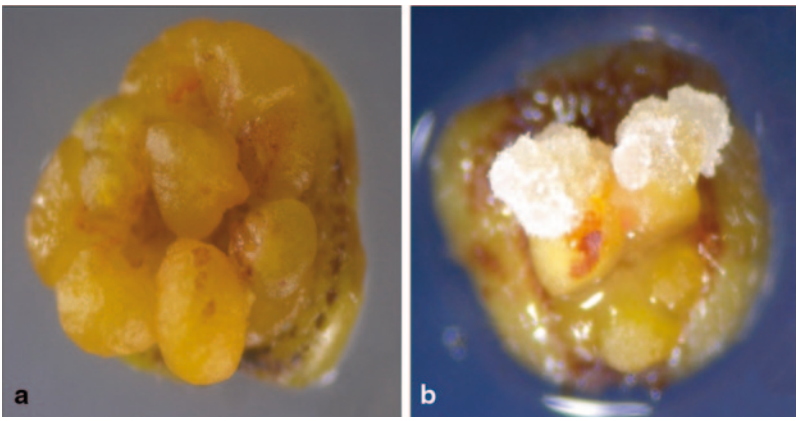

Fig. 8.3 **a** A slice section of tea ovary culture, showing swollen ovules, after two weeks of culture (8X). **b** Four-week-old cultures showing bursting of ovules and emergence of callus from within (8X)

Anther culture studies are quite limited in coffee. The first attempt to produce haploid coffee plants was made in *Coffea arabica* (Sharp et al. 1973). Successful reports of haploid and dihaploid plants from anther culture of coffee have been published (Montes 1981; Ascanio and Arcia 1987). Ascanio and Arcia (1987) reported the existence of a correlation between different developmental stages of anthers, the size of flower buds and the quantity of calli obtained after 90 days of culture. Further in 1994, the authors studied the effect of developmental stage and heat shock on the formation of embryogenic calli from *C. arabica* var. guernica anthers.

Unlike anther culture, isolated microspore culture allows haploid plant regeneration directly from microspores, assuring pure gametophytic origin. Colonies of haploid cells have been successfully obtained via mechanically isolated microspore cultures of *C. arabica* varieties Catuai and Catimor in either liquid or solid media (Carneiro 1997). Herrera et al. (2002) described, for the first time, a new approach for embryo induction and plant regeneration from *C. arabica* cv caturra isolated microspores, pre-treated with colchicine. Their study clearly demonstrated that colchicine could activate the androgenic response in coffee microspores. A positive androgenic development was observed only when late uninucleated or early binucleated microspores were cultured. Such response was evidenced by microspore divisions beginning after 25 days of colchicine exposure. The best androgenic response was found when microspores were pre-cultured in 100 mg/l colchicine for 48 h. Flow cytometry and morphological analyses revealed that 95 % of regenerated plants were dihaploids (2n = 2x = 22). However, some doubled dihaploid plants (2n = 4x = 44) were also obtained, suggesting that not only androgenic induction but also chromosome doubling could be expected as a result of colchicine exposure of coffee microspores.

Lanaud in 1981 reported somatic embryogenesis in ovules from *C. canephora* and established conditions for rapid multiplication and differentiation of embryo-

genic mass to produce haploid plants. In brief, anther and ovule cultures are least developed coffee tissue culture techniques despite their substantial potential in reducing the time required to produce new varieties.

4.4 Citrus sp. (Family: Rutaceae)

Citrus species represent the largest production of fruits worldwide. They are valued for their antioxidant properties and are one of the richest sources of vitamin C and essential oils.

The first report of development of haploid seedlings of *C. natsudaidai* was reported by Karasawa (1971) by the application of gamma rays. Thereafter, there have been several reports on production of haploids via in vivo crossing (Oiyama and Kobayashi 1993), gynogenesis induced by in vitro pollination with pollen from a triploid plant (Germanà and Chiancone 2001) and by in vivo parthenogenesis (Germanà 2006). Anther culture technique has been used to obtain haploid calli, embryoids and plantlets with limited efforts in a few *Citrus* species like *C. madurensis, C. limon, C. deliciosa* x *C. paradise* (Chen et al. 1980; Germanà et al. 1991; Germanà and Reforgiato 1997). However, extensive research has been carried out on *C. clementina*. Since the first embryogenic calli and haploid plantlets were obtained by anther culture in *C. clementina* Hort. Ex Tan. cv Nules (Germanà et al. 1994), many studies have been carried out to improve the androgenic response in *Citrus* species by the use of different combinations of plant growth regulators (Hidaka et al. 1979; Chaturvedi and Sharma 1985; Geraci and Starrantino 1990; Germanà et al. 1994, 2000a, b, 2005). Germanà and Chiancone (2003) proposed an improved and detailed protocol for haploid induction through anther culture of *C. clementina* Hort. Ex Tan. cv Nules by evaluating a number of factors that affect androgenesis. They observed that temperature pre-treatment of flower buds between 4 °C and 25 °C for 14 days was more favourable to induce embryogenic callus and embryoids in anther cultures. Anthers were excised from pre-treated flower buds and cultured on N_6 medium (Chu 1978) supplemented with Nitsch and Nitsch vitamins (Nitsch and Nitsch 1969), galactose (9000 mg l^{-1}), lactose (18,000 mg l^{-1}), coconut water (5 % v/v), casein hydrolysate (500 mg l^{-1}), L-glutamine (200 mg l^{-1}), biotin (0.5 mg l^{-1}), ascorbic acid (500 mg l^{-1}), NAA (0.11 μM), 2,4-D (0.09 μM), Kinetin (4.6 μM), BAP (2.2 μM), Zeatin (2.28 μM), TDZ (0.45 μM), and GA_3 (1.45 μM) in dark for 15 days before being shifted to diffuse light at 25 ± 2 °C. With this protocol, 1.9 % anther cultures showed embryoid development and a total of 570 and 1,000 embryoids developed in Nules and SRA 63 cultivars, respectively. Direct gametic embryogenesis without callus formation was observed in 7 % responsive anther cultures of the cv Nules and in 45 % of the responsive anther cultures of the cv SRA 63. The embryoids were later germinated on MS medium containing GA_3 (2.89 μM) and NAA (0.05 μM). Recently, Chiancone et al. (2006) studied the effect of polyamines, spermidine and putrescine, with an aim to further improve the rate of embryogenic callus and embryoid induction in anther cultures of *C. clementiana*

cv Nules. The addition of 2 mM spermidine to the suggested medium of Germanà and Chiancone (2003) stimulated gametic embryogenesis in 4 % cultures whereas putrescine did not influence embryo production. Flow cytometric analysis revealed that the regenerants were mostly trihaploids; few were doubled-haploids while none of them were haploids.

4.5 Malus domestica Borkh. (Family: Rosaceae)

Malus domestica Borkh. (Family: Rosaceae) or apple is an important temperate fruit tree. It is one of the most widely cultivated tree fruits in the world. In vitro approaches to induce haploids in apple have been rather limited in comparison to other plant species (Höfer and Lespinasse 1996). Anther culture in apple was pioneered by Japanese scientists at the beginning of the 1970s. They induced calli capable of root formation. Subsequently, several working groups initiated haploid induction in apple by anther culture. Induction of embryogenesis and plant formation has been reported in apple from anther cultures (Fei and Xue 1981; Xue and Niu 1984; Höfer 1995). Although regeneration from embryos is reproducible via adventitious shoot formation, the induction of embryogenesis from cultured apple anthers is still low and highly dependent on genotype (Höfer 1995, 1997). Kolova et al. (1994) obtained multinuclear structures and Bouvier (1993) obtained callus formation from isolated microspore cultures. Höfer et al. (1999) reported, for the first time, the induction of embryogenesis and plant formation from isolated apple microspores at late uninucleate stage of development. The authors reported that several factors were responsible for successful androgenesis in apple, especially a combination of starvation and cold treatment. The effect of starvation of buds or microspores for 1–2 days at 4 °C or 27 °C was found to be very important for androgenesis in apple. Starvation is an effective stress treatment which has yielded successful embryogenic induction in tobacco (Kyo and Harada 1986; Touraev et al. 1996a) and wheat microspores (Touraev et al. 1996b). The requirement of heat or cold pre-treatment has been shown to vary from plant to plant (Höfer et al. 1999). The induction medium and genotype of the donor plant strongly affect embryogenic capacity of isolated microspores. “Alkmene” and “Rene” are the cultivars of apple in which the highest embryo induction via anther culture was achieved. Success with “Rene” has been reflected in many experiments related to androgenesis in apple. They attained 17 % embryo induction from microspore cultures as compared to only 7 % induction from anther cultures on modified N_6 basal medium devised by Chu et al. (1990) in wheat. The embryos were germinated on MS + TDZ (0.45 μM). Höfer (2004) reported that an increase in the frequency of embryo induction is possible up to 10 times by microspore culture depending on the genotype. Starvation treatment, induction medium, maltose concentration, type of culture vessel, microspore density and genotype influenced embryo induction in apple Höfer (2004).

4.6 *Morus sp. (Family: Moraceae; Common name: Mulberry)*

Mulberry is a vital crop for sericulture industry and is native to warm temperate subtropical regions of Asia, Africa, Europe, and the Americas, with the majority of the species native to Asia alone. Mulberry leaves are ecologically important as the only food source for the silkworms (*Bombyx mori*), the pupa/cocoon which are used to make silk. Apart from its application as a food source for the silkworms, the medicinal properties of mulberry sp. are also widely acclaimed. Like many other woody genera, mulberry is highly heterozygous and, therefore, production of haploids and doubled haploids through anther culture is highly beneficial.

Several authors have reported androgenesis in mulberry which dealt with studies performed on induction of division in pollen culture (Katagiri 1989), effects of sugars and alcohols on pollen division (Katagiri and Modala 1991), embryo differentiation (Sethi et al. 1992) and production of haploid plantlets from anther culture (Shoukang et al. 1987). However, detailed studies were conducted by Jain et al. (1996) who evaluated the effect of temperature and Kinetin pre-treatment on induction of androgenic callus in anther cultures of mulberry. It was revealed that cold pre-treatment given to flower buds at 4 °C for 24 h increased the percentage of callus originating from anther cultures. The anthers split and produced embryogenic callus on Modified Bourgin (MB) medium (Qian et al. 1982) with 8 % sucrose and supplemented with NAA (2.68 μM) and BAP (4.44 μM). Upon transfer of the calli to MB basal medium supplemented with NAA (2.68 μM), BAP (2.22 μM), 2,4-D (4.12 μM) and Polyvinyl pyrrolidone (PVP) (1.0 mg/l), embryos were induced which later developed roots upon removal of 2, 4-D from the medium. Finally, the embryoids germinated precociously without developing cotyledons, but forming elongated shoots. Rhizogenesis was induced when calli were subcultured on MB medium containing NAA and BAP (each 0.5 mg/l with reduced myoinositol (75 mg/l). The cytological study of the induced roots from the calli revealed the haploid nature of the callus tissue.

To raise haploids from female clones of mulberry, ovary culture is the only possible approach because inbreeding and anther culture are not applicable (Thomas et al. 1999). In *ab initio* individual ovary cultures of *Morus alba,* the growth was very poor and gynogenic plants were never formed, suggesting that unfertilized ovaries required some stimulus from the parental tissue for the initial growth. This is in contrast to the observations of Lakshmi Sita and Ravindran in 1991 who observed gynogenesis in *ab initio* ovary cultures of *Morus indica*. These authors cultured individual ovaries before or after fusion to form sorosis under field conditions without taking any measure to prevent chance pollination. The inflorescence of mulberry is a catkin in which ovaries are loosely arranged. After fertilization, the ovaries enlarge and fuse to form a solid looking fruit called ‘sorosis’. It was possible that some of the ovaries cultured by them were fertilized. This could be the reason that some of the gynogenic plants were haploid, others were diploid. (Thomas et al. (1999) followed a two-step protocol for ovary culture of mulberry. They raised nodal segment cultures on MS+BAP (5 μM) which developed axil-

lary shoots and one or more inflorescence from the axil or leaves in three weeks. Inflorescence segments, each bearing 4–5 florets, from four-week-old cultures of nodal segments, were planted on MS + BAP (8.5 μM) + 2,4-D (4.5 μM) and after three weeks individual ovaries were transferred to MS + 2,4-D (4.5 μM) + Glycine (0.5 mg/l) + Proline (0.2 mg/l). In this treatment, 16 per cent of the ovaries developed a gynogenic seedling. After the gynogenic plants are transplanted in soil, out of 20 plants examined, 12 showed haploid number of chromosome (2n = x = 14) and the other eight were aneuploids with 13–17 chromosomes in their root tip cells.

4.7 Populus sp. (Family: Salicaceae)

Poplars form a very important part of basic forest biology and are economically important trees cultivated for their high wood quality that finds use in paper industry and energy production. The genus Populus consist of more than 30species, occurring throughout the forests of temperate and cold regions of northern hemisphere. The species *P. ciliata*, the only native poplar of India, is endemic to the Himalayan belt and has been an important tree for the forest breeders in India. In 1950, a large number of exotic clones of *Populus* were introduced and grown in North India, mainly, for increasing the wood availability for match and plywood industries. The study of poplars is essential to complement the ever increasing knowledge database of this important tree species.

First successful report on anther culture in *Populus* is by Wang et al. (1975), who observed callus proliferation from pollen and subsequent formation of haploid plants via organogenesis from the pollen-derived calli. Later, several reports were published on production of pollen plantlets in anther cultures of poplar (Zhu et al. 1980; Ho and Raj 1985; Kim et al. 1986; Mofidabadi et al. 1995). Induction of haploids via embryogenesis occurring from cultured anthers of *P. Maximowiczii* was obtained by Stoehr and Zsuffa (1990). The scientists applied cold pre-treatment to the flower buds (at 4 °C for four days) prior to culture the anthers at uninucleate stage of microspores, on MS medium containing 2,4-D (2.26 μM) and Kinetin (0.46 μM). Globular calli were developed from anthers after 4–8 weeks of dark incubation in the medium at 20 °C. When the anthers with globular calli were cultured on MS medium supplemented with NAA (0.54 μM) and BAP (4.4 μM), microspore division and embryoidal structures resembling globular–to-heart-shaped embryoids were obtained. However, the embryoids germinated precociously without developing cotyledons. After transfer to MS medium with BAP (4.4 μM), adventitious shoots developed mainly from the roots. Shoots were rooted on half strength MS medium supplemented with NAA (0.13 μM). Out of 34 plants analyzed cytologically, 22 showed haploid chromosome number (n = 19), one was aneuploid and the rest were diploids (Srivastava and Chaturvedi 2008). Kiss et al. (2001) reported further improvement in the rate of haploid plant regeneration by increasing the rate of induction from the anthers and with sustained shoot regeneration frequency in five

different genotypes of two poplar species, viz., *P. nigra* L. and *P. deltoides* Bartr. They reported that frequency of callus induction, shoot regeneration and number of shoots developed per calli are highly dependent on the genotype. The anthers were taken from floral buds which were subjected to cold pre-treatment at 4 °C for 8–14 days. For callus induction and shoot regeneration, they used the media suggested by Stoehr and Zsuffa (1990) with slight modifications. The anthers were incubated in dark for 6–7 weeks at 25 °C. Calli for shoot organogenesis were transferred to both MS or WPM medium (Lloyd and McCown 1980) supplemented with BAP (4.4 μM), NAA (0.54 μM), and 2.5 % sucrose and incubated in light at 23 °C. Shoots were rooted on hormone-free WPM. Of the 208 rooted plants, eight haploids, 179 diploids, four tetraploids and 17 aneuploid plants were obtained. Deutsch et al. (2004) reported haploid plant regeneration via embryogenesis from isolated immature pollen culture of two poplar hybrids (*Populus nigra* L.× hybrid 'Aue1' and 'Aue2') and employed microsatellite marker analysis to confirm the ploidy level of the regenerants.

5 Analysis of Ploidy Level

Analysis of Ploidy level is an integral part of haploid production programme which can be carried out efficiently either by chromosome counting during mitotic and meiotic cell division or by flow cytometry.

5.1 Chromosome Counting

Counting of mitotic chromosomes is easier and faster. Root tips are the most convenient source of mitotic cells. When roots are not available, young axillary buds, leaves or callus can be used. The cytological procedures of chromosome preparation and staining are different and need to be modified depending on the plant species. The cytological procedures for visualizing chromosomes in woody plants may not be the same as in herbaceous species. Nevertheless, basic principles for handling the mitotic chromosomes of all plant species are similar and consist of collection of material, fixation and chromosome staining. The most critical step for chromosome counting involves proper chromosome squash preparation. It is very important to obtain sufficient well spread metaphase plates and proper physical separation of the chromosomes. However, method of chromosome staining applied for ploidy level analysis depends on plant species and chromosome size (Maluszynski et al. 2003a).

Protocol for cytological investigation in neem (Chaturvedi et al. 2003)
Fixation:

- Healthy root-tips (1 cm) from plantlets were collected at 10.30 am and rinsed with tap water.

- The root-tips were pre-treated with 0.02 % 8-Hydroxyquinoline at 4 °C for 4 h.
- Following which, the root-tips were fixed in a modified Carnoy's fluid containing absolute alcohol-chloroform-Glacial acetic acid-Methanol (7:3:1:1) for 48 h.
- Finally, the root-tips were preserved in 70 % ethanol at 4 °C.

5.2 Chromosome Staining

- The root-tips from fixed roots were excised and placed in a mixture of nine drops of 2 % aceto-orcein and one drop of 1N HCl in a watch glass and warmed gently.
- After cooling, the individual root-tips were placed in a drop of aceto-orcein on a glass slide, covered with a cover slip, warmed gently and squashed.

5.3 Flow Cytometric Analysis

Flow cytometry using DNA selective flourochromes has been considered to be a fast and reliable method for the measurement of nuclear DNA content in recent years (Muirhead et al. 1985; Thorthwaite et al. 1985; Dolezel et al. 2007). Unfortunately, its application in plant biology has been overdue, largely owing to the fact that flow cytometry requires single cell suspension (Shapiro 1985). As plant cells usually exist in complex tissues, methods had to be developed for the preparation of such suspensions. Although flow cytometry is an extremely efficient technique with high degree of accuracy, the preparation of high quality plant samples for ploidy analysis remains a vital issue. For determination and interpretation of the haploid status of the regenerants, firstly tissue of known ploidy is analyzed followed by the unknown tissue whose ploidy is to be analyzed.

5.4 Protocol for Flow-Cytometric Analysis in Tea

5.4.1 Preparation of Nuclear Sample

- Well developed calli obtained after two months of culture initiation were used for ploidy analysis.
- Extraction of nuclei and the analysis were carried out via fine chopping of the hard calli placed in 1 ml ice-cold woody plant buffer. The woody plant buffer was prepared by mixing 0.2 M Tris HCl, 4mM $MgCl_2.6H_2O$, 2mM EDTA $Na_2.2H_2O$, 86mM NaCl, 10 mM Sodium Metabisulfite, 1 % Triton X-100 (v/v), and 2 % PVP-10 (w/v) according to the protocol of Loureiro et al. 2007 with

slight modifications. The pH of the buffer was adjusted to 7.5, filtered through 0.22 µm Polyvinylidene fluoride (PVDF) membrane filter and stored at 4 °C.
- The suspension containing the nuclei was mixed gently by pipetting up and down, softly, several times, followed by filtering of the homogenate through a 30 µm nylon mesh.
- The nuclear suspension was stained with Propidium iodide at a concentration of 50 µg/ml.
- Simultaneously RNAse at a concentration of 50 µg/ml was also added to the nuclei.

Ploidy analysis:

- The ploidy level was determined using a FACs Calibur cytometer (Becton-Dickinson, USA).
- All measurements were carried out in triplicate using fresh tea leaves as an external standard. Using instrument gain (photomultiplier voltage and amplitude gain), the position of peak G_1 nuclei of the reference sample was established on channel 200 on a 1,024 scale following which the instrument settings were kept constant and the unknown samples were run under the same parameters. The mean channel number of the unknown sample G_1 peak was determined and the DNA Ploidy was calculated according to the relationship:
  Sample Ploidy (integer) = Reference Ploidy X (mean position of the G_1 sample peak/mean position of the G_1 reference peak)

6 Diploidization of Haploids

Doubled haploids are of immense importance for genetic studies and in crop breeding programmes. Selected doubled haploid lines are used for production of commercial hybrids (Chase 1974). To obtain fertile, homozygous diploids for analyzing the progenies and the breeding behaviour of the pollen plants, the chromosome complement of the haploids must be duplicated. This is because haploids may grow normally up to the flowering stage, but in the absence of homologous chromosomes, meiosis is abnormal and consequently, viable gametes will not be formed and, hence, they are sterile. Spontaneous duplication of chromosomes in pollen-derived plants has been observed, but its frequency is very low.

In vegetable crops, doubled haploids are used prominently as parents for F_1 hybrid seed production. Similarly, in medicinal and aromatic plants, doubled haploids for F_1 production have the potential to make significant advances in providing high, stable and predictive yields of raw biochemicals to be processed by pharmaceutical and neutraceutical industries (Ferrie 2007). In case of cross pollinating species and hybrids, pure homozygous lines are highly desirable. Although conventional breeding method to produce homozygous diploids is well

established, it requires 7–8 repeated inbreeding cycles which in turn is time-consuming and labour intensive. Moreover, this approach is difficult in self-incompatible male sterile plants and tree species with long gestation period. Also, the pure lines obtained after several generations of self-pollination may not be 100 % homozygous (Germanà 2006). Production of fertile doubled haploid lines in *S. cereale* (Immonen and Anttila 1996) and *Festuca and Lolium* (Nitzche 1970) are of immense importance as these species suffer from inbreeding depression. One of the most efficient techniques for production of homozygous diploid plants in a single generation is through diploidization of the haploids. Doubled haploids have a key feature in establishing chromosome maps in a number of species, notably barley *Hordeum vulgare*, *Oryza sativa, Brassica napus, Triticum aestivum* (Forster and Thomas 2005), apart from providing a vast majority of mapped genetic markers. Molecular marker maps provide a platform for trait mapping, which is of particular interest to plant breeders. Doubled haploidy is also useful in rapid isolation and purification of selected mutants in subsequent generations. It is possible to create a population of homozygous mutant lines directly by targeting the mutation treatment at single gametic cells and then inducing embryogenesis, followed by subsequent chromosome doubling for homozygous diploid plant production (Szarejko and Forster 2006).

Doubled haploidy can be significantly enhanced by artificial means using chemicals such as colchicine, pronamide, trifluralin, oryzalin and amiprophos methyl (APM) (Wan et al. 1991). Colchicine treatment is one of the most preferred techniques for chromosome doubling, which in turn is one of the most critical steps in the doubled haploid breeding process. For *Nicotiana tabacum,* a 0.4 % solution of colchicine is recommended to diploidize the pollen plants. In practice, the young pollen derived plantlets are immersed in a filter sterilized solution of colchicine for about 96 h and then transferred to a culture medium to allow their further growth. Alternatively, the treatment is given in the form of a lanolin paste (Bhojwani and Razdan 1996). It is applied to the axils of the upper leaves and the main axis is decapitated to stimulate the axillary buds to grow into diploid and fertile branches. Besides bringing about chromosome duplication, colchicine treatment may also result in chromosome and gene instabilities (Burk 1970). Therefore, the frequent occurrence of spontaneous duplication of chromosomes in differentiated plant cells like cortex and pith and callus cells in long-term cultures has been exploited to raise homozygous, fertile diploids from haploid plants. In Apiaceae, for diploidization the plantlets grown in Petri dishes were taken out and the agar was removed from the roots. Subsequently, the roots were submerged in a 0.34 % (w/v) solution of colchicine for 1½ h. The roots were rinsed in water and the plantlets were transferred to a soil-less mix and grown in the greenhouse (Ferrie et al. 2005). For *Brassica* species, the roots of 25–30 pollen-derived plantswere immersed in a bunch in 0.25 % (w/v) colchicine solution for 5 h in light, for diploidization. After rinsing the treated roots with distilled water, the plants were transferred to a potting mix for hardening and further growth (Bhojwani and Dantu 2010).

7 Application of Haploid Production

7.1 Development of Pure Homozygous Lines

Homozygous true breeding cultivars are highly important for screening of high yielding lines and to produce hybrid vigour as a method of crop improvement. Obtaining homozygous diploid plants by conventional methods is difficult in perennials. From several decades to over a hundred years are required to obtain a pure line by means of successive inbreeding throughout many generations. The seed set by inbreeding in many trees is very low, usually only a few of ten thousandth or sometimes no seed can be obtained at all; therefore, it is impractical to obtain pure lines by inbreeding (Chen 1986). Moreover, conventional method of haploid production by inbreeding is impossible if the plant is strictly cross-pollinating in nature. On the other hand, homozygous diploid plants can be achieved in a single generation by diploidization of in vitro raised haploids through colchicine treatment.

7.2 Genetic Studies

Because of the lack of accurate material in research work, the progress in the study of genetics in trees is much slower than that in annual herbaceous plants. The genetics of a lot of important traits in economically important plant species has not been clearly demonstrated as yet. As a result, it is still unknown whether the desirable characters of the parents will appear in their progenies. Only when crossing between different homozygous diploid plants is carried out, we can gain a clear idea of dominance of genes controlling various characteristics and that these characteristics are either monogenic or polygenic (Chen 1986). Furthermore, if we can use the haploid plants as samples of gametes, then we can obtain directly the recombination value between genes. Moreover, we can also use the haploid plants to study chromosome homology within genome or between genomes.

7.3 Gametoclonal Variation

The "gametoclonal variation" arises among plants regenerated from cultured gametic cells consisting of differences in morphological and biochemical characteristics as well as in chromosome number and structures that are observed. Besides yielding haploids, in vitro androgenesis helps in the screening of gametophytic variation at plant level. Pollen grains within an anther form a highly heterogeneous population because they are the product of meiosis which involves recombination and segregation. Therefore, each pollen plant is genetically different from the other. The gametoclonal variants being hemizygous in nature, express also the recessive characteristics in the R_0 plants (Bhojwani and Razdan 1996). Different sources of

variation can explain gametoclonal variation such as new genetic variation induced by cell culture procedures, from segregation and independant assortment, chromosome doubling procedures, etc., (Morrison and Evans 1987; Huang 1996).

7.4 Induction of Mutations

In general, a majority of induced mutations are recessive and, therefore, are not expressed in diploid cells due to the presence of dominant allele. Since haploid plants have only one set of chromosomes, their dominant and recessive characteristics can be seen simultaneously on separate plants. It is extremely advantageous to provide a convenient system for the induction of mutations and selection of mutants with desirable traits in the absence of their dominant counterparts (Bhojwani and Razdan 1996).

7.5 Obtaining New Genotypes with Alien Chromosomes

The technique of interspecific and intergeneric hybridization can be combined with anther culture techniques (Thomas et al. 2003) for obtaining new genotypes with alien chromosomes. Thus new genotypes with various reconstructed chromosome complements can be obtained after their successful chromosome doubling.

7.6 Genetic Manipulation

As microspore culture is a single cell system, it makes selection at the single cell level possible and, furthermore, offers new prospects for genetic manipulation like mutagenesis and transformation. Direct gene transfer by microinjection offers the possibility of transgenic plant formation by using isolated pollen culture having high regeneration efficiency (Kasha and Maluszynski 2003). Moreover, if transgenes can be incorporated into the haploid microspore genome prior to DNA synthesis and chromosome doubling, the doubled haploids may also be homozygous for the transgenes. Thus isolated microspores not only provide a good target for bombardment, but also are readily amenable to transgene in vitro selection. Jahne et al. (1994) were the first to achieve plants homozygous for the transgenes using biolistic bombardment of barley microspores.

7.7 Genomics

Doubled haploids play a vital role in genomics, especially, in the integration of genetic and physical maps, thereby, providing precision in targeting candidate genes

(Kunzel et al. 2000; Wang et al. 2001). Doubled haploids, combined with marker-assisted selection, provide a short-cut in backcross conversion, a plant breeding method for improving an elite line defective in a particular trait (Toojinda et al. 1998). Expressed sequence tags may help in identification of genes that determine any trait of interest.

8 Conclusion

The haploid-derived true breeding lines provide a rapid means of achieving homozygosity, thereby speeding up the usually cumbersome and protracted conventional breeding methods for crop improvement. Today, haploids and doubled haploids have been reported in over 200 plant species (Forster et al. 2007), however, less than 10 % of these reports encompass tree species. Thus much work needs to be done in the field of in vitro haploid production. This may be feasible only with the development of novel genotype-independent methods through the study and improvement of existing protocols and by obtaining a better understanding of the haploid induction process, especially of the two main developmental switches: the induction of the male/female gametophyte to undergo division, and subsequent occurrence of embryogenesis. The recent spurt in the development of ultra high throughput technologies of advanced genomic, transcriptomic, proteomic and imaging tools hold great promise for identification and analysis of genes that might be playing important roles in the haploid induction process. This will immensely help in the understanding of these processes and towards development of highly efficient protocols for production of haploids, resulting in the overall improvement of highly desirable and recalcitrant plant species. Through this chapter, we intend to provide a clear and simple overview of haploidy with the purpose to stimulate interest among scientists working in the related area and the potential application of the various in vitro techniques used in haploid production intended for crop improvement.

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Chapter 9
Production of Abiotic Stress Tolerant Fertile Transgenic Plants using Androgenesis and Genetic Transformation Methods in Cereal Crops

S. M. Shahinul Islam and Narendra Tuteja

1 Introduction

Various abiotic stresses such as drought, cold, and soil salinity are some of the major environmental stress factors that adversely affect plant growth and productivity. Of these, high salinity and drought are the major causes affecting the crop yield in the world and increasing salinity stress is the main reason for reduced agricultural production in the available crop lands (Tuteja 2007; Amudha and Balasubramani 2011). Therefore, it is very important to understand the effects on abiotic stress response mechanisms and networks in plants. To minimize the crop losses, modern biotechnology can be used to generate genetically-engineered plants with new and improved characteristics. Genetic engineering is an attractive tool because of its potential to improve abiotic stress (cold, drought, salt, heat, starvation, etc.,) tolerance plant varieties more rapidly (Kasuga et al. 1999). The use of genetic engineering technology could lead to simpler and more effective gene-based approaches for improving crop tolerance. Thus, the application of biotechnology in combination with conventional breeding methods may help to increase food production properly. An endeavour to genetically improve the abiotic stress-tolerant crop plants, with respect to disease resistance, drought, heat and salinity tolerance with high yielding cultivars, may be helpful in boosting the major cereal crop production in developing countries. Breeders have often developed cultivars with superior adaptation to their environmental without the detailed knowledge of the underlying physiological mechanisms (Kuchel et al. 2006). Gene transfer technologies offer

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a suitable alternative for improving desirable gene(s) in a directed manner without the insertion of undesirable DNA fragments. The establishment of stable and regenerative tissue culture and transformation systems is a prerequisite for barley and other cereal crops. Different explants, immature embryos (Breiman, 1985), mature embryos (Lupotto 1984), apical meristems (Cheng and Smith 1975), anthers (Kao and Horn 1982), microspores (Köhler and Wenzel 1985), cell suspensions (Kott and Kasha 1984) and protoplasts (Lazzeri and Lörz 1990) have been used for this purpose. In practical breeding, to develop homozygous transgenic plants with a stable transgene is very interesting and important (Vyroubalová et al. 2011). Androgenesis is an elegant system for genetic transformation (Jähne et al. 1994; Stöger et al. 1995), and could provide a practical alternative for the production of transgenic doubled haploid plant species, in which regeneration from somatic cells is difficult, especially in the recalcitrant cereals. This protocol may be used to overcome genotypic limitations of doubled haploid formation in cultivars that had previously been found to be recalcitrant in anther culture (Sopory and Munshi 1996). To date, haploid offspring have been obtained from over 200 species, including all the major crops and hundreds of cereals (Floss et al. 2009). By using this system (transgenic doubled haploid plant formation), it has been possible to produce homozygous transgenic crop plants with targeted genes and crops (Kumlehn et al. 2006, Aionesei et al. 2006; Fukuoka et al. 1998). This chapter has been focused on combination of in vitro androgenesis and genetic transformation technology using different stress tolerance genes for the production of fertile transgenic/ homozygous plants rapidly.

2 Methods

Various approaches are employed in order to obtain fertile transgenic plants using androgenesis and genetic transformation systems in cereal crops. The successful development of transgenic plants necessitates a reliable tissue culture regeneration system, gene construct(s), suitable vector(s) for transformation and efficient procedures to introduce desired genes into target plants. Generally, some steps are common for almost all crop species which are mentioned here in the flow chart below.

2.1 Androgenesis (Anther and Microspore Culture)

General techniques of anther culture, media and other steps by the protocol of Schmid (1990) are followed. For isolated microspore culture, microspore isolation procedure, media and other steps by the protocol of Kunz et al. (2000) and Huang (1992) are followed.

2.1.1 General Steps of Androgenesis Methods

Plant grown in the field or/greenhouse

↓

Spikes are harvested from plants when microspores are at early to mid uninucleate stage

↓

Harvested spikes pre-treated under cold condition at 3–4 °C for 3–21 days

↓

Spikes sterilized with 70 % ethanol or mercuric chloride (0.1 %) for 2–3 minutes

↓ *Anther culture*

Anthers removed from the middle zone of spike

↓ *Microspore culture*

Microspores are isolated mechanically with the help of a sterile glass rod or blender

↓

In both cases, cultures are incubated at 28 °C for 3–6 weeks for embryo formation

↓

Suitable embryos are transferred to regeneration medium

↓

For regeneration, cultures are incubated at 26 °C under the light condition

↓

Regenerated plantlets are transferred to suitable medium for root and shoot development

↓

Ploidy levels are analyzed by flow cytometry/karyotypic methods

↓

If required colchicine treatment may be applied for chromosome doubling in haploid plants

↓

Treated plants are transferred to soil

↓

Plants are grown in field and/or greenhouse and DH seeds are harvested

2.2 Genetic Engineering to Develop Selected Abiotic Stress Tolerence Homozygous Plants

There are different ways of plant transformation and analysis of transgenes. Generally, some steps are common for almost all cereal crops which are mentioned herein. Protocol varies a little bit depending on the use of the crop and the explant. This chapter highlights mainly the *Agrobacterium* -mediated transformation. The transformation steps through androgenesis (anther or microspore-derived callus)

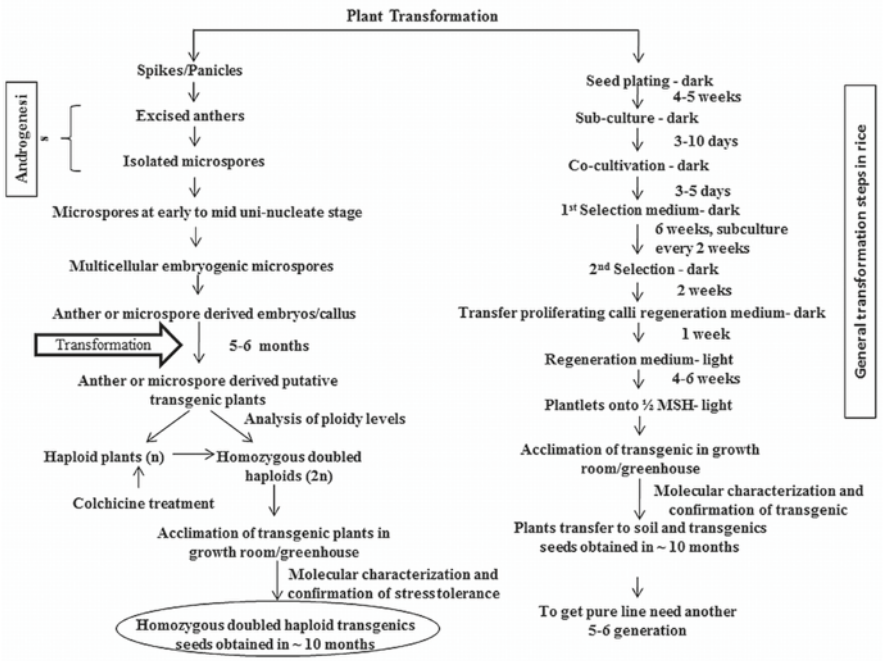

Fig. 9.1 A flow chart showing the comparative study of both plant transformation systems-androgenesis and general transformation (seed-derived callus), in rice

and general transformation (seed-derived callus) are shown in Fig. 9.1. Success of plant transformation and its analysis in different stages is mentioned in the following reports (Hiei et al. 1994; Hiei et al. 1997; Toki et al. 2006; Nishimura et al. 2006; Lemaux PG 2008; Hiei and Komari 2008; Chauhan and Khurana 2010). General steps of both the plant transformation systems are mentioned below accordingly.

2.2.1 Plasmid Construction of Targeted Genes

Plasmids are constructed harbouring the targated gene along the expression cassette of plant promoters accompanied by selectable marker gene. Constructs are used for anther/microspore-derived embryoids as explant for doubled haploid transgenic plant production.

2.2.2 Transformation

Plant transformation is performed via *Agrobacterium* strains of LBA4404 for co-cultivation using anther/microspore-derived embryos as explants. Transformation is performed with plasmids containing targeted genes separately and for both constructs, the selectable marker is GUS.

(i) Plating: *Agrobacterium* strains are inserted in the YM/YEP semi-solid medium containing antibiotics for two to three days before the transformation.
(ii) Inoculation: Inoculation of *Agrobacterium* using the targeted explants (microspores, protoplast isolation from microspore-derived cell suspension cultures, anther or microspore-derived embryos/callus) with co-cultivation medium and cultures are incubated at 26 °C for 2–3 days.
(iii) Disinfection of *Agrobacterium* and screening of transformed callus: Treated explants are washed properly with Claforan (250 mg/l). Blotting the calli with sterile filter paper and transferring the dry calli to the selected medium and incubated in dark at 28 °C for 4–6 weeks (The callus are transferred to a fresh medium after two weeks).
(iv) Regeneration and screening: Transformed embryoids/calluses are transferred to regeneration medium and incubated at 28 °C for 2–3 weeks in light for regeneration.
(v) Root and shoot development: To promote root and shoot development of regenerated plantlets, they are transferred to the rooting medium.
(vi) Flow cytometry analysis: The ploidy level of transgenic plants is estimated by flow cytometry. Nuclei are isolated from the young leaf. For the measuremet procedure, a PARTEC Ploidy Analyser II (Partec, Münster, Germany) flow cytometry equipped with the software (Partec, Münster, Germany) is used.
(vii) Transfer of plants to soil and colchicine treatment: For chromosome doubling, regenerated plants (if haploid) with good roots are immersed in 0.2 % colchine and 2 % DMSO (dimethyl sulfoxide) for 6 h and transferred to soil.
(viii) Homozygosity test: Testing of doubled haploid (DH) transgenic plants is done. Evaluation of stress tolerence is done and plants are grown in the field/greenhouse for DHs seed collection.

2.2.3 Molecular Analysis of Transgenic Plants

Molecular analysis of transgens is followed by the protocol of Sambrook et al. (1989), Datta et al. (1997), Vashisht et al. (2005), Tuteja (2010). Some common steps of molecular analysis of cereal crops are mentioned below.

(i) Confirmation of transgenic plants by PCR: Total genomic DNA is extracted from the putatively transgenic plants and control of untransformed plants is carried out using modified cetyltrimethylammonium bromide (CTAB) method (Tuteja 2010).
(ii) Southern blot analysis: For Southern blot analysis of transplastomic plants, total cellular DNA is extracted from leaf based on the CTAB method. A 2 µg total DNA is digested overnight with the restricted enzyme, separated on a 0.7 % agarose gel and blotted on to nylon membranes (Nytran N, Schleicher & Schuell, Dassel, Germany). Then, hybridization is performed using the protocol of Datta et al. (1997) and Sambrook et al. (1989).
(iii) Northern blot analysis: Total RNA is isolated from the leaves of transgenic plants as well as from the untransformed control. The subsequent RNA

hybridization is performed following the procedure used in Southern blot analysis. Then subsequent methods are performed using protocol of Vashisht et al. (2005).

(iv) Western blot analysis: Total soluble protein is obtained by homogenizing young leaves and stems of transgenic rice and control plants in the extraction buffer (200 mM Tris-HCl, pH 6.8, 20 % glycerol, 10 % 2-mercaptoethanol). Then, 10 µg of protein from each preparation is separated by 10 % SDS-PAGE, transferred to a hybond-C membrane (Amersham). Then the subsequent methods are performed using the protocol of Vashisht et al. (2005) and Sambrook et al. (1989).

(v) Bioassay analysis: In order to assess the stress tolerant doubled haploid transgenic plants, some work needs to be done using the following methods:

a) Confirmation of transgenic plants by overexpression of targeted genes: Selection, regeneration and growth of transgenic plants through overexpressing method is followed by the protocol of Tuteja (2010) and Mahajan and Tuteja (2005).
b) Confirmation of transgenic plants by histochemical, ß-glucuronidase (GUS) assay: This method is performed for screening the putative transgenic plants for the expression of GUS by following the protocol of Tuteja (2010).
c) Screening of transformants under the stress conditions: Transgenic plants along with wild type (WT) are evaluated for physical responses to drought stress by assessing their transpiration response to water deficit using the dry down techniques (Sinclair and Ludlow 1986).
d) Screening of transformants under the stress tolerance by leaf disk assay: Transgenic plants (T_0 generation) are checked by leaf disk assay for stress tolerance by following the protocol of Tuteja (2010).

Description of development of abiotic stress tolerant fertile transgenic plants using genetic transformation and androgenetic techniques is mentioned here in the following paragraphs:

2.2.4 Use of Biotechnology to Improve Abiotically Stressed Crop Plants

Major stresses (abiotic and biotic) have a considerable impact on crop growth, development, and productivity throughout the world (Zhao and Zhang 2007). Now a days, abiotic stresses have become a continuous hindrance in crop production. Therefore, it is important to elaborate on the injury and tolerance mechanisms, and improve the crop genotypes under abiotic stress conditions (Shariatpanahi et al. 2006; Roy and Basu 2009; Roy et al. 2011). Abiotic stresses such as sub- and supra optimal temperatures, excess salt levels, reduced water availability leading to drought stress, excess water resulting in flooding stress and oxidative stress caused by the combination of high light intensity, etc., showed adverse effects on almost all major crop plants (Grover and Minhas 2000; Gill and Tuteja 2010; Fleury et al.

2010). It has been estimated that about one-third of the world's potentially viable land suffers from inadequate supply of water, and of the remainder, crop yields are periodically decreasing due to drought and salinity (Kramer 1980). Most of the world's agriculturally important major cereal crops such as wheat, rice, maize and barley growing areas, are affected routinely by drought. Therefore, it is important to develop crops using suitable biotechnological methods which will be tolerable under adverse environmental conditions and breeders are educated on new technology.

2.2.5 Doubled Haploid Technology (Androgenesis) for Crop Improvement

The ability to transform and regenerate plants represents the most powerful tool and advancement in the field of plant biotechnology (Datta 2005). Through androgenesis (anther and microspore culture), complete homozygous plants can be produced within a year as compared to the long durational inbreeding method, which normally requires at least six inbreeding generations. The principle of androgenesis is to arrest the development of the pollen grains (male gametophytes) and to force them towards a somatic pathway (Datta 2005). It provides the most commonly used method for the production of doubled haploid plants that is eventually applied in breeding and crop improvement. Most of these lines exhibit wide adaptability, high resistance to drought, salinity and infertile soil, and luxuriant growth (Bikash and Mandal 2001). But even this widespread use of DH technology is impeded by the lack of a technique, which can satisfy all of the expected criteria for a successful system (Snape et al. 1986), e.g., (i) easy, consistent production of large numbers of DHs of all genotypes in the breeding program, (ii) DHs should be genetically normal and phenotypically stable, and (iii) recombinant DH populations should contain an adequate sample of the genetic variation in the parents.

2.2.6 Genetic Engineering for Crop Improvement

Davey et al. (2010) mentioned that gene manipulation, combined with the ability to induce cultured plant cells to express their totipotency leading to the regeneration of fertile plants, provides a unique opportunity to extend the genetic pool available to breeders for crop improvement. Cereal crops have been recalcitrant to recombinant techniques mainly because of problems in establishing regenerable cell and tissue cultures as well as due to efficient DNA delivery systems (Koprek 1996). There are different ways of introducing DNA into plant cells such as protoplast transformation/electroporation, microinjection, biolistics gun, and transfer from *Agrobacterium tumefaciens* (Dale and Schantz 2004). Potrykus (1990) reported that *Agrobacterium* method is not possible to be used for transformation of monocotyledonous tissues. But after the successful achievement of cereal crops by Hiei et al. (1994), Ishida et al. (1996), Cheng et al. (1997) his finding was questioned. The most distinct way of getting foreign DNA into a plant cell involves the bacterium *Agrobacterium tumefaciens*. Through particle bombardment, improvement of transgenic cereal crops

has been reported for rice (Christou et al. 1991), maize (Gordon-Kamm et al. 1990), wheat (Weeks et al. 1993), oat (Somers et al. 1992), rye (Castillo et al. 1994), barley (Harwood et al. 1995; Hagio et al. 1995; Kihara et al. 1998; Shim and Kasha 2003). Using isolated microspore as explants, the first homozygous transgenic Indica rice was reported by Datta et al. (1990).

Plant genetic engineering also holds the promise of circumventing the problems faced in wide hybridization programs, especially when sources of resistance are not available in taxonomically related species (Davey et al. 2010). Increasing research efforts through genetic engineering for abiotic stress tolerance in crops, are being employed (Holmberg and Bülow 1998; Umezawa et al. 2006). Certain genes are expressed at elevated levels when a plant encounters stress (Bray 1993). A cis-acting dehydration responsive element (DRE), identified in *Arabidopsis thaliana*, is involved in ABA-independent gene expression under drought, low temperature, and high salt stress conditions in many dehydration responsive genes (Nordin et al. 1991; Yamaguchi-Shinozaki and Shinozaki 2007; Iwasaki et al. 1997). It is generally agreed that in order to meet future challenges in food production, multi-disciplinary, multi-faceted approaches are needed. Solutions to the problem of how the developing world will meet its future food needs, are broader than producing more food, although the successes of the 'Green Revolution' demonstrate the importance of technology in generating the growth in food output in the past. Despite these successes, the world still faces continuing vulnerability to food shortages. It seems likely that conventional crop breeding, as well as emerging technologies based on molecular biology, genetic engineering and natural resource management, will continue to improve productivity in the coming decades (Huang et al. 2002). The use of genetic engineering technology could lead to simpler and more effective gene-based approaches for improving crop tolerance. Transcription factors have been shown to produce multiple phenotypic alterations, many of which are involved in stress responses.

2.2.7 Major Abiotic Stress Factors under Androgenesis and Combined with Genetic Engineering Research

The main targets for genetic engineering include modification of plants to enhance their tolerance to biotic stresses (herbicides used to control weeds and to confer resistance to insects, bacteria, fungi, and viruses). Baisakh et al. (2001) obtained homozygous transgenics in about a year from the start of transformation till the confirmation of the anther-derived line versus a minimum of 20–24 months required in the usual course of generation advancement. Very recently, Roy et al. (2011) reported that employing transgenic technology, functional validation of various target genes involve in diverse processes, such as signaling, transcription, ion homeostasis, antioxidant defense etc. to enhance abiotic stress tolerance crop plants. To date, different abiotic stresses to crop improvement are shown in Table 9.1. Crop improvement in some cereals using androgenesis and genetic transformation methods is shown in Table 9.2.

Table 9.1 Application of Androgenetic Method for Improving Abiotic Stressed Cereal Crops

Plant	Name of stress	Androgenesis (Explants)		Results (Embryos and Regeneration)	References
		AC	MC		
Barley	Cold	–	√	√	Davies and Morton (1998)
	Cold	√	–	√	Jacquard et al. (2003); Szarejko (2003)
	Cold and or/starvation	–	√	√	Kasha et al. (2001)
	Starvation	√	–	√	Castillo et al. (2000)
Durum	Cold	√	–	√	Jauhar (2003)
	Cold & osmotic stress			√	Slama-Ayed et al. (2010)
Maize	Cold	√	–	√	Barnabás (2003)
	Colchicine	√	–	√	Obert and Barnabás (2004)
	2-HNA and cold	-	√	√	Zheng et al. (2003)
Oats	Cold	√	–	√	Kiviharju and Pehu (1998)
Rice	Cold	√	–	√	Zapata-Arias (2003)
	Aluminum toxicity	√	–	√	Purwoko et al. (2010)
Rye	Cold	√	–	√	Wenzel et al. (1977)
	Osmotic stress	-	√	√	Guo and Pulli (2000)
Triticale	Cold	√	–	√	Tuvesson et al. (2000)
	Cold	–	√	√	Pauk et al. (2000)
Wheat	Cold	√	–	√	Tuvesson et al. (2000)
	Drought	√	–	√	Chauhan and Khurana (2010); Islam (2010a)
	Heat and/or starvation	–	√	√	Touraev et al. (1996)
	Cold and/or starvation	–	√	√	Kasha et al. (2003)
	Colchicine and heat	√	–	√	Barnabás (2003)
	Colchicine	-	√	√	Islam (2010b)
	2-HNA	-	√	√	Zheng et al. (2001); Liu et al. (2001)
	Gametocide	√	–	√	Schmid and Keller (1986)

AC Anther culture, *MC* Isolated microspore culture, *2-HNA* Hydroxynicotinic acid, *CGA* Gametocide, √ = Yes, – = No

3 Conclusion and Future Prospects

This review has been focused on combination of in vitro androgenesis and genetic transformation for the production of fertile transgenic crops rapidly, using different stress tolerance genes. Various stress pre-treatments, such as high temperature, cold, heat shock, salt, drought, oxidative stress, reduced atmospheric pressure, osmotic shock and starvation, during developmental of pollen grains are known to be essential for the induction of androgenesis in several crop plants, including cereals. Using anther and microspore-derived embryos/callus/microspores/protoplast for genetic transformation is an elegant system and could provide a practical alternative for the

Table 9.2 Application of androgenesis and genetic transformation methods to develop transgenic doubled haploids in cereal crops

Plant	Androgenesis (Explants)		Genetic transformation methods	Transformation freq. (%): fertile plants/targets	References
	AC	MC			
Barley	–	MC	*Agrobacterium*	n.d	Kumlehn and Hensel (2009)
	–	MDEs	Particle bombardment	FTPs (0–40.0 %)	Shim et al. (2009)
	–	MDEs	Particle bombardment	n.d.	Obert et al. (2008)
	–	MC	*Agrobacterium*	FTPs (0.3–2.2 %)	Kumlehn et al. (2006)
	–	MC	*Agrobacterium*	FTPs (17.64 %)	Coronado et al. (2005)
	–	MC	*Agrobacterium*	n.d	Hansel and Kumlehn (2004)
	–	MC	*Agrobacterium*	n.d	Kumlehn et al. (2004)
	–	MC	Biolistic transformation	n.d	Chen et al. (2003)
	–	MDEs	Particle bombardment	FTPs (43–67.0 %)	Thmás (2003)
		MDEs	Biolistic transformation	FTPs (one plant per 10^{-6} microspores)	Scholz et al. (2001)
	–	MDEs	Biolistic transformation	FT (3.3 %)	Carlson et al. (2001)
	–	MDEs	*Agrobacterium*	FTPs (0.3–2.9 %)	Wu et al. (1998)
	–	MDEs	Biolistic transformation	FTPs (one plant per 10^{-7} microspores)	Yao et al. (1997)
	ADEs	–	Particle bombardment	n.d.	Koprek et al. (1996)
	–	MC	Particle bombardment	FTPs (one plant per 10^{-7} microspores)	Jähne et al. (1994)
	–	MDEs	Particle bombardment	TDHs (0.4 %)	Ritala et al. (1994)
	–	MDEs	*Agrobacterium*	FTPs (0.3–7.9 %)	Wan and Lemaux (1994)
	–	MDEs	Electroporation	FTPs (2.5 % plants per 10^{-6} microspores)	Salmenkallio-Marttila (1994)
	–	MDEs	*Agrobacterium*	n.d	Kuhlmann et al. (1991)
	–	MDEs	Microinjection	n.d	Bolik and Hu, (1991)
	–	MDEs	*Agrobacterium* and microprojectile	n.d	Creissen et al. (1990)
Maize	ADEs	–	Biolistic transformation	FTPs (45–61.0 %)	Aulinger et al. (2003)
	–	MC	Electroporation	n.d	Fennell and Hanptmann (1992)

Table 9.2 (continued)

Plant	Androgenesis (Explants)		Genetic transformation methods	Transformation freq. (%): fertile plants/targets	References
	AC	MC			
	–	MC	Microinjection	n.d	Gaillard et al. (1992)
	ADEs	–	Electroporation	FTPs (5 %)	Sukhapinda et al. (1993)
Rice	–	MDEs	Particle bombardment	FTPs (0–9.0 %)	Otani et al. (2005)
	ADEs	–	Particle bombardment	n.d	Baisakh et al. (2001)
	–	MDEs	PGE transformation	FTPs (0–47.0 %)	Chair et al. (1996)
	–	MC	*Agrobacterium*	FTPs (2.2 %)	Rashid et al. (1996)
Triticale	–	MDEs	Particle bombardment	FTPs (43–67.0 %)	Thmás (2003)
	–	MDEs	*Agrobacterium*	FTPs (3.3 %)	Zimny et al. (1995)
Wheat	ADEs	-	*Agrobacterium*	FTPs (2.38 %)	Chauhan and Khurana (2010)
	–	MDEs	Particle bombardment	n.d	Gharanjik et al. (2008)
	ADEs	–	Electroporation	n.d	Holiloglu et al. (2004)
	–	MDEs	Particle bombardment	n.d	Folling and Olesen (2002)
	ADEs	–	*Agrobacterium*	n.d	Pauk et al. (2003)
	ADEs	–	*Agrobacterium*	FTPs (1.0–3.4 %)	Massiah et al. (2001)
	ADEs	–	*Agrobacterium*	n.d	He et al. (1993)
Rye	–	MDEs	*Agrobacterium*	n.d	Kumlehn and Hensel (2009)

AC Anther culture, *MC* Microspore culture, *ADEs* Anther-derived embryos, *MDEs* Protoplast isolation for microspore-derived cell suspension cultures/Microspore-derived embryos, *FTPs* Fertile transgenic plants, *TF* Transformation frequency: fertile plants/targets, n.d. = no data, √ = Yes, – = No

production of transgenic doubled haploid plant species in which regeneration from somatic cells is difficult, especially in the recalcitrant cereals. Till now suitable genetic transformation methods using different explants such as immature embryos, mature embryos, scutellum-derived calli, anthers, isolated microspores, ovules, cell suspensions, protoplast, etc., have been established for some cereal crops, especially for barley. Recent reports also focused on different cereal crops viz. rice, maize, wheat, etc., which are mentioned under this review. By this time, many abiotic stress inducible genes with various functions have been identified. Some researchers reported that transgenic plants that harbour these genes have shown increased tolerance to cold, freezing, drought, salinity, heat, etc. Many reports have been published on barley, but not enough on other cereal crops. It is time to work using standard protocols and need to develop specific abiotic stress tolerance cereal crops for stressful soil and environments.

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Chapter 10
Plant Diseases—Control and Remedy Through Nanotechnology

Remya Nair and D. Sakthi Kumar

1 Introduction

Agriculture is the backbone of most of the developing countries, in which a major part of their income comes from agriculture sector and more than half of the population depends on it for their livelihood. Even though nanotechnology has revolutionized the field of electronics, textiles, separation technology, material science and healthcare (Feiner 2006; Hu et al. 2007; Caruthers et al. 2007), its wide scope in agriculture and crop protection has not been explored much. Today, agriculture sector is facing several challenges such as increasing demand for food under changing climatic conditions and increasing risk of pests and diseases. Nanotechnology could provide possible solutions to many of the major risks in agriculture. It could improve our understanding of the biology of different crops, thus potentially enhance yields and nutritional values with greater control over various plant diseases and pest incidences. It could also help in developing improved systems for monitoring environmental conditions and delivering nutrients and/or plant protecting chemicals in the needed concentrations, thus controlling various plant diseases in the right manner at the right time (Sharon et al. 2010).

Development of nanodevices and nanomaterials for agriculture and plant research would allow various novel applications, ranging from treatments with agrochemicals to delivery of nucleic acids for genetic transformation (Scrinis and Lyons 2007). Nanosensors and nano-based delivery systems help in efficient use of water, chemicals and nutrients through precision farming and will help the agricultural industry combat viruses and other crop pathogens. Research is still a long way for us until such nanodevices could be widely used. Nanotechnology has the potential to revolutionize the agricultural and food industry with new tools for the molecular

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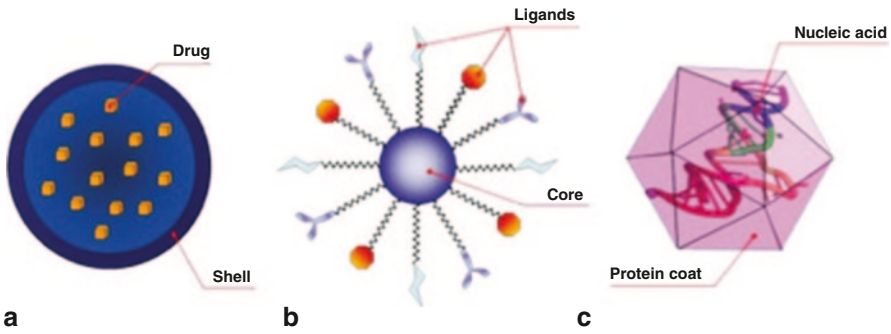

Fig. 10.1 Schematic representation of different nanodevices that could be successfully used for the control of various plant diseases. (Adopted from Perez-de-Luque and Diego 2009)

treatment of diseases, rapid disease detection, enhancing the ability of plants to absorb nutrients, etc., (Carmen et al. 2003; Perez-de-Luque and Diego 2009).

2 Nanoformulations for the Control of Plant Diseases

Nanotechnology provides new ways for improving and modifying existing crop management techniques. Plant nutrients and plant protecting chemicals are conventionally applied to crops either by spraying or broadcasting. Due to problems such as leaching of chemicals, degradation by photolysis, hydrolysis and microbial degradation, only a very low concentration of chemicals, which is much below the required minimal effective concentration, reach the target site of crops. Hence, repeated application is necessary to have an effective control, which might cause some unfavorable effects such as soil and water pollution. Nanoformulated agrochemicals should be designed with necessary properties such as effective concentration, time-controlled release in response to certain stimuli, enhanced targeted activity and less ecotoxicity with safe and easy mode of delivery, thus avoiding repeated application (Green and Beetsman 2007; Tsuji 2001; Nair et al. 2010) (Fig. 10.1).

As in the case of delivery of nanodrugs in humans, controlled release (CR) of agrochemicals has gained increased attention now a days. Controlled release is an important technique utilized in various fields for supplying a prescribed amount of necessary material at desired time. The encapsulated particles are the most commonly used functional particles for controlled release because the active component occupies large volume fraction, and the release rate of core material is of zero order, which is governed by the diffusion of dissolved material in the coating layer (Ito et al. 2003; Li et al. 2006). The interactions between the matrix and the active substances are important and this in turn may control the CR properties of the formulation such as diffusion, reaction rates or other physicochemical parameters. In addition, manipulation of the appropriate barriers, the target and the fate of the

active agent once beyond these barriers, are equally important and contribute to the success of the CR system designed. CR formulations provide advantages over their counterparts such as prolonged duration of action of an active agent, minimized adverse reactions or maximized efficacy, with tailor-made properties and higher stability of the active agents in the formulation.

A considerable number of nanoparticle formulation methods are based on nanoemulsion templates, which in turn are generated in various ways. It must therefore be taken into account that active principles and drugs encapsulated in nanoparticles can potentially be affected by these nanoemulsion formulation processes. Such potential differences may include drug sensitivity to temperature, high-shear devices, or even contact with organic solvents. Likewise, nanoemulsion formulation processes must be chosen in function of the selected therapeutic goals of the nanocarrier suspension and its administration route (Gutiérrez et al. 2008; Solans et al. 2005). This requires the nanoparticle formulation processes (and thus the nanoemulsion formation methods) to be more adapted to the nature of the encapsulated drugs, as well as to the chosen route of administration.

3 Nanoparticles for the Control of Disease and Pest Incidences in Plants

Nanoparticles of defined concentrations could be successfully used for the control of various plant diseases caused by several phytopathogens.

3.1 Silver Nanoparticles [AgNPs] and Nano-Silver-Silica Composites

Silver nanoparticles have found several applications in the field of medicine as successful antifungal and antibacterial agents (Panacek et al. 2009; Singh et al. 2008). This paved the way for their use as broad-spectrum antimicrobial agents in controlling various phytopathogens causing fungal and bacterial diseases in plants. AgNPs were found to be highly effective against sclerotium forming fungal phytopathogens such as *Rhizoctonia solani, Sclerotinia sclerotiorum* and *S. minor* causing several diseases in a wide range of host plants resulting in severe economic losses. AgNPs with large surface area could increase their contact and permeability with microbes, thus arresting fungal growth and sclerotial germination even with low concentrations of nanoparticles. The effectiveness of nanoparticles could be enhanced by applying them well before the penetration and colonization of fungal spores in plant tissues (Jo and Kim 2009). Antifungal activities of AgNPs were also investigated against ascomycetous fungus *Raffaelea sp*. causing oak wilt disease (Kim et al. 2009). Fungal hyphal growth and conidial germination significantly

decreased in a dose-dependent manner on the application of AgNPs. Nano-silver treatments were also found to be effective against bacterial growth in vase solution and at cut stem ends, thus extending the vase life of *Gerbera jamesonii* cv. Ruikou flowers (Solgi et al. 2009; Liu et al. 2009).

Nano-silver-silica composite obtained by mixing a silver salt with silicate and a water-soluble polymer followed by exposing the mixture to radioactive rays,is reported to have excellent antimicrobial effects even at its low concentrations (Oh et al. 2006). Such nano-sized silver-silica composite could be successfully used against plant pathogens. It was reported that plant pathogenic fungi such as *Phytophthora spp*., *Rhizoctonia spp.*, *Colletotrichum spp*., *Botrytis spp*., *Magnaporthe spp*. and *Pythium spp*. could be successfully controlled using nano-silver-silica composite (Park et al. 2006). Various experimental results have shown that this nanocomposite at a concentration lower than 3.0 ppm is effective enough to control various plant diseases caused by the above said plant pathogenic fungi. On absorbing such nano-silver silica by fungal cells, AgNPs increase disinfecting activity whereas silica nanoparticles provide physical barrier to pathogenic fungi, thus inducing increased resistance to diseases by preventing the recurrence of diseases for extended periods after disinfection of phytopathogens. Nanocomposition was also found to be effective against phytopathogenic bacteria at a concentration higher than 10 ppm. Hence, it is necessary to optimize the minimal effective concentrations of nanoformulations, that is, effective enough to fight against each phytopathogen. The best thing regarding the use of nano-silver-silica is that it could provide long-term control of microorganisms very selectively depending upon its concentration with single application and is safe for growers also since it does not cause chemical injuries and is non-toxic to humans. Such nanoparticle formulations are effective against phytopathogens that are less sensitive to antibiotics due to their poor penetration into microbial cells.

3.2 Nano-Silica

There were reports regarding the use of amorphous nanosilica as a nanobiopesticide. Use of nanosilica opens up novel research area for the biological control of various plant pests. Nanosilica controls insect pests by their absorption on cuticular lipids by physiosorption. They do not cause alterations on genetic make-up of insect pests and hence the process is purely physical (Barik et al. 2008; Mewis and Ulrichs 2001).

3.3 Titanium Dioxide Nanoparticles (TiO_2)

Titanium dioxide (TiO_2) is a non-toxic white pigment widely used in the manufacture of paints, paper, ink, cosmetics, ceramics, leather, etc., and is a very strong

disinfectant as compared to chlorine and ozone. Since TiO_2 is harmless, it is approved for use in food products up to 1 % of product final weight. TiO_2 photocatalyst technique has great potential in various agricultural applications, including plant protection since it does not form toxic and dangerous compounds and possesses great pathogen disinfection efficiency. Scientists have been trying to improve the phytopathogenic disinfection efficiency of TiO_2 thin films by dye doping and other suitable methods (Yao et al. 2007). Several research papers have already reported the positive effects of nano-TiO_2 on the germination and growth of spinach seeds. Nano-anatase TiO_2 enhances plant growth by increased nitrogen metabolism, improved light absorbance and enhanced activity of Rubisco activase and light harvesting complex II, thus promoting accelerated spinach growth (Zheng et al. 2005; Gao et al. 2006; Lei et al. 2007a, b). It was reported that the application of TiO_2 reduced the effects of *Curvularia* leaf spot and bacterial leaf blight in rice and maize plants and also decreased the incidence and severity of rice blast disease and tomato spray molds (Chao et al. 2005). TiO_2 plays the function of antibiotics in *Vigna ungiculata* Walp (cowpea) production in cowpea. Field trials conducted at the Institute of Agricultural Research and Training, Ibadan, Nigeria, evaluated the effects of TiO_2 on the development, yield and foliar and pod diseases of cowpea. There was an increase in the yield of cowpea, and the incidence and severity of *Cercospora* leaf spot and brown blotch disease got reduced on the application of TiO_2 and best results were obtained with two sprays of TiO_2 at 125 cc ha^{-1}. Hence, nano-TiO_2 could be successfully used for controlling various diseases in cowpea plant (Owolade and Ogunleti 2008). The effects of nano-TiO_2 semiconductor sol (nano-TiO_2 sol, with an average size of 30.6 nm) in controlling plant pathogenic bacteria; *Pseudomonas syringae pv lachrymans* and *Xanthomonas vesicatoria* infecting cucumber plants were studied (Zhang et al. 2007), and both artificial inoculation experiment and field experiments showed that spraying cucumber leaves with nano-TiO_2 sol controlled phytopathogenic bacteria by forming adhesive and transparent film on leaf surfaces, thus controlling bacterial angular spot and downy mildew diseases of cucumber. Hence, nano-TiO_2 could be successfully used as environment-friendly fungicide and/or bactericide thus preventing and inhibiting various fungal and bacterial diseases in plants.

3.4 Carbon Nanomaterials

Among the various engineered nanomaterials, carbon based nanomaterials (such as single walled carbon nanotubes (SWCNTs), multi walled carbon nanotubes (MWCNTs), buckyballs, graphene, etc.), occupy a prominent position in various nano-biotechnology applications. Increased use and exposure to carbon nanomaterials could cause environmental concerns. Hence, it is extremely important to systematically study the effects that carbon nanomaterials have in plants, which occupy a major component of the food chain. Recently, researchers reported the effects of MWCNTs of different concentration on the germination and growth of

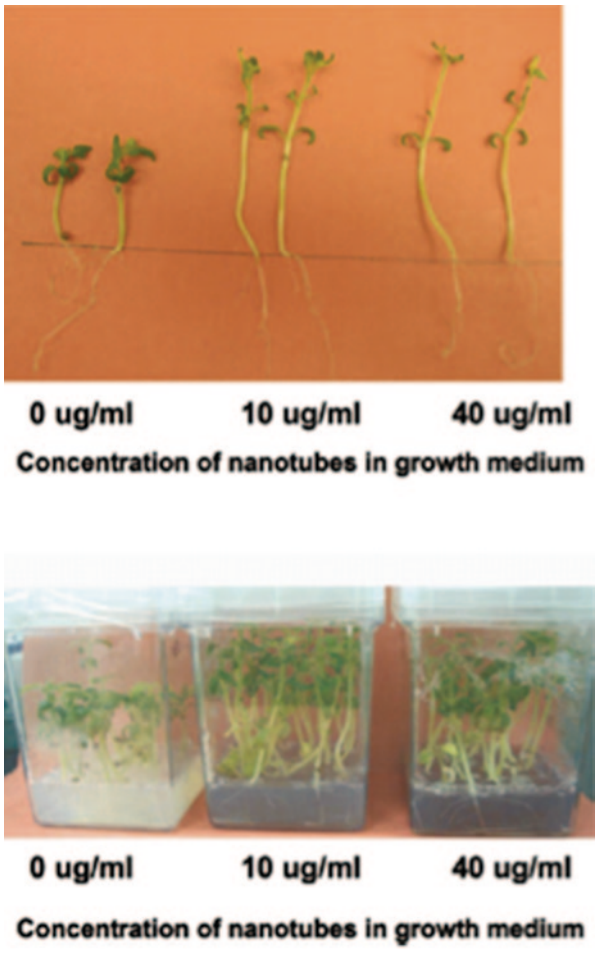

Fig. 10.2 Effect of carbon nanotubes on growth and development of tomato seedlings. Carbon nanotubes showed positive response on growth and development of tomato seedlings. (Adopted from Khodakovskaya et al. 2009)

tomato plants (Khodakovskaya et al. 2009). Experimental results showed enhanced germination of tomato seeds with increased water uptake (Fig. 10.2). Significant changes in total gene expression of tomato due to the interaction of MWCNTs with cells of tomato seedlings have been studied and it was reported that activation of several stress related genes, including the gene for tomato water protein promoted increased water uptake (Khodakovskaya et al. 2011). Similar results were noticed in experiments in our labwith different types of carbon nanomaterials such as SWCNTs, MWCNTs and buckyballs on germination of rice seeds, having harder seed coat than the tomato seeds (Nair et al. 2010). An improved root development and shoot establishment was noticed for rice seedlings, grown in carbon nanomaterial-enriched medium compared to the control seedlings. The reason for such an enhanced growth of plants in the presence of carbon nanomaterials is still mysterious. It is not easy for any external agent to enter into plant cells due to its cell wall. However, our findings showed the presence of nanotubes in the region of germina-

tion, which supports the hypothesis for the induction of new pores or enlargement of seed coat, pores upon interaction with these nanostructures. These findings open the wide possibilities of using carbon nanomaterials as delivery vehicles of various chemicals and desired molecules to seeds and/or plant itself, to protect them from various diseases and pest attack.

3.5 Magnetic Nanoparticles

The scope of magnetic nanoparticles for site-targeted delivery of drugs, has been exploited widely in biomedicine for the treatment of various diseases (Mornet et al. 2004; Jurgons et al. 2006). However, in plant biology, such an application is still in its budding stage. Magnetic-based nanomaterials could be utilized for site-targeted delivery of systemic plant protection chemicals for the treatment of diseases that affect only specific regions of plants. If the movement of internalized magnetic nanomaterials could be tracked externally using high power external magnets, then it would be possible to direct them to specific areas where the chemicals need to be released. The advantage of using carbon-based nanomaterials (such as SWCNTs and MWCNTs) functionalized with magnetic nanoparticles is that the internal space allows filling of suitable plant protecting chemicals and the functionalized magnetic nanoparticles allow external control of the movement of nanocarriers inside the plant system. The magnetic nanoparticles should also be externally functionalized for making them more biocompatible. The presence of magnetic nanoparticles inside the plant system, might cause changes in various metabolic and enzymatic functions of plants. Even though some works have already reported regarding changes in assimilatory pigment and nucleic acid levels (Racuciu et al. 2007; Racuciu et al. 2009), more research is needed to understand the overall physiological and metabolic changes that occur in plants on the uptake of magnetic nanoparticles.

4 Nanoparticles as Transgene Vehicles for Developing Transgenic Plants with Novel Properties

The major challenge for plant gene delivery is the presence of cell wall. The main constraints associated with conventional gene transfer methods in plants such as *Agrobacterium*-mediated gene transfer, electroporation, PEG-mediated gene transfer, particle gun bombardment, etc., are high cost, labor extensiveness and significant perturbation to the growth of cells. Hence, it is high time to utilize novel delivery systems for the development of successful transformants. Nanotechnology has shown its ability in modifying the genetic constitution of plants by introducing novel genes, thus resulting in crop improvement. Besides the use of different nanoparticle formulations for controlling the incidence of various plant diseases,

there is a huge scope in using nanoparticles for transgene delivery in plant cells resulting in transgenic plants with novel properties, such as disease resistance, stress resistance, drought tolerance, etc.

The application of fluorescent labeled starch-nanoparticles as plant transgenic vehicle, was reported in which the nanoparticle biomaterial was designed in such a way that it binded the gene and transported it across the cell wall of plant cells by inducing instantaneous pore channels in cell wall, cell membrane and nuclear membrane with the help of ultrasound (Jun et al. 2008). It is possible to integrate different genes on the nanoparticle at the same time and the imaging of fluorescent nanoparticle is possible with fluorescence microscope, thus understanding the movement of exterior genes along with the expression of transferred genes. Hence, successful generation of pores on cell wall and cell membrane by suitable agents, helps in nanoparticle mediated DNA transfer that might be more successful in regenerative calli and soft tissues.

Dendrimers are synthetic polymers with highly symmetric architecture in which their well-defined structures and high ratios of multivalent surface moieties to molecular volumes, make them highly suitable to function as vectors for gene and drug delivery. Several publications have reported the successful use of these specialized nanoparticles as drug delivery system in medicine, however their use for direct and non-invasive gene transfer in plants has been a novel idea. It was reported that polyamidoamine (PAMAM) dendrimer acts as a nanocarrier for delivering genes into plant cells with intact cell wall. Supramolecular complexes of PAMAM dendrimer-DNA are formed through electrostatic interactions and these complexes are penetrated through the cell walls of turf grass calli, expressing foreign genes within the cells (Pasupathy et al. 2008).

Surface functionalized mesoporous silica nanoparticles (MSNs) provide new ways to precisely manipulate gene expression at single cell level by delivering DNA and its activators in a controlled fashion (Torney et al. 2007) by penetrating through plant cell wall (Fig. 10.3). MSNs are loaded with gene and its chemical inducer and the ends are capped with gold nanoparticles to protect the molecules from leaching out. Uncapping of capping agents results in stimuli responsive release of chemicals, thus triggering gene expression in plants. It is found that surface modification of MSNs with triethylene glycol promotes their easy penetration into cells and also allows plasmid DNA to absorb on MSN surface. In this method, the minimum amount of DNA required to detect marker expression is 1000-fold less than that required for the conventional delivery method and such delivery method has significant applications in various gene expression studies. Such nanoparticle-mediated plant transformation allows site-targeted simultaneous delivery of both DNA and effector molecules. Future possibilities include enlargement of pore size and multifunction-

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uncapped by incubating the plant cells with dithiothreitol (DTT). This releases the β-estradiol effector molecules and activates the expression of plasmid DNA in the nucleus. Surface functionalized MSNsare successfully used for the intracellular-controlled release of genes and chemicals into plant cells. This will help in the future investigations of plant genomics and gene function as well as improvement of crops. (Adopted from Torney et al. 2007)

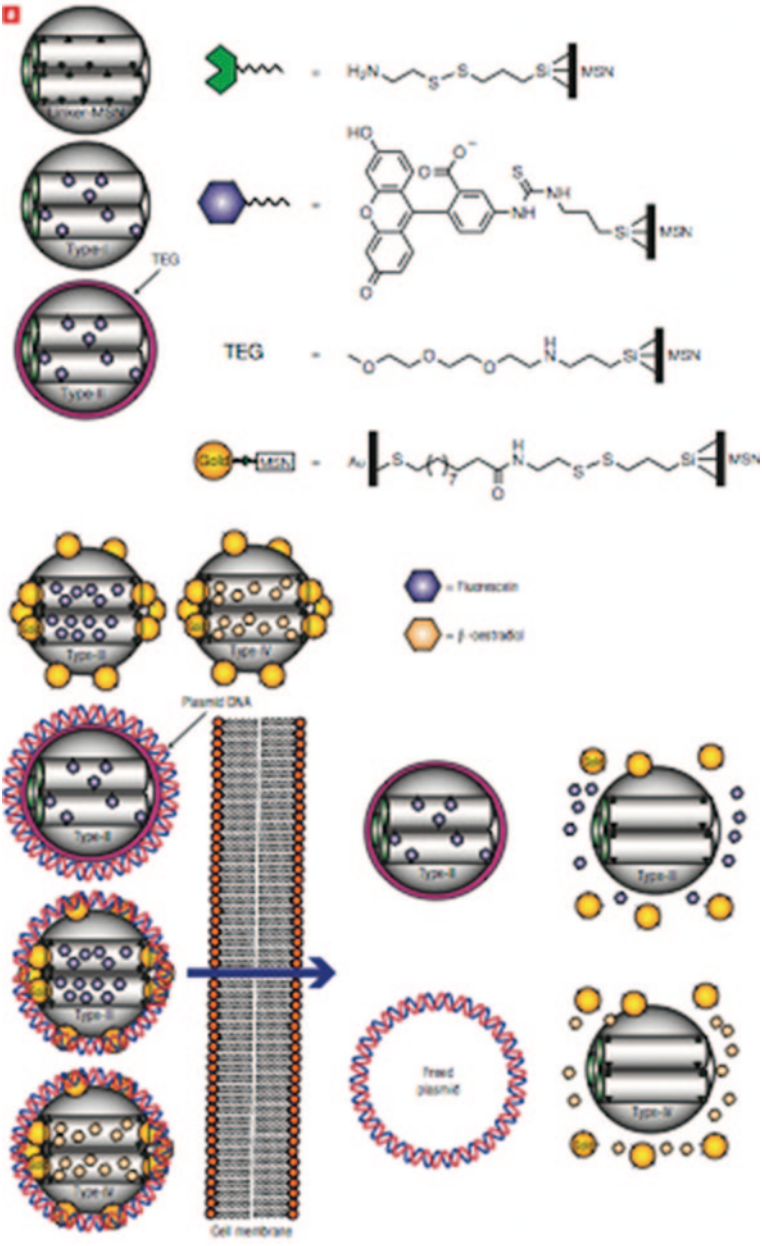

Fig. 10.3 Internalization of mesoporous silica nanoparticle [MSN] by plant cell. Type I: Fluorescein-labeled MSN; Type II: Type I MSN coated with Triethylene Glycol (TEG) polymer; Type III: Fluorescein-labeled MSN capped by gold nanoparticles; Type IV: β-estradiol-loaded MSN with gold nanoparticle capping. After action of the gene gun, MSNs [small circles], carrying the small effector molecule (β-estradiol) within the gold-capped structure and externally coated with plasmid DNA, penetrate the cell wall and, in some cases, enter the cytoplasm. The MSNs are

alization of MSNs, which support site-targeted delivery of proteins, nucleotides and chemicals in plant biotechnology.

The ability of carbon nanotubes to penetrate intact plant cell wall and cell membrane has already been reported (Liu et al. 2009). Cellular uptake of SWCNT/fluorescein isothiocyanate and SWNT/DNA conjugates revealed the ability of nanotubes to act as nanotransporters in walled plant cells. Utilizing carbon nanotubes as nanotransporters for intact plant cells has significant importance in plant intracellular labeling and imaging, genetic transformation, and also in enhancing our knowledge of plant cell biology. Thus the nanomaterial-mediated transformation methods will provide great possibilities in developing disease resistant transgenic plants.

5 Detection of Plant Diseases Through Nanotechnology

One of the major problems associated with plant disease management is the detection of correct stage of disease. Most of the plant diseases are noticed at late stages only, and so their prevention and control becomes a major challenge. Most of the times, plant protection chemicals such as fungicides and pesticides are applied only after the appearance of symptoms thus causing some significant crop losses. Hence, it is essential to detect and diagnose plant diseases at their early stage itself, so that plant protecting chemicals could be applied at correct dose at the right time thus avoiding residual toxicity and environmental hazards. A smart collaboration between plant pathology and nanotechnology could help in the early detection of various fungal, bacterial and viral diseases in plants. Current detection techniques take several days to identify plant diseases and hence, researchers are focusing on simple detection techniques that give better results within a short period of time. Also, many of the molecular systems for the detection of microorganisms are primarily based on specific nucleotide probe detection or on specific antibodies and such systems are highly sensitive and selective and hence mostly suited for laboratory uses only. In sustainable farming, detailed knowledge of the distribution of diseases in the field is necessary, however it is very difficult to inspect each and every plant in a large field area since it is highly laborious, time-consuming and expensive too. Proper sensing systems that could detect and quantify pathogens in defined positions of the field would help the growers in site-targeted and optimized application of agrochemicals with minimal environmental hazards. In this scenario, nano-biosensors, once installed in the field, could detect pathogens with high sensitivity and specificity. Such nanosensors are highly potable systems with ‘real-time’ monitoring of results. They do not need extensive sample preparations and detection is label-free also (Sadanandom and Napier 2010).

Cell biologists of Cornell University are investigating on nanofabrication technologies to understand how the fungi and bacteria feel their way on plant surface, to induce infection (Mccandless 2011). Fungi distinguish minute differences on leaf surface to decide where and when to infect. Researchers have simulated leaf surface by microfabricating ridges on silicon wafers using electron beam lithography and

microorganisms are made to orient themselves on ridges, which is very similar to the leaf surface. Fungi sense the topographical surface mimicking a particular characteristic on leaf surface and this signals them to develop primary infection structure and such information could help the breeders in near future to develop better plants that makes them more resistant to fungal attack. Among the various plant diseases, viral diseases are hard to control since the spread of diseases by vectors needs to be prevented and disease symptoms appear only late, in which case, pesticide application after the appearance of diseases has not been successful. So, detection of exact stage of disease's viral DNA replication and viral protein production is important for the control of viral diseases. Use of nano-based viral diagnostics could help in the detection of exact viral strain and the stage of application of chemicals to control viral diseases. Such nano-based diagnostic kits make detection at a faster pace and also increase the power of detection.

6 Conclusion

One of the major factors limiting crop production and productivity is the incidence of pests and diseases. Among various crop protection strategies, control and remedies for plant diseases and pest attacks through nanotechnology has gained increased attention. Nanoparticles could be used for delivery of suitable plant protecting chemicals and agrochemicals, which help in the control of various pathological sufferings of plants. Site-targeted delivery of nanoparticles can be done to avoid collateral damage to plant parts that might happen by various conventional agrochemical application methods, and also to support environmental protection by avoiding soil and water pollution. Nanoparticle-mediated plant genetic engineering and use of nanosensors in agriculture are frontier areas to increase crop production by avoiding pest and disease incidences, which will be a boon to farmers. Even though nanotechnology application in food and agriculture is in its budding stage, we can hope to see increasing uses of tools and techniques developed by nanotechnology in agricultural field in the next few years.

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Chapter 11
Nanobiotechnology: Scope and Potential for Crop Improvement

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1 Introduction

1.1 Nanotechnology

Nanotechnology is manufacturing at the molecular level—building things from nanoscale components, where unique phenomena enable novel applications. Nanos: Greek term for dwarf, Technology: visualize, characterize, produce and manipulate matter of the size of 1–100 nm (Ball et al. 2002). In layman's language, nanotechnology is the science behind the intentional creation, manipulation, and characterization of extremely small particles and macro molecules. Nanotechnology proposes the construction of novel nanoscale devices possessing extraordinary properties. The chemical, physical, and biological properties of materials differ in fundamental and valuable ways from those of individual atoms, molecules, or bulk matter (Nel et al. 2006). To get an idea of the size of particles that nanotechnology encompasses, consider some comparisons. A nanometer (nm) is one-billionth of a meter. A typical sheet of paper is about 100,000 nm thick, a red blood cell is about 2,000–5,000 nm in size, and the diameter of DNA is in the range of 2.5 nm. The size range of highest interest in the field of nanotechnology is from 1–100 nm (Maynard et al. 2006), so nanotechnology deals with matter that ranges from one-

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half the diameter of DNA up to 1/20 the size of a red blood cell. This size range is comparable to that of viruses and is one-fourth the wavelength of visible light. The beginning of nanoscience was mainly devoted to the study and fabrication of materials at the nanoscale, where much effort was dedicated to shrink the dimension of fabricated materials. It was the same time when the two basic fabrication approaches were defined: "bottom-up" and "top-down." The bottom-up approach aims at building things by combining smallest possible building materials such as single molecules and atoms, which are held together by covalent forces. The advantage of the bottom-up design is that the covalent bonds that hold a single molecule together, are far stronger than the weak interactions that hold more than one molecule together. A top-down approach (also known as step-wise design) is essentially the moulding, carving, and fabricating of small materials and components by using bigger objects such as mechanical tools and lasers. Recently, application of nanomaterials prepared using techniques involving both the approaches have evolved. However, the bottom-up approach has far more practical and future applications. Thus, nanotechnology is a multi-disciplinary field that seeks to combine mature nanoscale technology of fields such as physics, biology, engineering, chemistry, computer science, and material science. Potential applications include agricultural production (plant and animal), food processing, and manufacturing in areas such as pathogen detection, food engineering, packaging, and equipment (Perez-de-Luque et al. 2009; Torney et al. 2007).

1.2 The "Nano-Bio" Interface

For last many years, nanoscale processes and structures have been optimized in order to govern the biosystems. Biologists have been working for several years at the molecular level, in the range of nanometers (DNA and proteins) to micrometers (cells). A typical protein such as hemoglobin has a diameter of about 5 nm, DNA's double helix is about 2 nm wide, and a mitochondrion spans a few hundred nanometers (Whitesides et al. 2003). Consequently, the study of any subcellular entity can be considered "nanobiology". Moreover, today, a living cell having its hundreds of nanomachines is considered to be the essential nanoscale fabrication system. Nanosized molecular building blocks have been the basis of each and every biological system that cooperates to produce living entities. These elements have enlightened the imagination of nanotechnologists for many years, resulting in the birth of the new science "Nanobiotechnology: The combination of nano and biotechnology".

Nanotechnology provides the tools and technology platforms for the investigation and transformation of biological systems, whereas biology offers inspirational models and bio-assembled components to nanotechnology (Fortina et al. 2005; Lowe et al. 2000; Bohr et al. 2002). The difference between "nanobiology" and "nanobiotechnology" exists in the technology part as anything that is "man-made" falls into the technology section of nanobiotechnology. Nanobiotechnology will lead to the design of entirely newclasses of micro- and nanofabricated devices and machines, for which the inspiration will be based on bio-structured machines.

1.3 Nanobiotechnology

The term "nanobiotechnology" was first given by Lynn W. Jelinski, a biophysicist at Cornell University, USA. Nanobiotechnology is a promising area that combines nanofabrication and biosystems to the benefit of both, for all applications of genomics, including mammalian, plants and microbials (Robinson et al. 2009). As mentioned earlier, there are two basic fabrication approaches to create nanostructures—top-down and bottom-up. In top-down approach, nanobiotechnology utilizes tools and methods of nano/microfabrication to manufacture nanostructures and nanodevices. However, the bottom-up approach exploits biological structures and processes via a collection of molecular tool kits of atomic resolution, to create novel functional materials, biosensors, and bioelectronics for different applications. Nanobiotechnology helps in achieving many essential goals that are rather difficult to achieve by other means. For instance, a DNA's ladder structure provides a natural framework for assembling nanostructures instead of fabricating silicon scaffolding for nanostructures as DNA's ladder provides highly specific bonding which brings atoms together to create a nanostructure. It provides the tools and technology for gathering information and designing novel devices to investigate questions related to the biological importance of the genomic information and its implementation in various fields, particularly medicine and agriculture. Applications of nanobiotechnology in agriculture are gradually moving from the theoretical possibilities into the applicable area and play an important role in improving the existing crop management techniques. Nanoscale devices with novel properties are capable of responding to different conditions by themselves, and therefore taking appropriate remedial action. These systems help in delivering chemicals in a controlled and targeted manner through genetic improvement of plants (Kuzma et al. 2007; Scott et al. 2007), delivery of genes and drug molecules to specific sites at cellular levels (Maysinger et al. 2007), and nano-array-based gene technologies for gene expressions in plants under stress conditions. The interest is increasing with suitable techniques and sensors for precision in agriculture, natural resource management, early detection of pathogens and contaminants in food products, smart delivery systems for agrochemicals like fertilizers and pesticides. Agrochemicals are conventionally applied to crops by spraying and/or broadcasting. In order to avoid the problems such as leaching of chemicals, degradation by photolysis, hydrolysis and microbial degradation, a concentration of chemicals lower than minimum effective concentration to reach the target site of crops is required. Hence, the nanocapsulated agrochemicals should be designed in such a manner that they hold all essential properties such as effective concentration, time-controlled release in response to certain stimuli, enhanced targeted activity and less ecotoxicity with safe and easy mode of delivery, thus avoiding repeated application (Green et al. 2007; Wang et al. 2007; Boehm et al. 2003; Tsuji et al. 2001). The best example is the reduction of phytotoxicity of herbicides on crops by controlling the parasitic weeds with nanocapsulated herbicides (Perez-de-Luque et al. 2009). Proper functionalization of nanocapsules ensures better penetration and allows slow and controlled release of active ingredients on reaching the target weed and also makes the concentrated active ingredients,

safe and easy to handle. Besides these, plants and/or their extracts help in biological synthesis of some metallic nanoparticles which is more ecofriendly and gives a controlled synthesis with well-defined size and shape (Kumar et al. 2009; Sharma et al. 2009). Therefore, with increased advances made by using nanobiotechnology in the agricultural sector, it can be expected to become a major economic driving force and benefit consumers as well as farmers with no harmful effect on the ecosystem.

2 Types of Nanomaterials

Depending on their existence in nature, nanomaterial is a term that includes all nanosized materials, including natural, incidental and engineered nanomaterials.

2.1 Natural Nanomaterials

Natural nanomaterials have been in existence since the beginning of the earth' history, and still occur in the environment. Materials that are a result of natural process with a structure approximately 1–100 nm are called natural nanomaterials. For example, particles arising from volcanic eruptions, sea spray, and atmospheric gas-to-particle conversion. Many important functions of living organisms also take place at the nanoscale level. The human body uses natural nanoscale materials, such as proteins and other molecules, to control many systems and processes of the body.

2.2 Incidental Nanomaterials

Incidental nanomaterials are defined as the materials with a structure approximately 1-100 nm that are produced as a result of manmade industrial processes such as diesel exhaust, coal combustion, welding fumes, etc.

2.3 Engineered Nanomaterials

Materials that are purposefully manufactured with nanoscale dimensions (1 and 100 nm), can be termed as engineered nanomaterials. Engineered particles of very small dimension attract enormous interest of researchers and are of potential benefit to society due to their properties which are different from larger particles of the same chemical composition. Engineered nanomaterials have received a particular attention for their positive impact in improving many sectors of economy, including

consumer products, pharmaceuticals, cosmetics, transportation, energy and agriculture (Nowack et al. 2007; Roco et al. 2003). The properties of engineered nanomaterials are essentially important due to their aggregation behavior and mobility in aquatic and terrestrial systems and also for their interaction with algae, plants and fungi (Enrique et al. 2008). Engineered nanomaterials can be categorized as:

- Carbon-based nanomaterials
- Metal-based nanomaterials
- Dendrimers
- Composites

2.3.1 Carbon-Based Nanomaterials

These types of nanomaterials mainly consist of carbon having the most common form of hollow spheres, ellipsoids, or tubes. Spherical and ellipsoidal carbon nanomaterials are referred to as fullerenes, while cylindrical ones are called nanotubes such as single-walled carbon nanotube (SWCNT) and multi-walled carbon nanotubes (MWCNT). These materials have various potential applications, such as improved films and coatings, stronger and lighter materials, and applications in electronics, agriculture and food (Remya et al. 2010). Recently, it was found that fullerenes may act as antioxidants, preventing lipid peroxidation induced by super oxide and hydroxyl radicals (Wang et al. 1999).

2.3.2 Metal-Based Nanomaterials

Metal-based nanomaterials have received considerable attention in science and technology in the last decade. These include quantum dots, gold, silver, palladium and metal oxides (TiO_2 and ZnO) nanomaterials. There is a huge scope for applying nanomaterials to plants for agricultural use (Liu et al. 2002; Pavel et al. 1999; Pavel et al. 2005; Joseph et al. 2006). TiO_2 nanoparticles have been found to induce spinach seed germination of aged seeds and its vigor (Zheng et al. 2005). It was also observed that the presence of TiO_2 nanoparticles increases the dry weight, chlorophyll synthesis, and metabolism in photosynthetic organisms. Due to the antimicrobial properties of engineered nanomaterials, the strength and resistance of plants to stress can be increased. Gene transfer by bombardment of DNA-absorbed gold particles has been successfully used to generate transgenic plants in a species-independent manner (Christou et al. 1988).

2.3.3 Dendrimers

Dendrimers are nanosized polymers composed of branched units having the capability to be customized to perform a specific chemical function. The surface of a

dendrimer has numerous chain ends; this property could also be useful for catalysis. In addition, since three-dimensional dendrimers contain interior cavities into which other molecules could be placed, they may be useful for drug delivery.

2.3.4 Composites

The combination of two different nanomaterials or nanomaterials with bulk-type materials (Lin et al. 2007) is called composites. It can be of different morphologies such as spheres, tubes, rods and prisms (Yu-Nam et al. 2008). For example, nanosized clays are being used now-a-days for products ranging from auto parts to packaging materials, to enhance mechanical, thermal, barrier, and flame-retardant properties.

3 Preparation of Different Nanomaterials

The synthesis of metal and metal oxide nanomaterials is a growing research area due to their potential applications in the development of novel technologies. The scientific and technological importance of metal (Au, Ag, Pd etc.) and metal oxide (ZnO, SiO_2, TiO_2, etc.) nanomaterials has made them the subject of intensive research owing to their wonderful physical and chemical properties and also their important applications in physical, chemical and biological field such as in nanobiotechnology. Generally, nanomaterials are prepared by a variety of chemical methods, including sol gel, template method, wet chemical synthesis, electrochemical method, photochemical method, and sonochemical synthesis. However, eco-friendly and cost-effective procedures for the synthesis of nanomaterials are of great interest to biologists, chemists and materials scientists using non-toxic chemicals, environmentally benign solvents, and renewable materials. Currently, there is growing need to develop eco-friendly and body benign nanomaterials synthesis methods without the use of toxic chemicals in the synthesis protocols to avoid adverse effects in biomedical and agricultural applications. Recently, the green chemistry which aims at reducing or eliminating substances hazardous to human health and the environment, is becoming more and more important (Poliakoff et al. 2001, 2002). Various types of nanomaterials have been successfully prepared by our research group using green chemistry route.

3.1 Gold Nanoparticles

Gold nanoparticles have been synthesized by simple and cost-effective microwave irradiation processes with an irradiation time of 40–70 s.

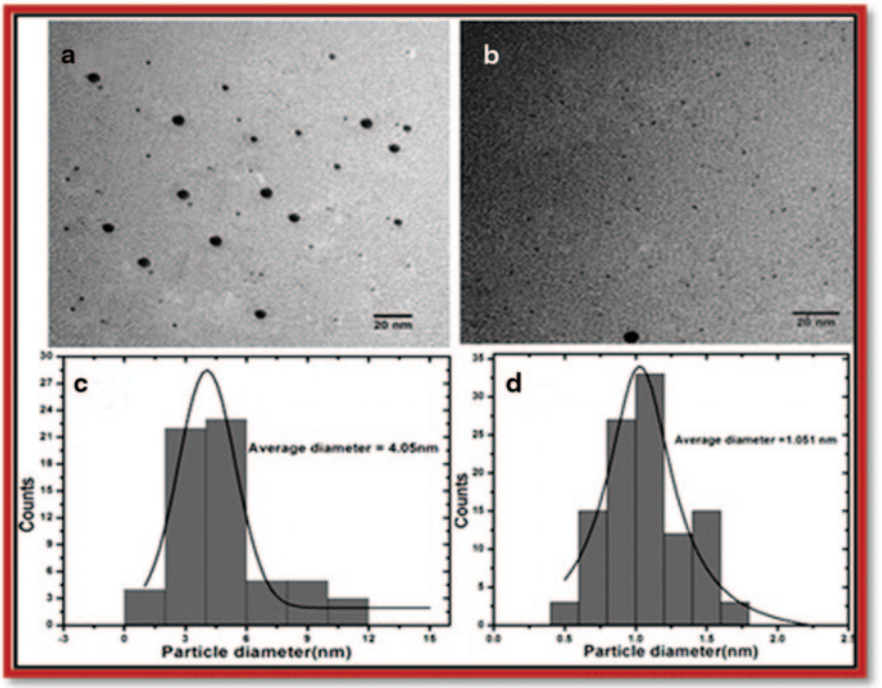

Fig. 11.1 Transmission Electron Microscopy (TEM) images of gold nanoparticles for **a** 40 s **b** 70 s and their corresponding size distribution for **c** 40 s **d** 70 s, respectively. (Adopted with kind permission from Arshi et al. 2011a)

Microwave irradiation is an efficient and distinct heating method, and has attracted interest of researchers owing to its unique features such as short reaction time, rapid volumetric heating, energy saving, environmental friendly and high reaction rate (Ela et al. 2009; Ahmed et al. 2011a, b). The synthesized gold nanoparticles were characterized by Transmission Electron Microscopy (TEM) and UV/Vis spectroscopy. The TEM images (Fig. 11.1) reflect stable and nearly spherical nanoparticles with an average diameter of 4.05 nm and 1.05 nm for samples at 40 s and 70 s irradiation time, respectively. Particle size calculated by using approximately 100 randomly selected individual nanoparticles from TEM micrograph shows that the size of the nanoparticles for 40 s and 70 s ranges from 1–10 nm (see Fig. 11.1a) and 1–2 nm (see Fig. 11.1b), respectively.

Figure 11.2 shows the UV/Vis absorption spectra of gold nanoparticles. Surface plasmon resonance peaking at 590 nm for 40 s and 560 nm for 70 s samples respectively, confirms the presence of gold nanoparticles. Generally, the broadness of the peak is a clear indicator of the size of the nanoparticles. There is a blueshift in the absorption peak to 560 nm which shows that the particle's size is decreasing as the heating time exceeds from 40 to 70s (Shahverdi et al. 2007).

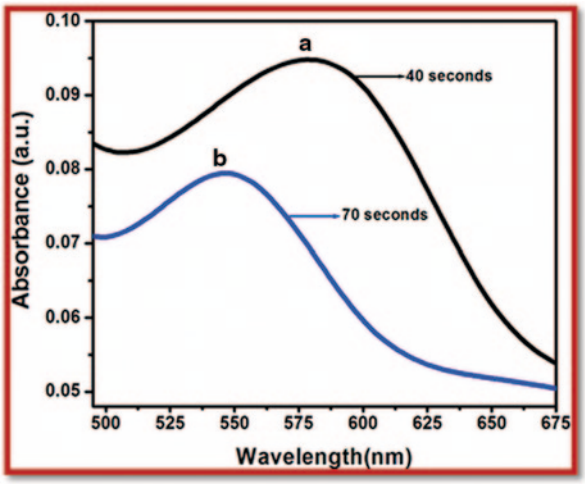

Fig. 11.2 UV/Vis spectra of gold nanoparticles heated for **a** 40 s **b** 70 s. (Adopted with kind permission from Arshi et al. 2011a)

3.2 Silver Nanoparticles

We presented a simplest, cheapest and environmentally benign synthesis of silver nanocrystals using sugar as the reducing-cum-stabilizing agent in ambient conditions without any solvent. The structural analyses of the as-synthesized nanocrystals were performed using X-ray diffraction (XRD). The morphological study of the sample was done using Atomic force microscopy (AFM) and TEM. The optical study of the synthesized product was performed using UV/Vis spectroscopy.

Figure 11.3 shows the XRD pattern of the as-synthesized silver nanocrystals. The XRD pattern of silver nanocrystals showed a single phase nature with face-centered cubic (FCC) structure. No secondary phase was detected and the high intensity of peaks revealed the high crystalline nature of the as-synthesized silver nanocrystals. The average grain size calculated by using Debye-Scherrer formula (Cullity and Stock 2001) was found to be ~19 nm.

The UV/Vis absorption spectrum of the silver nanocrystals is shown in Fig. 11.4. A strong absorption peak at approximately 426 nm of nanosized silver nanocrystals was observed, which is the characteristic of the surface plasmon resonance of Ag materials. Figure 11.5a, b depict the topographical 2D and 3D AFM images of silver nanocrystals. It is clear from Fig. 11.5a, b that most of the grains are in the size ranging from 10–20 nm with average diameter of ~18 nm.

This is evidently in accordance with the results obtained by XRD. Figure 11.6a shows the TEM micrograph of homogeneous silver nanocrystals with particle size in the range of about 10–30 nm. It is clear from the TEM micrograph that the particles are nearly cubic in shape. A statistical distribution of particle size is shown in Fig. 11.6b, which shows average particle size of a silver nanocrystal to be about ~22 nm.

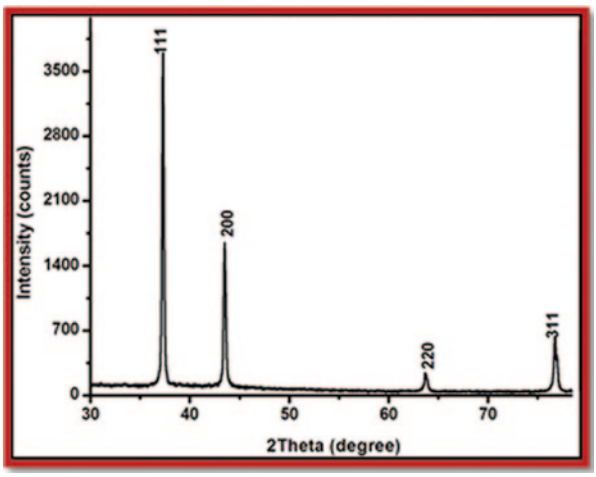

Fig. 11.3 XRD pattern of silver nanocrystals. (Adopted with kind permission from Arshi et al. 2011b)

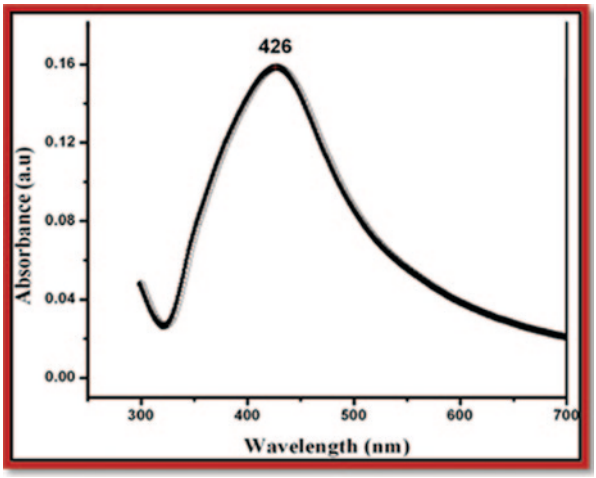

Fig. 11.4 UV-Vis spectrum of silver nanocrystals. (Adopted with kind permission from Arshi et al. 2011b)

3.3 ZnO Nanostructures

ZnO nanoflowers consisting of nanorods have been prepared using efficient, cost-effective and energy-saving microwave-assisted solution method. The as-synthesized nanorods were characterized by using XRD, FESEM and HRTEM measurements. These ZnO nanorods exhibit room temperature ferromagnetism which may be due to the presence of defects (oxygen vacancies) in the rods. A schematic representation of the possible growth mechanism of ZnO nanoflowers is shown in Fig. 11.7.

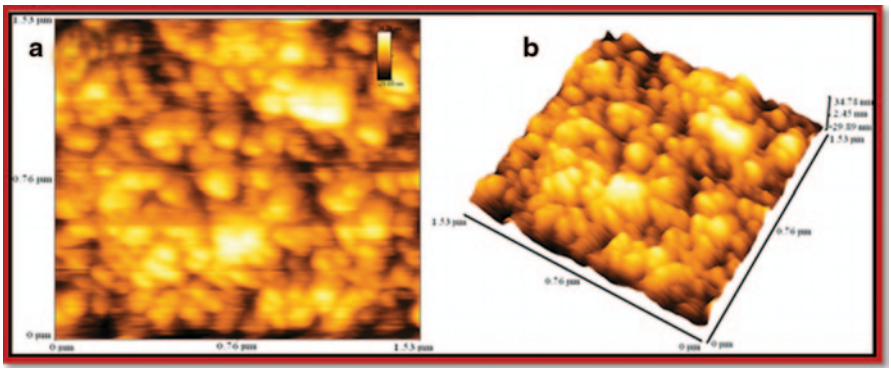

Fig. 11.5 AFM images of silver nanocrystals **a** 2D preview **b** 3D preview. (Adopted with kind permission from Arshi et al. 2011b)

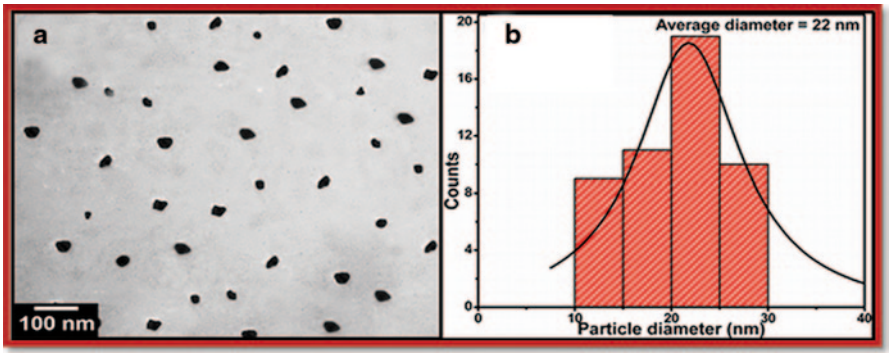

Fig. 11.6 **a** TEM image of silver nanocrystals **b** corresponding statistical size distribution histogram. (Adopted with kind permission from Arshi et al. 2011b)

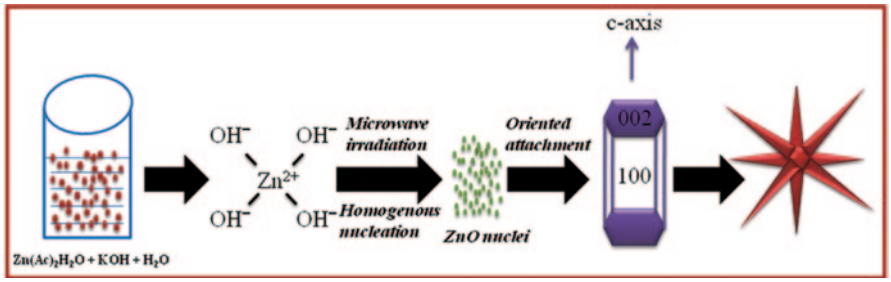

Fig. 11.7 Schematic diagram of the formation process of flowerlike ZnO nanostructures. (Adopted with kind permission from Ahmed et al. 2011a)

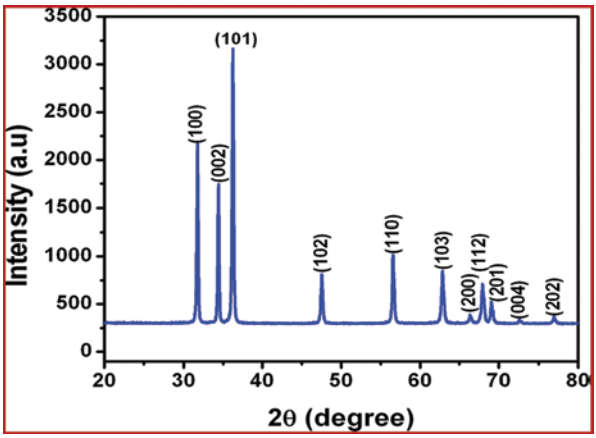

Fig. 11.8 XRD pattern of ZnO nanorods. (Adopted with kind permission from Ahmed et al. 2011a)

Figure 11.8 shows the typical XRD pattern of as-prepared ZnO nanopowder, which was indexed using POWDER-X software as the pure hexagonal phase ZnO with the lattice parameters a = 3.254 Å and c = 5.197 Å. No diffraction peaks from any other impurities are detected and the sharpness of the peaks implies the good crystallinity of the as-prepared ZnO nanorods. Figure 11.9a shows the typical FESEM images of the ZnO nanostructures. It can clearly be seen from the image that the as-synthesized ZnO nanorods are flowerlike clusters. Complementary morphological description is achieved through the TEM equipped with the SAED, as shown in Figs. 11.9b, c. The diameter of the nanorods is within 150–190 nm (tip diameter ~15 nm) with a length of about 2 μm. Figure 11.9c shows a typical TEM image of a single ZnO nanorod to confirm the crystal quality and growth direction. Well-resolved lattice spacing of 0.265 nm corresponding to the d spacing of the wurtzite ZnO (002) plane also indicates that the ZnO nanorod is of a single crystal in nature and referentially grows along the [001] direction (c-axis), which is further confirmed by the SAED pattern. Figure 11.9d shows the chemical composition of the nanorods determined by EDS. Only oxygen and zinc signals have been detected, which confirms that the nanorods are primarily ZnO.

4 Mechanism of Nanomaterials-Plants Interaction

Nowadays, a lot of attention is being given to the effect of different nanoparticles on plant growth and their metabolic functions. Plants cell walls, having a primary site for interaction, serve as a barrier for the entry of any external agent, including nanoparticles into plant cells. Major cell wall components include carbohydrates and proteins (Heredia et al. 1993; Knox et al. 1995), and these walls are semi-permeable in nature which permits the entry of small molecules and blocks the larger ones.

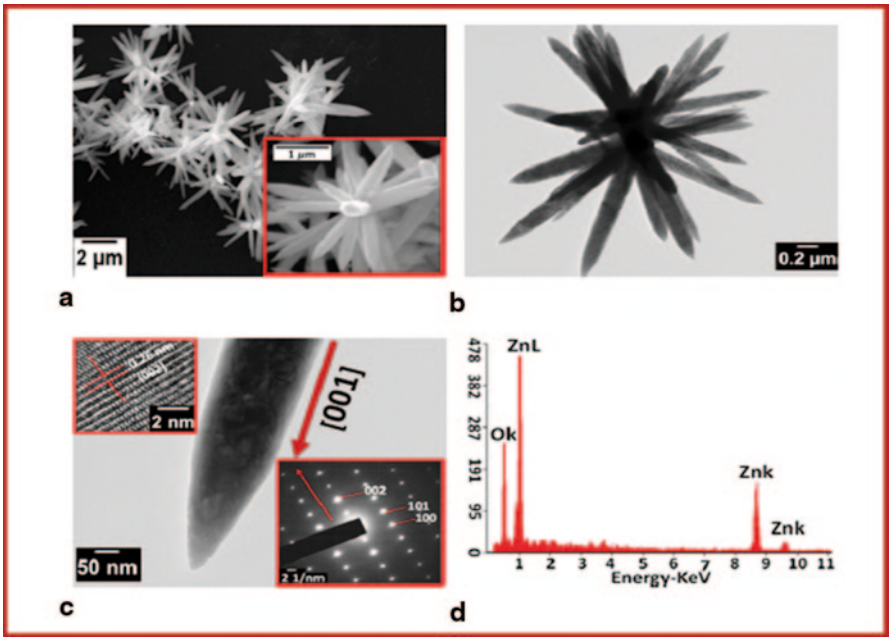

Fig. 11.9 **a** FESEM images of ZnO nanorods, TEM micrographs of **b** an individual ZnO flower and **c** focused image of a single ZnO nanorod. The upper left and lower right insets in **c** correspond to the HRTEM image and SAED pattern of a single nanorod, respectively, **d** EDS spectra of ZnO nanorods. (Adopted with kind permission from Ahmed et al. 2011a)

The pore diameter of the cell walls having a thickness ranging from 5 to 20 nm, determines its sieving properties (Fleischer et al. 1999; Fujino et al. 1998; Madigan et al. 2003; Zemke-White et al. 2000). Consequently, nanoparticles having a size smaller than that of the largest pore can easily pass through the cell wall and reach the plasma membrane. The enlargement of pores or induction of new cell wall pores might be possible upon interaction with nanoparticles, thus increasing the uptake of the nanoparticles through the cell wall. For example, ZnO nanoparticles have been reported to increase permeability and even create "holes" in bacterial cell walls (Brayner et al. 2006; Sondi et al. 2004; Stoimenov et al. 2002) with pore size similar to plant cell walls (Carpita et al. 1979). As the nanoparticles pass the cell wall, they reach the plasma membrane. This plasma membrane forms a cavity-like structure which surrounds the nanoparticles and pulls it into the cell during the endocytic process. The nanoparticles may also cross the cell membranes using embedded transport carrier proteins or ion channels. As the nanoparticles enter the cell, they may bind with different types of organelles (e.g., endoplasmic reticulum, Golgi, and endo-lysosomal system), and interfere with the metabolic processes at that site, possibly as a result of the production of reactive oxygen species (ROS) (Jia et al. 2005).

Plants also get exposed to nanomaterials in atmospheric and terrestrial environments. Nanomaterials present in air are attached to leaves and other aerial parts

of plants while the soil-material-associated nanomaterials interact with the roots. Therefore, it is expected that the plants having higher leaf area indexes (LAI) also have a higher interception potential for air-borne nanomaterials, which in turn increase their entrance into trophic webs. Once the nanomaterials are applied on the leaf surface, they might penetrate the plants via the bases of trichomes or through stomatal openings and get translocated to different tissues. Due to stomata obstruction, the accumulation of nanomaterials in photosynthetic surfaces might provoke foliar heating which results in alteration of the gas exchange (Da Silva et al. 2006). This heating might produce changes in various physiological and cellular functions of plants (Da Silva et al. 2006).

5 Nanomaterials for Crop Improvement

5.1 Carbon-Based Nanomaterials

Carbon-based nanomaterials such as single-walled carbon nanotubes (SWCNTs), multi-walled carbon nanotube (MWCNTs), carbon buckyballs, etc., have found vast applications in the field of agriculture and food. Canas et al. (2008) reported the effects of functionalized SWCNTs and non-functionalized SWCNTs on root elongation of six different crop species, such as cabbage (Brassica oleracea), cucumber (Cucumissativus), carrot (Daucuscarota), onion (Allium cepa), lettuce (Lactuca sativa), and tomato (Solanumlycopersicum). They showed that the root elongation in onion and cucumber was enhanced by non-functionalized SWCNTs, and the interaction of both functionalized SWCNTs and non-functionalized SWCNTs with root surface, resulted in the formation of nanotube sheets on cucumber root surface, without entering into the roots. However, cabbage and carrot remained unaffected by either form of nanotubes. Furthermore, functionalized SWCNTs inhibited the root elongation of lettuce, while tomato was found to be most sensitive to non-functionalized SWCNTs with significant root length reduction, whereas a positive response has been shown on the seed germination and growth of tomato plants upon interaction with MWCNTs (Khodakovskaya et al. 2009). They showed that the presence of MWCNTs increased water uptake by seeds which in turn enhanced the germination process (Fig. 11.10a, b). Tomato seeds placed on medium with different concentrations of MWCNTs germinated on the third day, while the tomato seeds placed on regular MS (Murashige and Skoog) medium did not germinate at that time (Fig. 11.10b).

Similar positive effects of MWCNTs on seed germination and root growth of six different crop species—radish (Raphanussativus), rye grass (Loliumperenne), rape (Brassica napus), lettuce (Lactuca sativa), corn (Zea mays) and cucumber (Cucumissativus)—was also reported (Lin et al. 2007). Very recently, Remya et al. (2010) also reported the positive effects of both SWCNTs and MWCNTs on the germination of rice seeds and observed an enhanced germination for seeds germinated in the presence of nanotubes. But, the interaction of different nanomaterials with plants and their mechanism for genetic and molecular modification of plants

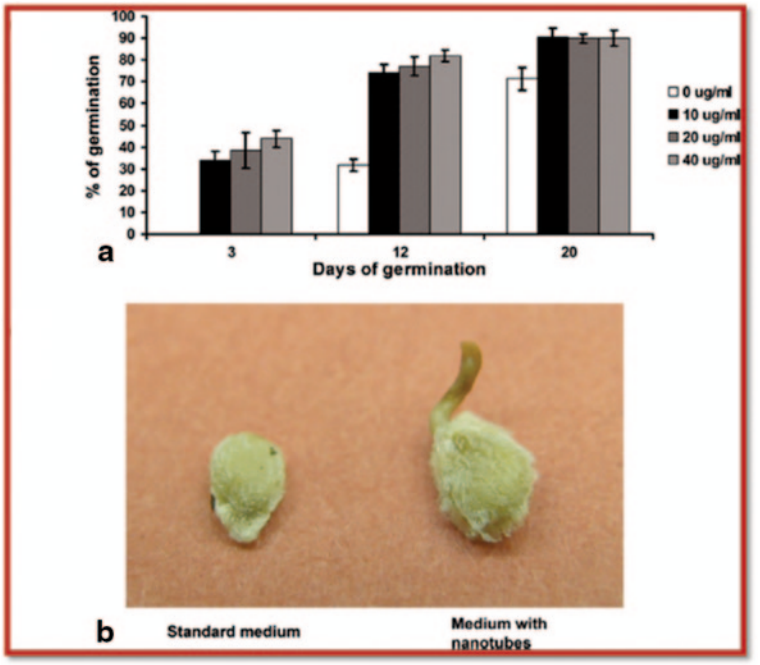

Fig. 11.10 Effect of CNTs on tomato seed germination. **a** Time of germination and germination percentages of seeds incubated with and without CNTs during 20 days. Seedlings with developed cotyledons and root system were recognized as fully germinated in this experiment. **b** Phenotype of tomato seeds incubated during three days without (*left*) or with (*right*) CNTs on MS medium. (Adopted with kind permission from Khodakovskaya et al. 2009)

are still unpredictable. The interaction of nanomaterials with plants differs with type and time of exposure to nanomaterials, so these facts should be kept in mind while performing nanotoxicity studies. Additionally, the orientation of nanotubes with respect to the plant cell wall might be important for their penetration, but the mode of entry of nanotubes through the cell wall remains mysterious which still needs more studies.

5.2 Magnetic Nanomaterials

Magnetic nanoparticles have received enormous attention because they allow specific localization of the particles to release their load, which plays a crucial role in the applications of nanoparticulate delivery for plants. Some research groups (Gonzalez-Melendi et al. 2008; Zhu et al. 2008; Corredor et al. 2009) have reported the uptake, translocation and specific localization of magnetic nanoparticles in pumpkin plants. No toxicity detected on plant growth which suggested that these kinds of

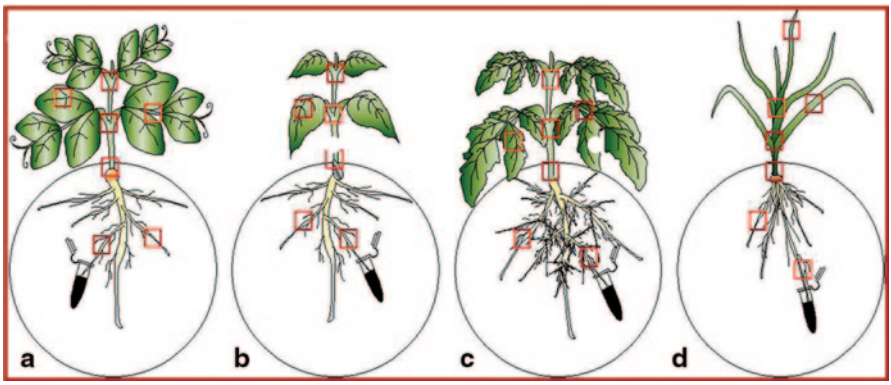

Fig. 11.11 Schematic representation of the Petri dish rizhotron with the four crops: **a** pea; **b** sunflower; **c** tomato; **d** wheat. Squares indicates sampling points of plant tissues. (Adopted from kind permission of Cifuentes et al. 2010)

nanoparticles are safe for nanoparticulate delivery in plants. Recently, genetic effect of ferrofluids has been a subject of great interest to nanobiologists as it leads to chromosomal aberrations in young plants (Racuciu et al. 2007a, 2009; Pavel et al. 1999, 2005). Racuciu et al. (2007b) analysed the influence of magnetic nanoparticles coated with tetramethylammonium hydroxide on the growth of *Zea mays* plant in early ontogenetic stages. They showed that these nanoparticles not only have chemical but also magnetic effect on the enzymatic structures in the different stages of photosynthesis. At low concentration of ferrofluid, the level of 'chlorophyll' was increased while higher concentrations of ferrofluid led to its inhibition. Also, the magnetic nanoparticles have the possibility to create some magnetic effects on the enzymatic entities involved in different photosynthetic and developmental stages. Therefore, in order to design the biotechnological tools for plant cultures, it is important to know the suitable ranges of ferrofluid concentration, so that a better yield of biochemical mutant types with improved photosynthetic pigment levels can be achieved. Zhu et al. (2010) reported that in an aqueous medium containing magnetite nanoparticles for the growth of Cucurbita maxima, particles can absorb, move and accumulate in the plant tissues. On the other hand, Phaseoluslimensis is not able to absorb and move particles. Therefore, different plants have different response to the same nanoparticles.

Cifuentes et al. (2010) studied the absorption and translocation of carbon-coated magnetic (iron) nanoparticles through the root in four crop plants (Fig. 11.11) belonging to different families—sunflower (*Helianthus annuus*) from the family Compositae; tomato (*Lycopersicum sculentum*) from the Solanaceae; pea (*Pisum sativum*) from the Fabaceae; and wheat (*Triticumae sativum*), from theTriticeae. They showed that after only 24 hours of exposure to the bioferro fluid, nanoparticles were able to leak into the vascular tissues of the tested crops (Fig. 11.12). This indicates that in order to get big amounts of nanoparticles inside the plant, the immersion of the roots into nanoparticle solutions is faster and more reliable than

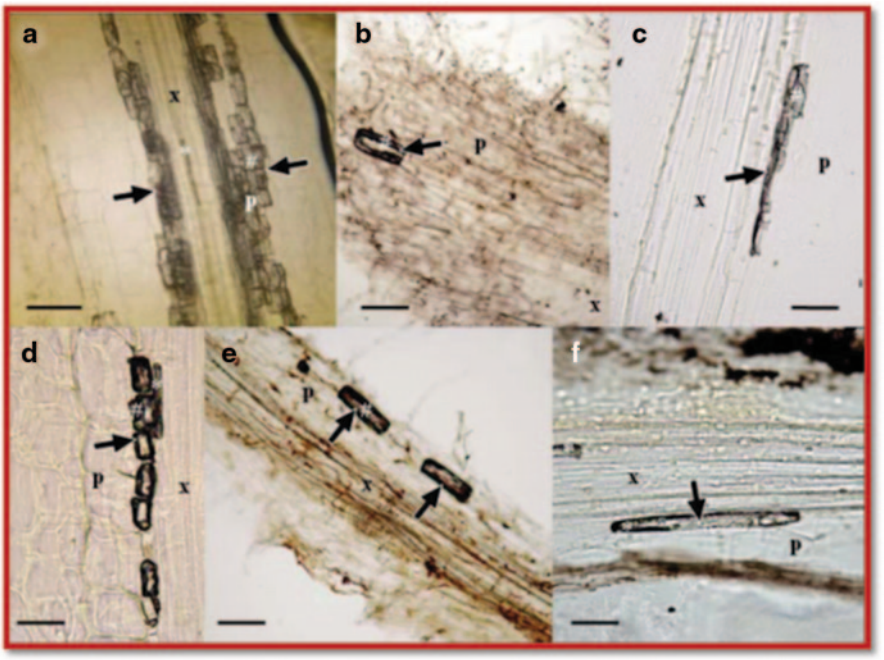

Fig. 11.12 Longitudinal sections of roots of pea (**a**, **d**), sunflower (**b**, **e**) and wheat (**c**, **f**). Arrows indicate accumulation of bioferrofluid in the cells; *, xylem-containing ferrofluid; #, parenchimatic cell-containing ferrofluid; p, parenchimatic cells; x, xylem vessels. Scale bars: (**a**) and (**f**), 50 μm; (**b**) and (**e**), 100 μm; (**c**) and (**d**), 25 μm. (Adopted with kind permission from Cifuentes et al. 2010)

applying the bioferrofluid through the leaves and aerial parts by pulverization or injection (Corredor et al. 2009; Ghosh et al. 2008).

For example, Pea roots accumulated higher contents of bioferrofluid (Fig. 11.12a) than sunflower or wheat which remain unchanged even after 48 h of exposure to bioferro fluid (Fig. 11.12d–f). This shows that pea roots could be more permeable to nanoparticle penetration. Therefore, the speed of absorption and distribution of the nanoparticles is faster in pea and wheat than in tomato and sunflower. This fast movement of the nanoparticles inside the plants can be an important factor in the development of nanoparticles as smart delivery systems inside the plants.

5.3 Metal-Based Nanomaterials

5.3.1 Gold Nanoparticles

In recent years, gold nanoparticles have been used in many biomedical and agricultural applications (Paciotti et al. 2004; Rosi et al. 2005; Peer et al. 2007; El-Sayed et al. 2006; Shukla et al. 2005; Arshi et al. 2011a). In most of these appli-

cations, it is essential that nanoparticles should pass cell plasma membranes either by endocytosis (Onelly et al. 2008) or by direct penetration to reach target cellular compartments. Onelly et al. (2008) reported the internalization of gold nanoparticles using tobacco protoplasts. In their report, they showed the penetration of gold nanoparticles into the protoplasts by endocytosis linking to different pathways upon their charge.Therefore, endocytosis appears as a reasonable way for internalization of nanoparticles. A recent report deals with penetration of gold nanoparticles through lipid membranes bypassing endocytosis (Lin et al. 2010). Their mean force calculations showed a significant gain of energy upon adhesion and penetration. In the case of penetration, it was found that defective areas were induced across the entire surface of the upper leaflet of the bilayer and a hydrophilic pore that transports water molecule was formed with its surrounding lipids highly disordered. It was also found that the increase in charge density of gold nanoparticles increased the level of penetration and membrane disruption. These findings suggest a way of controlling the gold nanoparticles–cell interactions by manipulating surface charge densities of gold nanoparticles to achieve designated goal in their biological applications, such as a delivery agent.

5.3.2 PalladiumNanoparticles

Since the geogenic background, a significantly lower concentration of palladium (Pd) than the concentration of other non-essential toxic elements such as mercury, lead or cadmium, Pd may not yet have affected biological systems. Earlier studies (Battke et al. 2008; Jo et al. 2009) showed a higher mobility and uptake rates of Pd in soil than platinum, e.g., in grass samples from roadsides. Battke et al. (2008) studied the uptake of Pd in barley and behavior of Pd nanoparticles in nutrient solutions used to grow plants, in order to develop a model of Pd exposure of plant systems. Their results showed that smaller and larger Pd particles were comparatively assessed and the Pd uptake, via the roots, depends on its particle diameter. Pd nanoparticles of smaller diameter cause significant effects on leaf length growth. As the concentration of Pd increased in the nutrient solution, leaf length decreased significantly with the increased variability of leaf lengths. Moreover, with increasing Pd concentration in the nutrient solution, leaves become rigid and slightly convoluted.

5.3.3 Silver Nanoparticles

Owing to their several antimicrobial functions, silver nanoparticles have been widely used to control various phytopathogens (Park et al. 2006; Min et al. 2009; Kim et al. 2009; Stampoulis et al. 2009; Arshi et al. 2011c). Harris et al. (2008) studied the uptake limits and the distribution of silver nanoparticles in *Brassica juncea* and *Medicago sativa*. They observed that *Medicago sativa* showed an increase in metal uptake with a corresponding increase in the substrate of metal concentration and exposure time as compared with *Brassica juncea*. Study of hydroponic solution

mended with Ag nanoaprticles for the seed germination and root growth of zucchini plants, showed no negative effects, whereas on prolonging their growth in the presence of Ag NPs, a decrease in plant biomass and transpiration was observed (Rehm et al. 1997).

5.4 Metal Oxide-Based Nanomaterials

5.4.1 ZnO Nanoparticles

Zinc (Zn) is one of the necessary micronutrients required for the optimum growth of plants. It plays an important role in driving many metabolic reactions within the plants. Zn is also a part of several other enzymes such as super oxides dismutase and catalase, which prevent oxidative stress in plant cells. Growth and development would stop if specific enzymes were not present in the plant tissues (Vitosh et al. 1994). Important role of Zn can be decided as it controls the synthesis of indole acetic acid (IAA), a phytohormone which significantly regulates the plant growth. It also helps in the chlorophyll synthesis and carbohydrate formation (Adiloglu et al. 2002) and enables the plants to withstand lower air temperatures. Zn helps in the biosynthesis of cytochrome, a pigment and maintains the plasma membrane integrity, and the synthesis of leaf cuticle. Additionally, the enhancement of Zn nutritional status helps in reducing harmful heavy metals uptake which hinders their toxicity in plants, such as Cd (Pandey et al. 2010). Pandey et al. (2010) studied the effect of ZnO nanoparticles consisting of oxygen vacancies on the seed germination and root growth of *C. arietinum* seeds. They observed that due to oxygen vacancies, the oxygen deficient, i.e., Zn-rich ZnO nanoparticles increased the level of IAA in roots (sprouts), which in turn indicated the increase in the growth rate of plants.

Due to the presence of very small quantity of Zn, the amount of Zn utilized byplant is very less, this ensures a controlled delivery of Zn to the plant, hence the excess of Zn that may toxicate the plant is avoided. Furthermore, its excess amount would least spoil the soil quality as ZnO is an eco-friendly and bio-friendly material which can be used as a green reagent.

5.4.2 TiO_2 Nanoparticles

Owing to its photocatalytic nature, TiO_2 nanoparticles under light are able to generate an oxidation–reduction reaction, and produce superoxide anion radical and hydroxide (Crabtree et al. 1998; Hong et al. 2005a). Photosterilization by TiO_2 nanoparticles improves the growth and development of plants. Hong et al. (2005b) reported the effects of TiO_2 nanoparticles (rutile phase) on the photochemical reaction of chloroplasts of Spinaciaoleracea. They found that the treatment of TiO_2 nanoparticles enhanced the Hill reaction and chloroplasts activity, which accelerated FeCy

Fig. 11.13 Effect of nanoanatase TiO_2 on the growth of spinach. The picture was taken after four weeks of cultivation. Lane 1 Control, lane 2 treated with bulk-TiO_2, lane 3 treated with nano TiO_2 (anatase). (Adopted with kind permission from Linglan et al. 2008)

reduction and oxygen evolution. Furthermore, noncyclic photophosphorylation activity was higher than that of cyclic photophosphorylation activity. The possible reason, according to author, could be due to the penetration of TiO_2 nanoparticles into the chloroplast and their oxidation-reduction reactions which accelerate electron transport and oxygen evolution. TiO_2 nanoparticles (anatase) have also been found to induce spinach seed germination and plant growth (Fig. 11.13) by regulating the germination of aged seeds and their vigor indexes (Linglan et al. 2008; Yang et al. 2006). An increase of these indexes was observed at 0.25–4 % TiO_2 nanoparticles treatments. Furthermore, it was observed that during the growth stage, the presence of TiO_2 nanoparticles increased the dry weight, chlorophyll synthesis, and metabolism in photosynthetic organisms. These results confirmed that the nanometer-size particles have remarkable effects on physiological processes. These positive effects are assumed to be due to the antimicrobial properties of nanoparticles, which in turn can increase strength and resistance of plants to stress. Additionally, nanoparticles could also sequester nutrients on their surfaces and thus serve as a nutrient stock to the organisms, especially those nanoparticles having high specific surface area. The authors also reported that the effects of bulk-TiO_2 particles were not significant. In another report, it was shown that TiO_2 nanoparticles (anatase) improved plant growth by enhanced nitrogen metabolism (Yang et al. 2007) which promotes the absorption of nitrate in spinach, and helps in accelerating the conversion of inorganic nitrogen into organic nitrogen, consequently increasing the fresh and dry weights. Other studies also showed the effects of nitrogen photoreduction on the improved growth of treated spinach plant (Mingyu et al. 2007). It has also been reported that

TiO_2 nanoparticles (anatase) enhanced antioxidant stress by decreasing the accumulation of superoxide radicals, hydrogen peroxide, malonyldialdehyde content and increase the activities of superoxide dismutase, catalase, ascorbate peroxidase, guaiacol peroxidase and thus increase the evolution oxygen rate in spinach chloroplasts under UV-B radiation (Lei et al. 2008).

6 Conclusion and Future Scenario

Nanobiotechnology being studied since several years is still in the early stages of advancement, however, the development is multi-directional and spreading rapidly. Moreover, the increasing interest in nanobiotechnology has attracted enormous attention which led to the rapid development of commercial applications involving utilization of manufactured nanomaterials for crop improvement. In order to reduce the collateral damage in plants, nanomaterials are proved to be a promising tool to distribute pesticides and fertilizers in a controlled manner. In the framework of plant–pathogen interaction, nanomaterial-based tools and their efficient transportation to specific sites provides novel solutions for the plants treatment. As compared to bulk materials, size of nanoparticles plays key role in the behavior, reactivity and toxicity of nanoparticles. With these characteristic, it is obvious to discover both positive and negative effects of nanoparticles on plants. Therefore, for assessing toxicity and trophic transport of nanoparticles, an indepth understanding of plant interactions with the nanoparticles is very important. Recently, Sabo-Attwood et al. (2011) reported that gold nanoparticles, AuNPs, enter plants through size-dependent mechanisms, translocate to cells and tissues and cause biotoxicity. We also need to be very careful of the presence of engineered nanoparticles in our environment which may be of potential risk to the ecosystem. Recently, Dey et al. (2011) have studied the effect of nanomullite (NMu) and their metal-amended derivatives on the growth of mung bean plants and found that the metal-amended NMu exerts adverse effects on the growth and biomass production of plants compared to NMu. The plant system can also be used to test the phytotoxicity of the nanoparticles as Ma et al. (2010) have investigated the phytotoxicity of four rare earth oxide nanoparticles—nano-CeO(2), nano-La(2)O(3), nano-Gd(2)O(3) and nano-Yb(2)O(3)—on seven higher plant species (radish, rape, tomato, lettuce, wheat, cabbage and cucumber) by means of root elongation experiments. Their results were helpful in understanding phytotoxicity of rare earth oxide nanoparticles (Ma et al. 2010). Recently, the phytotoxic and genotoxic effects of ZnO nanoparticles on garlic (*Allium sativum* L.) have also been reported (Shaymurat et al. 2011).

Can metal nanoparticles be a threat to microbial decomposers of plant litter in streams is a big question for which we need to be worried. Recently, Pradhan et al. (2011) have suggested that the extensive use of nanometal-based products can increase the chance of their release into aquatic environments, which can pose a risk to aquatic biota and the associated ecological processes. If there is a possibility to distribute and guide the well functionalized nanoparticles all over the plant vascular

system to targeted sites, the subsequent unloading of chemicals (fungicides, insecticides, etc.) can be achieved by using these nanoparticles. Plant cell-nanoparticles interaction modifies the plant gene expression and its biological pathways, which consequently affects plant growth and development.

It is well known that nanobiotechnology industry is spreading rapidly; nevertheless, there is a crucial urgency to perform further studies on the subject. Hence, future work is needed to evaluate how the nanoparticles penetrate and are transported within the plants, and also the mechanism of intracellular internalization to explore the potential use of nanoparticles. However, in spite of the fact that plants have the capability to endure the presence of nanoparticles inside their tissues, an important issue that arises is what happens when such nanoparticles move into the food chain.

Exploitation of the biological machinery of nature for designing a 'smart' biomaterial such as forisomes could also be used to develop stress tolerant plants. Forisomes are spindle-like bodies that are composed of ATP-independent, calcium-powered, mechanically active proteins which are present in sieve tubes in legumes (Tuteja et al. 2010a–c). When legumes experience mechanical injury, forisomes disperse and occlude sieve tubes to hinder leakage of photoassimilates or invasion of phytopathogens (Tuteja et al. 2010c). The interesting properties of the forisomes could be exploited in biomimetics and in nanobiotechnological devices (Shen et al. 2005; Knoblauch et al. 2004a, b; Peters et al. 2008). The overexpression of forisomes in crops may also lead to the development of the insect injury resistant plants.

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Chapter 12
Role of Nematode-Trapping Fungi for Crop Improvement under Adverse Conditions

Rakesh Kumar Singh, Dipesh Kumar Trivedi and Amit Srivastava

1 Introduction

Nematodes are small round worms mostly found in all the natural habitats (soil, aquatic and marine). A large number of nematodes are saprophytic free living and feed on decaying plant and animal matter and sustain themselves by consuming bacteria or other microscopic organisms. Some other nematodes are phytonematodes which are plant parasitic, attacking mainly the roots of the plants. More than 20 phytonematode species acting as obligate parasites of higher plants, have been recorded and majority of them belong to the order *Tylenchida*. Phytonematodes that cause biotic stresses are migratory ectoparasites, migratory endoparasites, semi-endoparasites and sedentary endoparasites. These sedentary endoparasitic nematodes are characterized by their ability to produce specialized organs as feeding cells within plant tissues, which mainly suppresses photosynthesis and process of respiration in plant tissues (Schans 1991). These parasitic nematodes possess certain structural characteristics which ensure their existence in plant tissues. These include the stylet adopted for penetration of plant cell walls and esophageal glands and phasmids which discharge some enzymatic secretions into the root tissues that help in the establishment of pathogenesis of nematode-induced plant diseases. Phytonematodes create stress factors, to which plants respond in more or less the same way as abiotic stress. The biotic stress induced by parasitic nematodes have several characteristics distinguishing it from stress reactions caused by other pathogens.

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These differences are mainly due to the high level of development of the trophic, reproductive, excretory and response of nervous systems in phytonematodes. Compared to other phytopathogens, plant nematodes exhibit a higher degree of motility and have a more sensitive sensory system to make them safe from foreign invasion (Zinov'eva et al. 2004).

In general, the most widespread and economically-important phytonematodes are root knot and cyst nematodes. These phytonematodes cause heavy losses in some economically-important crops viz., 18–25 % loss in vegetables, 20–25 % in pulses and 18–23 % in oil seed crops. Global annual yield loss of major crops by nematodes damage is estimated to be 12.3 % (Sasser 1989), causing annual losses of nearly $ 100 billion worldwide (Nordmeyer 1992) and considered as a serious constraint to agricultural production, particularly in intensive cultivation of vegetable and cereal crops. The damage is generally not recognized at first sight because of their presence beneath the soil and plants infected by nematodes resemble those suffering from water or nutrient stress, showing chlorosis and poor or stunted growth.

Even judicious use of chemical nematicides is highly hazardous to human health and the environment another disadvantage of chemical nematicides is their persistence in the environment which favours development of resistance in nematodes, leading to the use of more toxic chemicals. Because of increase in awareness and cautious approach among people on health and environmental concerns, there has been now a paradigm shift in the strategies towards adoption of novel ecofriendly means. Several nematode management strategies such as nematicides, resistant varieties, crop rotation, fallowing and summer ploughing, intercropping with non-hosts/antagonistic crops, organic amendments have been used conventionally. However, each management strategy has some limitations in its implementation. The use of nematode-trapping fungi as potential biocontrol agent is a novel approach whose success mainly depends upon the basic knowledge and research required for better understanding of the ecology and biology of nematode–fungal interaction for their successful adoption in field.

In this chapter, we have described and discussed some of the researches performed by us for augmentation of mass culture of nematode-trapping fungi *Arthrobotrys oligospora* effective in reducing root knots and inducing growth, along with organic substrates in tomato (*Lycopersicon esculentum*), brinjal (*Solanum melongena*) and rice (*Oryza sativa*) plants.

2 Overview of Nematode-Trapping Fungi

The nematophagous fungi which is also known are predaceous fungi comprises more than 200 species (Table 12.1), which show the characteristic of parasitism of nematodes at different stages. They constitute endoparasitic fungi, toxin-producing fungi, egg and female parasitic fungi and nematode-trapping fungi. As the name implies, nematode-trapping fungi capture nematodes with the formation of various trapping devices, i.e., adhesive hyphae, adhesive two-three dimensional networks,

Table 12.1 Nematophagous fungi and their taxonomic positions (Nordbring-Hertz et al. 2006)

Infection structure	Species	Taxonomic classification
Adhesive nets	*Arthrobotrys oligospora*	Deuteromycetes
	A. conoides	
	A. musiformis	
	A. superba Duddingtonia flagrans	
Adhesive branches	*Monacrosporium gephyropagum*	Deuteromycetes
Adhesive knobs	*M. ellipsosporum*	Deuteromycetes
	M. haptotylum	
Constricting rings	*A. dactyloides*	Deuteromycetes
	A. brochopaga	
Adhesive knobs and adhesive spores	*Nematoctonus concurrens*	Basidiomycetes
Adhesive spores	*N. leiosporus Drechmeria coniospora*	Basidiomycetes
	Hirsutella rhossoliensis	Deuteromycetes
Ingested spores	*Harposporium anguillulae*	Deuteromycetes
Zoospores	*Catenaria anguillulae*	Chytridiomycetes
	Haptoglossa dickii	Oomycetes
Adhesive hyphae	*Stylopage hadra Cystopage cladospora*	Zygomycetes
Toxic droplets	*Pleurotus ostreatus*	Basidiomycetes
Appressoria	*Pochonia chlamydosporia*	Deuteromycetes

adhesive stalks or unstalked knobs, non-constricting rings as well as constricting rings.

The nematode-trapping fungi generally grow as saprophytic phase, growing in the form of simple vegetative mycelium and switching over to parasitic phase through induction of specific hyphal traps stimulated by nematodes. During the parasitic phase, these specialized morphological structures, i.e., traps of nematode-trapping fungi, infect and parasitize nematodes (Duddington 1951; Barron 1977; Scholler et al. 1999; Ahren et al. 2004) and therefore are important from biological control point of view (Dong et al. 2004).

The majority of nematode-trapping fungi are hyphomycetes, placed within the Orbiliales (Ascomycetes) based on morphological or molecular studies. The genus *Nematoctonus* whose teleomorph belongs to the genus *Hohenbuehelia* (Basidiomycetes), shows an interlink between nematode-trapping fungi and endoparasitic fungi which possess both adhesive traps and adhesive spores to kill the nematodes. Since the pioneer work done by Drechsler (1937) and Haard (1968), nematode-trapping fungi have been classified in a number of genera based on morphology of conidia (size, shape and septa) and conidiophores (branching, modifications of the apex). Traditional taxonomic concepts were mainly based on the morphology of conidia and conidiophores, irrespective of trapping devices formed by them. This has led to a situation where species with diverse types of trapping devices have been assigned to one genus, while others with similar trapping devices can be found in different genera (Glockling and Dick 1994; Liu and Zhang 1994, 2003; Zhang et al. 1996a, b). With the advancement of molecular technology, traditional generic classification based on the morphology of conidial characters, was challenged and justified by the molecular data. Phylogenies based on rDNA sequences have indicated

that trapping devices are more informative than other morphological characters to understand these nematode-trapping fungi in a better way (Liou and Tzean 1997; Pfister 1997; Ahren et al. 1998; Scholler et al. 1999; Kano et al. 2004). Ahren et al. (1998) clustered nematode-trapping fungi into three lineages: Species with constricting rings, species with various adhesive structures (net, hyphae, knobs and nonconstricting rings) and species having no trapping devices. Based on the assimilation of results obtained from morphological and molecular characters, Hagedorn and Scholler (1999) and Scholler et al. (1999) classified nematode-trapping fungi into four genera: *Dactylellina* species have stalked adhesive knobs characterized by non-constricting rings with stalked adhesive knobs, *Gamsylella* species have adhesive branches and unstalked knobs, *Arthrobotrys* species which is the largest group comprising all type of adhesive networks and *Drechslerella* have only constricting rings. Li et al. (2005) further converged the nematode-trapping fungi into three groups where constricting rings forming fungi are placed in *Drechslerella,*adhesive networks and unstalked adhesive knobs producing fungi are placed in *Arthrobotrys* and stalked adhesive knobs or unstalked adhesive knobs and stalked adhesive knobs with non-constricting rings producing fungi are placed in *Dactylellina*.

3 Nematode-Trapping Fungi as Biocontrol Agents

The initial studies of these nematode-trapping fungi were carried out for hyphal bail formation, attraction and predacity, mostly with bacteria feeder saprophytic nematode *Panagrellus redivivus*. Under normal conditions, when nematodetrapping fungi grow, they form normal hyphae, and when these hyphae interact with phytonematodes, some parts of hyphae are transformed into different types of trapping devices. This transformation in the hyphae takes place either due to 'nemin' secreted by nematodes, some amino acids like valine, or sometimes even formed spontaneously. This transformation of normal hyphae to trapping devices indicates switching over of such fungi from saprophytic phase to predaceous phase.

According to these changes, nematode-trapping fungi are classified into two groups, one which is sensitive group, that is fungi forming constricting rings e.g., *Drechslerella brochopaga* where predacious ability is more but growth rate and colonization ability is low, while the other is insensitive group fungi forming adhesive networks like *A. oligospora* where potential of predaceous ability is less but their saprophytic phase and colonization ability to substrates are very good. Nematodestrapping fungi have been considered as promising biological agents for control of nematodes for a long time. Their spectacular predacious behaviour on agar plates makes them to translate those results in the field conditions. A method to assess the variability in the predacity of nematode-trapping fungi in vitro was developed by Heintz (1978). In order to select the most effective predacious fungi for soil and field tests, the nematode capturing ability among nematode-trapping fungi is tested first in vitro in 1:10 corn meal agar medium and most potential isolates screened out for further testing in microcosm, pot, or field experiments.

Earlier results obtained with these nematode-trapping fungi in the soil were erratic and more confusing. The work done and reviewed by Cooke (1964), Dudding-

ton (1962) and Mankau (1980) with a view to establish these biocontrol agents in soil, found somewhat negative results and concluded that biocontrol efficiency of nematode-trapping fungi can be only satisfactory if the fungus has ability to tolerate ill-effects of soil fungistasis and has high degree of competitive ability. Their interaction in soil with nematodes, plants, other organisms and the soil environment is little understood because of difficulties of working in the complex soil matrix. Growth of mycelium or trap formation requires energy which can be supplied readily available carbon and nitrogen sources consequently they concluded that addition of organic amendment to soil results in a reduction in the predaceous activity of the fungus. This occurs because of the intensified soil microbial population competing with the predaceous fungi for nutrients. They observed lysis of fungal spores of these fungi and found none of them colonized or exploited the soil microhabitat when placed in the soil.

In nematode-trapping fungi, germination by germ tube was hampered which gave rise to conidial traps which induced the parasitic ability of nematode-trapping fungi. This trapping structure formed directly with the spore, was recognized for the first time by Dackman and Nordbring-Hertz (1992) as conidial trap (CT) (Fig. 12.1a, c, e) which has the similar ability to capture nematode, as trapping structure formed on normal hyphae (Fig. 12.1b, d, g, f). They believed that the CT formation in response to soil fungistasis is a boon for nematode-trapping fungi where fungistasis hampered the growth of many other pathogenic and non-pathogenic fungi, conidial trap formation promotes and acts as a survival structure which gets energy from the captured nematodes and further growth of the spores takes place in the soil.

The first attempt to use them as successful biological control was carried out by Linford (1937) in his classical work where chopped green pineapple tops were placed in nematode-infested soil in pots. They estimated the nematode population and activity of predatory fungi after application of pineapple tops and noticed increase in the number of free living nematodes in the soil as well as increase in the stimulation of predatory fungi which killed the nematodes and brought their population below their original level. The basic hypothesis was that the decomposition of organic substrates added to the soil increased food supply which resulted in the multiplication of free living nematodes. These nematodes were captured by nematode-trapping fungi, and if the capturing rate is faster than the rate of its multiplication, then the effect of nematode-trapping fungi declined and in the meantime the population of plant parasitic nematodes also get reduced.

Despite their generally negative or erratic results in the past, a few recent examples of successful results have been obtained with the application of nematode-trapping fungi. The biology, ecology and potential of these biological control agents for nematodes have been extensively reviewed (Kerry 1987; Stirling 1995; Sayre 1986; Sikora 1992) and found that in future these nematode-trapping fungi have very good ability to replace nematicides for the management of nematodes in different crops.

There are two general ways of applying nematode-trapping fungi for biological control of nematodes—addition of large amount of inoculum in the form of mass culture to the soil, and stimulation of activity of the pre-existing fungi using

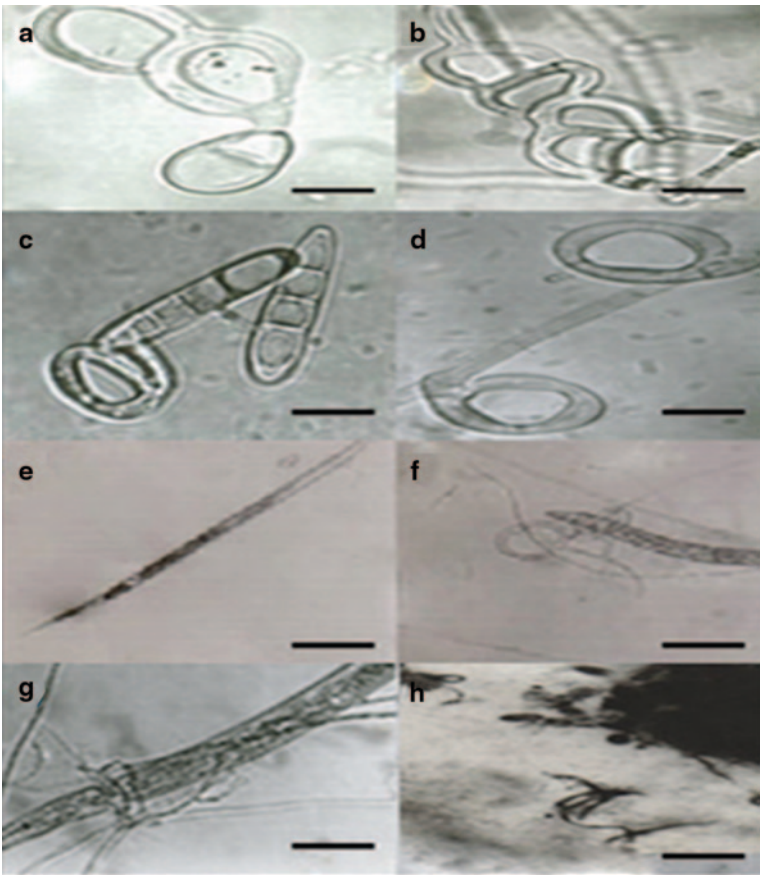

Fig. 12.1 **a** Conidial traps of *Arthrobotrys oligospora* **b** Hyphal traps of *A. oligospora* **c** Conidial traps of *Drechslerella* brochopaga **d** Constricting rings of *D. brochopaga* **e** Capturing of phytonematodes by conidial trap of *A. oligospora* **f** Capturing of phytonematode by hyphal trap formed by *A. oligospora* **g** Capturing of phytonematode by *D. brochopaga.* **h** Capturing of phytonematodes released from the eggmasses *Bars a, b,c, d* 10 µm, *e* 40 µm, *f, g,* 20 µm, and h 60 µm

various organic amendments. Here, a renewed interest evolves in using nematode-trapping fungi, partly due to an increased knowledge on the biology of these fungi and partly due to better methods of formulation and application of fungal biocontrol agents to the soil. Commercial production of some of these fungi was started and used in France. These are *Arthrobrotrys robusta* variant "Antipolis" marketed as 'Royal 300' (Cayrol et al. 1978) and 'Royal 350' of *A. irregularies* for controlling root knot nematodes on tomato (Cayrol and Frankowski 1979). Stirling and Smith (1998a) developed formulation of nematode-trapping fungi for the control of root knot nematodes in microcosm. Our laboratory experience also showed that it is possible to develop mass culture of nematode-trapping fungi on some cheap solid substrates with long shelf life (unpublished data).

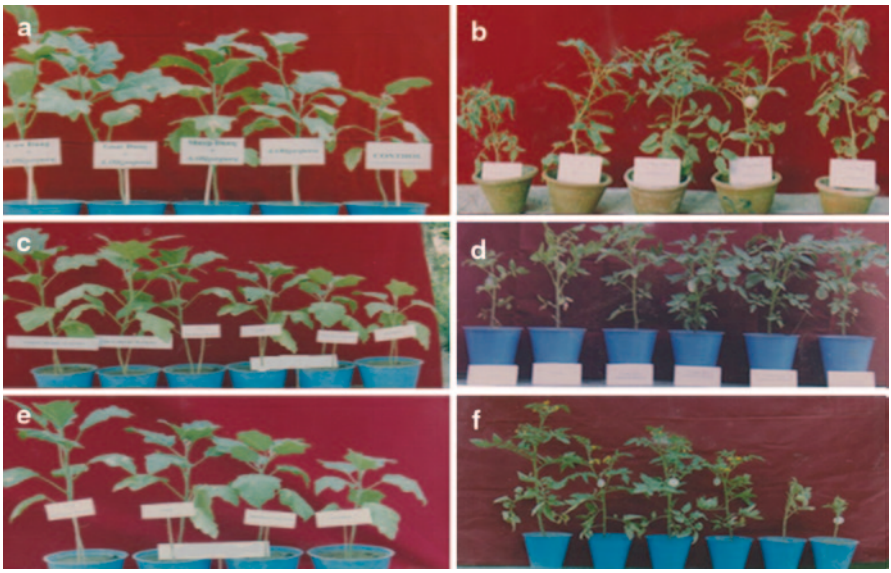

Fig. 12.2 a, b Effect of mass culture of *A. oligospora* with dung of different animals on the growth of brinjal and tomato plants in root knot-infested soil. **c, d** Effect of different amounts of mass culture of *A. oligospora* on the growth of brinjal and tomato plants in root knot-infested soil. **e, f** Effect of time of application of mass culture of *A. oligospora* on the growth of brinjal and tomato plants in root knot infested soil

Among all the nematode-trapping fungi, the most extensively studied fungus is *Arthrobotrys oligospora* due to its fast growth as well as good saprophytic and colonization ability. We have also used *A. oligospora* to determine its biocontrol potential in pot experiments with a view to understand the basic criteria for its establishment and efficacy in soil. Our study was conducted to determine whether organic substrates in any form improve its potential, to determine optimum level of mass culture for application and to determine the consequence and efficacy of *A. oligospora* when applied either prior to planting or same day of planting of the seedlings. We have tested the same set of experiments on different crops like brinjal, tomato and rice with mass cultures of *A. oligospora* prepared on sorghum grains and applied in nematode-infested soil for 30 days and 60 days after transplanting.

In the first experiment when mass culture of *A. oligospora* was applied to fully rotten animal dung, the biotic stress caused by *Meloidogyne incongnita* was altered, and the effect was also visible on the growth parameters of brinjal and tomato plants (Fig. 12.2a,b) in terms of shoot and root biomass and chlorophyll content in the plants. The increased growth of plants in response to mass culture of nematode-trapping fungi combined with farm yard manure or other animal dung might be because of reduction of infective juveniles due to capturing and killing by nematode-trapping fungi. Effective control of root knot diseases of plants by application of nematode-trapping fungi have been found, particularly when these fungi were applied in combination with organic substrates or compost (Jansson et al. 1980; Stirling et al. 1998b;

Jaffee et al. 1998; Jaffee 2002; Jaffee and other animal dung might Strong 2005). Recently, Kumar and Singh (2006) reported that application of *A. dactyloides* reduced the number of root knots in tomato and increased plant growth in pots. The effect of *A. dactyloides* was enhanced when its mass culture was applied in combination with cow dung manure. Similar results on reduction of root knot nematodes in rice plants were observed by Singh et al. (2006) by nematode-trapping fungi *A. oligospora* with cow dung manure. Similar type of biocontrol efficacy of nematode-trapping fungi was also observed by several researchers when applied in combination with compost (Matskevich et al. 1978) and organic substrates (Gueye and Duponnois 1997; Duponnois 1995; Ashour 1999). Sayre and Walter (1991) remarked that where biological control has been achieved through use of trapping fungi, the results may have been confounded by the abundance of organic material that was added as food base for these fungi.

In another experiment (Fig. 12.2c,d), biotic stress reduced significantly when the amount of mass culture of *A. oligospora* was increased in the nematode-infested soil. This clearly indicates that application of mass culture at higher concentration reduces more number of root knots which indirectly helped in plant growth. This is quite expected as the predaceous activity would be increased with increase in amount of mass culture inoculum.

Similar experiments on the time of application of mass culture of *A. oligospora* showed even more reduction in root knots in both tomato and brinjal when mass culture was applied one or two weeks prior to planting in the nematode-infested soil (Fig. 12.2e,f).

From the observation, it is obvious that irrespective of treatments in different experiments, reduction in number of root knots was higher in 30-day-old plants in comparison to 60-day-old plants, which clearly indicates that nematode-trapping fungi is more effective during the early growth stages of plants. The higher percentage of reduction at early growth stage of plants may be attributed to increase in predaceous activity of the nematode-trapping fungi. It is a well-established fact that the early infection in plants by root knot nematode causes biotic stress as acute stunting of the plants whereas delayed infection at later stage of plants may not show even perceptible stunting. This type of reaction may be due to varying ratio of nematode biomass and root biomass of the plants.

3.1 Mycoparasitism

Mycoparasitism is a characteristic of fungi that obtain nutrition either directly or indirectly from another fungi through various mechanisms (Jeffries 1997). The ability of nematode-trapping fungi to attack other fungi was first time noticed by Tzean and Estey (1978). Nematode-trapping fungi such as *A. oligospora* formed hyphal coiling around the fungus *Rhizoctonia solani* in a similar manner as formed by *Trichoderma* species (Chet et al. 1981). It has been confirmed by using radioactive phosphorus tracing that considerable amount of phosphorus transfer takes place between the hyphae of *R. solani* to nematode-trapping fungi *A. oligospora* (Ols-

son and Persson 1994). This mycoparasitic behaviour of *A. oligospora* showed the biocontrol capability of nematode-trapping fungi as biocontrol agents to nematodes as well as fungal parasites. Many other nematode-trapping fungi, including *A. oligospora, A. superba, A. conoides*, *Monacrosporium globosporum* and *M. sinense* actively colonize the sclerotia of *Sclerotinia sclerotiorum* in natural soils (Li et al. 2001). This group also identified a new fungal species *Monacrosporium janus* sp. nov. parasitizing sclerotia and hyphae of *Sclerotinia sclerotiorum* along with *R. solani*, *Fusarium solani* f.sp. *pisi* and *Phytophthora cactorum*. More investigation of this kind of characteristic of nematode-trapping fungi may result in discovering more effective strains for control of both soil-borne plant pathogenic fungi and phytonematodes in a single application.

3.2 Root Endophytes

The term endophyte was always closely associated with beneficial organisms colonizing the phyllospore. However, the definition of an endophyte is now broadened and now includes any organisms that live in plant tissues whether neutral, beneficial or detrimental (Sikora et al. 2007). Nematode-trapping fungi also have the ability to infect and colonize plant roots (Jansson and Lopez Llorca 2004). From the biological control point of view, the presence of nematode-trapping fungi in the rhizosphere of agricultural plants shows their potential bicontrol ability. Persmark and Jansson (1997) noticed maximum number of nematode-trapping fungi from the pea rhizosphere with maximum frequency of *A. oligospora*. Bordallo et al. (2002) compared *A. oligospora* with an egg parasitic fungus *Pochania chlamydospora* in the rhizosphere of barley and tomato and observed that both the fungi formed appressoria during penetration of plant cell walls and colonized epidermis and cortex. They suggested that colonization of plant roots and formation of appresoria by these fungi as root endophytes, may render the plants more resistant to plant parasitic nematodes and have profound implication for their suitability as biocontrol agents of plant parasitic nematodes. Our observation also showed that the colonized root of plants with *A. oligospora* when placed in agar plates, the juveniles released from the egg masses present on root knot captured on its release (Fig. 12.1h) which might also occur in the soil during disintegration of infected roots.

4 Improvement of Nematode-Trapping Fungi by Genetic Engineering

There is an important arena of basic and applied research opportunities in the genetic tailoring of organisms for effectiveness in specific environmental and cultural situations. Advances in the techniques of biotechnology introduce additional

possibilities of biological engineering of nematode-trapping fungi. Identification of new potential virulence factors is important and new technologies such as functional genomics, proteomics and metabolomics will make us to identify the real cause of infection process and to elucidate the signals which switch on the process in the nematode-trapping fungus so that its biocontrol potential may be improved to overcome the biotic stresses caused by phytonematodes.

Improvement of biological control agents has involved the overexpression of lytic enzymes which is an important virulence factor in the process of infection. Ahman et al. (2002) investigated the role of protease II encoding serine proteases in host infection by generating several PII mutants. Deletion of the PII gene had a limited effect on pathogenicity while overexpression of the gene resulted in a higher capacity to kill nematodes. Deletion of mutant produced less traps while multicopy transformants produced more. The strategy of biotechnology would involve selection or induction of variants of potential biological control agents with characteristics which could enhance their effectiveness. New information on host resistance genes and nematode virulence genes provides additional insights into the problem. Recently, several gene products have been identified which are secreted by nematode during parasitism. In situ hybridization has been applied extensively to study the tissue specificity and developmental expression of nematodes genes. Though RNA interference (RNAi) has been a challenge in phytonematodes due to thick cuticle, obligate parasitic feeding and lack of selection of transformants. Recent demonstration of gene silencing with double-stranded RNA in cyst nematode holds promises for biocontrol of these phytonematodes.

5 Concluding Remarks

A key belief to use nematode-trapping fungi for the control of phytonematodes has largely been neglected due to its inconsistent results and less effectiveness than chemical control. Every management practice has some limitations, and no single approach is fool-proof. Although use of nematode-trapping fungi shows promising results, it is better to integrate some of the management practices for the control of phytonematodes and development of microbial consortia to overcome biotic stress caused by them. We have also presented our conducted experiments and figures which indicate that these nematode-trapping fungi have very good biocontrol potential. Their performance can be improved from more research work and better understanding among weak links of phytonematodes lifecycle and strong attributes of nematode-trapping fungi during fungal and nematode interactions.

The host plant is after all the most important living entity in an agro ecosystem and our experiments conducted on tomato, brinjal and rice show that nematode-trapping fungi work effectively in the soil. There may be some difference in potential among isolates or different nematode-trapping fungi on the basis of sensitivity but once these are augmented in the rhizosphere, then colonization itself reveals their potential for restricting the damage or losses caused by phytonematodes.

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Chapter 13
Sugars as Antioxidants in Plants

Darin Peshev and Wim Van den Ende

1 Plant Carbohydrates: An Introduction

Typical plant carbohydrates consist of carbon, hydrogen and oxygen. Their empirical formula is $C_m(H_2O)_n$. (m and $n \geq 3$, $m \geq n$). They are polyhydroxy aldehydes and ketones with different degrees of polymerization (DP). On this basis, monosaccharides (DP1), disaccharides (DP2), oligosaccharides ($DP \leq 10$), and polysaccharides ($DP > 10$) can be distinguished. Some biomolecules do not strictly obey the empirical formula (e.g., chitin), but they are still considered as carbohydrates.

Carbohydrates are the most abundant biomolecules on the planet. They fulfill numerous functions in living beings (He and Liu 2002), especially in plants (Lewis 1984). Polysaccharides are often used to store energy (e.g., starch, glycogen, fructans), or to make up structural components (e.g., cellulose and chitin). Ribose and deoxyribose form an integral part of RNA and DNA. Ribose is also a part of NAD(P)H and ATP which are important cellular metabolites.

Numerous carbohydrates are recognized participants in stress responses in plants and other living beings. This chapter will discuss in detail the relations between carbohydrates and plant stress responses, particularly focusing on their role in the plant antioxidant network. First, the plant stresses are introduced. Second, the metabolism of stress-related sugars is highlighted. Third, the actual functions of different types of sugars in plants will be discussed, with special attention on the emerging "sugar as antioxidant" concept in which sugars act as true ROS scavengers in plants. Finally, how these insights might be exploited to create stress-tolerant crops in the near future is discussed.

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2 Threats in the Field: The ROS-Stress Connection

Plants as sessile organisms are subjected to various forms of environmental stresses such as salinity, UV radiation, drought, heavy metals, temperature variations (heat-shock, chilling, frost), nutrient deficiency, air pollution, herbicides and pathogen attacks, from which they cannot escape. They need to be able to develop defense mechanisms to cope with such unfavorable factors (Nishizawa et al. 2008). It is generally accepted that the imposition of the above mentioned stresses leads to excess concentrations of reactive oxygen species (ROS; Nishizawa et al. 2008). Crop yield and quality are negatively-affected by all these different types of stresses, potentially leading to oxidative damage (Bolouri-Moghaddam et al. 2010). In other words, oxidative damage is likely the main reason for yield and quality losses under stress.

2.1 ROS Identity, Origin and Function

In plants, ROS are continuously produced in chloroplasts during the process of photosynthesis, during the respiration process in mitochondria, in peroxisomes and at the plasma membrane (NADPH oxidases). ROS include hydroxyl radicals ($^{\bullet}OH$), superoxide radicals ($O_2^{\bullet-}$), singlet oxygen ($^1O_2$) and hydrogen peroxide (H_2O_2). Among these, the $^{\bullet}OH$ is the most reactive (*in vivo* half-life 10^{-9} s) and dangerous species, immediately attacking virtually any molecule in its neighbourhood. By contrast, H_2O_2 is much more stable and it is able to cross membranes. To deal with ROS, plants develop mechanisms that keep the equilibrium between the production and the scavenging of ROS in the cell: ROS homeostasis (Gill and Tuteja 2010). In general, two antioxidant protective mechanisms are discriminated: enzymatic and non-enzymatic mechanisms. The former includes the action of superoxide dismutase (SOD), ascorbate (AsA) peroxidase (APX), glutathione (GSH) peroxidase (GPX), thioredoxin peroxidase (TPX) and catalase (CAT) (Nishizawa et al. 2008). Non-enzymatic antioxidants include polyphenols, AsA, GSH, flavonoids, carotenoids, α-tocopherol, sugar-sterols and sugar-phenols and soluble carbohydrates, such as fructans and Raffinose Family Oligosaccharides (RFOs) (Stoyanova et al. 2011).

The ROS equilibrium determines whether it causes damage or rather acts as a signal to induce defense responses under abiotic and biotic stresses (Gill and Tuteja 2010). Therefore, spatio-temporal variations in ROS are greatly important to understand whether ROS (e.g., H_2O_2) could act as a signal or not (Gill and Tuteja 2010). ROS levels likely differ among different plant organelles, being intimately linked to the specific metabolism and antioxidant mechanisms taking place at these different locations. Under stress, the delicate balance is disturbed leading to temporal ROS accumulation (ROS overshoot) in one or more organelles, and finally also in the whole cell, including the cytosol, nucleus and the vacuole (see below; Gill and Tuteja 2010). This might lead to oxidative damage of biomolecules, including nucleic acids, proteins and lipids (Gill and Tuteja 2010; Nishizawa et al. 2008).

2.2 Abiotic Threats and Their Connection with ROS

Often, plants are challenged with different types of stresses at the same time. Such combined stresses can cause severe harm or decrease a plant's ability to resist consequential stresses (Tester and Bacic 2005). For example, low water supply is often accompanied by high temperature stress, high photon irradiance and soil mineral toxicities constraining root growth (Tester and Bacic 2005). More severe drought stresses can make a plant more susceptible to damage from high irradiance (Tester and Basic 2005). Another example is chilling stress going along with water deficits by disturbed water transport, leading to ROS accumulation and damage to cell membranes (Hodges et al. 1997; Kawakami et al. 2008). In fact, the first harmful effects in case of hypothermia in cold-tolerant and, especially, cold-sensitive plants are caused by increased oxidative stress. The generation of the superoxide radical seems to occur in an initial step (Sinkevich et al. 2010).

Other stress conditions also lead to increased ROS production, such as high photosynthetic activity in source leaves leading to temporal sugar accumulation. However, these sugars are believed to counteract (directly or indirectly) oxidative stress, perhaps contributing to re-establishing cellular ROS homeostasis (see below). Sugar starvation too can lead to ROS accumulation (Bolouri-Moghaddam et al. 2010). Taken together, both sugar shortage and excesses might lead to a disturbance of respiratory metabolism, leading to excess ROS during mitochondrial electron transport (Xiang et al. 2011). In rice, cytoplasmic male sterility (CMS) was found to be correlated with ROS overproduction and ATP depletion, leading to mitochondrial failure and disturbance of pollen development (Nguyen et al. 2010).

3 Stress-Related Carbohydrates and Their Metabolism

Some soluble carbohydrates (glucose, fructose and sucrose) in concert with hormone-signaling pathways are crucial in signaling events controlling plant growth and development (Smeekens et al. 2010; see also below). There are three main water-soluble carbohydrate types (collectively referred to as "sugars" from this point on) that play essential roles in plant stress responses: disaccharides (sucrose, trehalose), RFOs and fructans. Typically, such sugars can accumulate under (mild) stresses when growth is restricted but photosynthesis is not (or only partially) inhibited (De Roover et al. 2000; Muller et al. 2011; Skirycz et al. 2011).

3.1 Disaccharides

Sucrose (Glcα(alfa)1,2β(beta)Fru) is one of the most widespread disaccharides in nature (Salerno and Curatti 2003). In higher plants, it represents the major transport compound bringing carbon skeletons from source (photosynthetically active leaves) to sink tissues (roots, young leaves, flowers, seeds, etc.)(Koch 2004). Although

sucrose is a major reserve compound in some plants (e.g., sugar beet, sugarcane), it should be noted that starch is the most prominent reserve carbohydrate in most plants. It is a glucose polymer consisting of linear amylose (α(alfa)1,4 linkages) and branched amylopectin chains (α(alfa)1,4 and α(alfa)1,6 linkages). In contrast to sucrose which only occurs in plants and some algae, trehalose (Glcα(alfa)1,1Glc) is found in all domains of the tree of life. It accumulates for instance in fungi and in insects (Elbein et al. 2003; Muller et al. 1995). Upon drying, only a few so-called resurrection plants are known to accumulate trehalose to a great extent. Most higher plants contain different trehalases that prevent trehalose accumulation (Muller et al. 1995), but they keep on synthesizing low amounts of trehalose 6-phosphate (T6P via Trehalose 6-Phosphate Synthase) and trehalose (via Trehalose 6-Phosphate Phosphatase), the former most probably involved in sugar signaling (Zhang et al. 2009; see also below).

3.2 Raffinose Family of Oligosaccharides

Raffinose family of oligosaccharides (RFOs) are α(alfa)(1,6) galactosyl extensions of sucrose, such as raffinose (DP3), stachyose (DP4) and verbascose (DP5). Raffinose is nearly ubiquitous in plants (Vanhaecke et al. 2008). Lychnose and its derivatives can be considered as alternative RFOs, derived from raffinose (Vanhaecke et al. 2010). The metabolism of classic RFOs is well known. First, galactinol synthase (GolS) synthesizes galactinol from UDPGal and myoinositol. Next, raffinose synthase (RafS) transfers the galactose residue to sucrose to form raffinose. Stachyose synthase (StaS) fulfils a similar reaction with raffinose as acceptor substrate. With the synthesis of higher DP (>DP4), RFOs occur independent of galactinol. Galactan:galactan galactosyl transferases (GGTs) are used for this purpose (Tapernoux-Luthi et al. 2004). Raffinose and stachyose are synthesized in the cytoplasm (Schneider and Keller 2009). Recently, it has been reported that RFO gene expression and enzymatic activities and RFO accumulation are closely associated with responses to environmental stress (Nishizawa et al. 2008; Peters and Keller 2009). RFO biosynthesis in *Arabidopsis* seems to be mainly regulated at the level of transcription (Espinoza et al. 2010). Stachyose typically accumulates in *Arabidopsis* seeds (Taji et al. 2002). RFOs play a main role in many other seeds too (Blochl et al. 2008).

3.3 Fructans

Fructans are water-soluble sucrose-derived fructose polymers accumulating in about 15 % of the angiosperm flora (Hendry 1993), but they also occur in a wide range of bacteria and in some fungi. Some economically-important plant groups are known to accumulate fructans, such as *Poales*, *Liliales* and *Asterales* (Hendry

1993). The fructan syndrome probably arose some 30 ± 15 million years ago during a climatologic shift to seasonal drought (Hendry 1993). Thus, drought might have been an important evolutionary trigger and fructans may have played a role in drought resistance (De Roover et al. 2000). Plants with the ability to synthesize fructans are important components of ecosystems that experience frequent environmental changes (Albrecht et al. 1997). Typically, the DP of fructans is modified during such changes (Amiard et al. 2003).

The fructans have been classified in five different types depending on the type of linkages between the fructosyl units and their branching: Inulin consisting of β(beta)(2,1) linkages, levan containing β(beta)(2,6) linkages and graminan having both β(beta)(2,1) and β(beta)(2,6) linkages. Neo-inulin and neo-levan type of fructans as occurring in *Lolium*, *Asparagus* and *Allium* contain an internal glucose residue (Van den Ende et al. 2002). Fructans are believed to be synthesized in the central vacuole. The first step in fructan synthesis is catalyzed by sucrose: sucrose 1-fructosyltransferase (1-SST), synthesizing glucose and 1-kestotriose from two sucrose molecules. Depending on the species, further elongation occurs through the action of a specific number of other fructosyltransferases (FTs; 1-FFT, 6G-FFT and 6-SFT) by further adding β(beta)(2,1)- and/or β(beta)(2,6)-linked fructosyl moieties (Van den Ende et al. 2011 and references therein). Fructan breakdown is accomplished by fructan exohydrolases (FEHs). 1-FEH, 6-FEH, 6-KEH (6-kestose exohydrolase), and 6&1-FEH type of enzymes have been described in fructan plants (Kawakami et al. 2005 and references therein). However, such FEHs also occur in non-fructan accumulating plants but their functions in such plants remain unclear (De Coninck et al. 2005). The regulation of fructan biosynthetic and breakdown genes is mainly controlled at the transcriptional level (Van Laere and Van den Ende 2002). Dicot FT genes are specifically induced by sucrose and dicot FEH genes are induced by cold (Michiels et al. 2004). However, many FEHs are also inhibited by sucrose at the posttranslational level (Van den Ende et al. 2001). Bacterial fructans (levans, inulins) are much longer than plant fructans and involve the activity of levansucrases and inulosucrases (Banguela et al. 2011).

Fructans can act as long-term (inulins, dicots) and as short-term (other fructan types, grasses) reserve carbohydrates in different organs such as roots, stems, grains and sometimes in leaves (Pollock and Cairns 1991; Van Laere and Van den Ende 2002). Similar to invertases splitting sucrose into glucose and fructose (Koch 2004; Roitsch and Gonzalez 2004), the sucrose splitting capacities of some FTs can be used to establish sucrose gradients and regulate sink strength (Ji et al. 2010). It can be speculated that storing fructans might be advantageous compared to starch. First, FTs are less inhibited by cold compared to starch synthases (Pollock et al. 1999). Second, remobilization of water soluble fructans is likely to occur at a faster rate compared to the insoluble starch (Van Laere and Van den Ende 2002).

Furthermore, fructans and FEH regulate osmosis during flower opening in *Campanula rapunculoides* and other species (Vergauwen et al. 2000). Fructans fuel rapid regrowth in grasses and act as membrane stabilizers under stress (Valluru and Van den Ende 2008; Lothier et al. 2010).

Inulin-type fructans, derived from chicory, are widely used as prebiotics in functional food leading to improved health and well-being (Roberfroid 2007). Recent insights suggest that they could counteract oxidative stress in the human body as well (Stoyanova et al. 2011).

4 Sugars as Osmoprotectants

One of the mechanisms that plants use to combat the detrimental effects of environmental stress is to synthesise different kinds of protective compounds such as compatible solutes and antioxidants. Soluble carbohydrates (e.g., trehalose, sucrose, raffinose and fructans) along with certain amino acids (e.g., proline), quaternary ammonium compounds (e.g., glycinebetaine), and polyols (e.g., mannitol) are thought to be compatible solutes. They are synthesized in response to osmotic stress by definition and can occur at high intracellular concentrations without interfering with normal cellular metabolism (Shen et al. 1997; Mundree et al. 2002; Parvanova et al. 2004). They act as osmoprotectants and some of them (e.g. fructans) are also storage carbohydrates (Kawakami et al. 2008). Compatible solutes facilitate osmotic adjustments during water stress and in addition may serve as protective agents by stabilizing proteins and membranes (Hincha et al. 2002). It should be noted that most compatible solutes show excellent capabilities to scavenge ROS *in vitro,* suggesting that they have similar roles in plants (Van den Ende and Valluru 2009; see also below).

4.1 General Mode of Action under Stress

The primary cause of injury during freezing is the destabilization of cellular membranes (Uemera and Steponkus 1999). Also during desiccation, the cell needs to keep all its membranes (plasma membrane, tonoplast membrane, organellar membranes) and proteins in proper functional state. In general, soluble sugar levels contribute to the increased cryostability of cellular membranes (Ma et al. 2009), keeping membranes in their proper state which is a prerequisite for survival under unfavorable conditions. Resurrection plants develop an array of mechanisms to survive complete dehydration, sugar accumulation being one of them (Djilianov et al. 2011). Sugars can replace water under drought stress. As such, they keep membrane surfaces “hydrated” and prevent membrane fusion by maintaining the space between phospholipid molecules (Mundree et al. 2002; Valluru and Van den Ende 2008). This can be explained in terms of ‘sugar vitrification’. It includes the formation of a solid, amorphous glass that prevents membrane fusion. Hydrogen bonds are not present in such glassy states. The magnitude of sugar vitrification depends on the temperature at which the glass devitrifies (T_g), which itself depends on the molecular weight of the sugar and on the water content (Levine and Slade 1991).

The model of Wolfe and Bryant (1999) strongly suggests that sugar vitrification is not only necessary but sufficient for the preservation of a dry membrane (Valluru and Van den Ende 2008). In summary, sugars may contribute to an efficient membrane protection in the dry state because of reducing the phase transition temperature (T_m) and forming an amorphous carbohydrate glass with high melting temperature T_g (Valluru and Van den Ende 2008). Both may operate in parallel. The ratio of about 1.5 glucose/sucrose per lipid molecule is thought to be optimal for membrane protection (Lenne et al. 2007) and was also found effective in reducing the T_m. This is supported with data obtained for trehalose. More details are described in Valluru and Van den Ende (2008).

Sucrose is thought to function as a typical osmoprotectant, stabilizing cellular membranes and maintaining turgor. As an easily metabolisable sugar, sucrose may serve as an immediate energy source upon rehydration (Mundree et al. 2002). In most cases (excluding trehalose type of resurrection species), sucrose accumulation is observed under drought stress, albeit at varying levels among species, implicating a role for sucrose in the acquisition of desiccation tolerance in these plants. Many kinds of soluble, sucrose-derived sugars can arise during the course of cold acclimation (see also below: RFOs in *Arabidopsis*). For instance, some mosses accumulate such soluble sugars (Nagao et al. 2006) in association with development of freezing tolerance. Also woody plants (mainly RFO accumulators) and cereals (mainly fructan accumulators) are known to develop high degrees of freezing tolerance in winter, associated with the increased levels of such compounds during cold acclimation (Sauter et al. 1996; Livingston et al. 2009). It is also proposed that the role of some oligosaccharides (e.g., raffinose) is to prevent crystallization of sucrose (Peters et al. 2007). Therefore, the sucrose/raffinose ratio might be more important than the absolute sucrose and raffinose concentrations as such (Djilianov et al. 2011).

In the following two sections, we will focus on transgenic plants carrying extra genes related to RFO and fructan metabolism. In general, the reactions of these plants under stress corroborate the protective nature of these sugars.

4.2 RFOs in Transgenic Arabidopsis

As already indicated above, RFOs are implicated in freezing tolerance in the model plant *Arabidopsis thaliana*. Both galactinol and raffinose increase in stressed *Arabidopsis* plants (Taji et al. 2002). Accordingly, downregulation of an α(alfa)-galactosidase in *Petunia* resulted in increased raffinose levels and cold tolerance (Pennycooke et al. 2003). *Arabidopsis* contains seven GolS genes, one of which is induced by cold (GolS3), two other ones by drought (GolS1 and GolS2; Taji et al. 2002). Two ecotypes with different freezing tolerance (C24 and Columbia) also showed clearly different raffinose levels, suggesting a positive correlation between raffinose levels and freezing tolerance (Klotke et al. 2004). However, *Arabidopsis* RafS knockout plants affected in raffinose synthesis were not more sensitive to

frost, suggesting that the increased galactinol levels in these lines could compensate for the impaired raffinose levels (Korn et al. 2010).

Overexpression of drought-inducible GolS1 and GolS2 genes in *Arabidopsis* also led to increased galactinol and raffinose levels, and showed reduced transpiration from leaves to improve drought tolerance (Taji et al. 2002) . Moreover, the intracellular levels of galactinol and raffinose in these transgenic plants were correlated with increased tolerance to paraquat treatment and salinity or chilling (Nishizawa et al. 2008). However, the debate is still on over the exact mechanism of action of these metabolites. They might act as signals but more likely they act as true ROS scavengers when they accumulate at high concentrations at particular locations (e.g., in the vicinity of chloroplast thylakoid membranes; Foyer and Shigeoka 2011). Intriguingly, introduction of a StaS from adzuki bean in *Arabidopsis* did not lead to increased freezing tolerance (Iftime et al. 2011). Probably stachyose is not at the correct place (cytosol) to provide protection. It can be speculated that *Arabidopsis* lacks a stachyose transporter in the chloroplastic envelope. However, recent evidence was generated for the presence of a raffinose transporter in the chloroplastic envelope (Schneider and Keller 2009).

4.3 Transgenic Fructan Plants

The reviews of Cairns (2003) and Banguela and Hernández (2006) summarize the efforts to introduce fructan metabolism in (mostly) non-fructan accumulating species. In a first phase, focus was on introducing bacterial levansucrases in such species (Cairns 2003). However, much debate arose on the correct delivery of these levansucrases to the vacuole and to plastids (mistargeting to the ER; putative ER contamination in plastid preparations, etc.). In many cases, only low levels of fructan were found (max 10 % on dry weight basis) since higher levels appeared to become toxic for these plants (Cairns 2003). Recently, Banguela et al. (2011) fused the preproprotein of onion 1-SST to the levansucrase of *Gluconacetobacter diazotrophicus* and introduced this in tobacco, in order to obtain a correct vacuolar delivery. Some transgenic lines accumulated levans up to 70 % on dry weight basis, and they showed phenotypic changes (leaf bleaching) during plant development. However, only slight leaf bleaching was observed in plants accumulating up to 30 % levans on dry weight basis (Banguela et al. 2011).

In the second phase, interest shifted to introducing plant FTs in non-fructan plants (Sevenier et al. 1998; Hellwege et al. 2000; Stoop et al. 2007; Li et al. 2007; Kawakami et al. 2008; Pan et al. 2009) or in plants accumulating other types of fructans (Vijn et al. 1997; Hisano et al. 2004; Gadegaard et al. 2008). In most cases, no detrimental effects on the plants phenotype were reported in these experiments. This might be explained by the correct vacuolar targeting of these enzymes and, perhaps, by the presence of endogenous FEHs being able to degrade the fructans at specific phases of plant development (e.g., pre-flowering stages).

More importantly, for some of these fructan accumulating transgenic plants, it was reported that they were more tolerant to stress. Konstantinova et al. (2002) showed that transgenic tobacco carrying the *Bacillus subtilis* levansucrase gene Sac B survived freezing stress both in controlled and in field conditions. Transgenic *Lolium* plants carrying wheat FT genes (1-SST or 6-SFT) and accumulating increased amounts of fructan also demonstrated enhanced freezing resistance at the cellular level (Hisano et al. 2004). Rice (*Oryza sativa* L.) is a non-fructan accumulating plant which is highly sensitive to chilling (Kawakami et al. 2008). Transgenic seedlings carrying wheat 1-SST and accumulating fructan oligo- and polysaccharides showed enhanced chilling tolerance. Introduction of the 1-SST of lettuce (*Lactuca sativa* L; Li et al. 2007) also led to increased freezing tolerance and reduced oxidation of membranes, similarly as observed in the *Arabidopsis* GolS overexpression plants (Nishizawa et al. 2008). Taken together, these data strongly suggest that sugars can contribute to stress tolerance by protecting membranes.

4.4 Fructan-Membrane Interactions

Sucrose and trehalose are well-known membrane stabilizers, and also fructans and RFOs have such properties, while hydroxyethyl starch, glucan and dextran have not. It is believed that fructans can replace water molecules at the membrane. Sucrose and trehalose can replace around 18 water molecules while 1-kestose has a volume equal to about 21 water molecules. For raffinose, this number is about 30 (Valluru and Van den Ende 2008). The Fru-Fru linkage (CH_2-O) in fructans is longer than the O-linkage in hydroxyethyl starch, glucan and dextran, creating an extra flexibility to interact with and stabilize membranes (Valluru and Van den Ende 2008). Inulin chains are even more flexible than levans (Vereyken et al. 2003). Using liposomes as a model system, five fructan classes (DP3, DP4, DP5, DP6 and DP7) and two DP>7 fractions were isolated from oat and rye and tested as membrane stabilizators *in vitro* (Hincha et al. 2007). The two DP>7 fractions from both species were unable to protect liposomes, while the fractions containing smaller fructans were protective to different degrees. Protection showed an optimum at DP4. Intriguingly, synergistic effects were found when low DP fructans were combined with DP>7 fructans, suggesting that mixtures of fructans, as they occur in living cells, may have protective properties that differ significantly from those of the purified fractions. However, no mechanistic insights are yet available to explain these observations. Accordingly, the capacity to accumulate higher DP fructans has been found in many stress-tolerant species, such as *Echinops*, *Viguiera*, *Dactylis*, *Lolium*, *Poa* and *Pachysandra* (Van den Ende et al. 2011 and references therein).

Next to protecting the tonoplast, fructans might also protect the plasma membrane. Tonoplast vesicle-derived exocytosis (TVE, see also below in Fig. 13.1) was proposed as a mechanism to transport fructans from the vacuole to the apoplast under stress (Valluru et al. 2008). No fructan transporters have yet been reported in

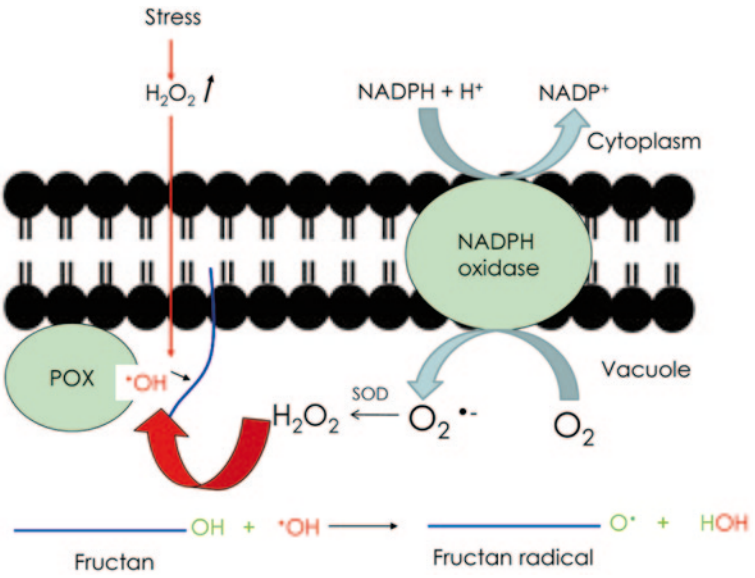

Fig. 13.1 A dual role for vacuolar fructans in the vicinity of the tonoplast under stress? Abiotic and biotic stresses can lead to increased concentrations of cytosolic H_2O_2, which can enter the vacuole via diffusion and/or through aquaporins. Alternatively, the oxidative stress can be transmitted through the action of a putative vacuolar NADPH oxidase. Vacuolar fructans can insert deeply between the headgroups of the tonoplastic membranes, stabilizing them under stress. Type III peroxidases (POX) associate intimately with the inner side of the tonoplast. Peroxidases also produce •OH radicals. Fructans are well-positioned to scavenge these radicals, a process in which fructan radicals and water are formed. Fructan radicals might be generated back into fructans with the help of phenolic compounds. (see Fig. 13.2)

the chloroplast envelope. Yet, the finding of raffinose transporters at this location urges further research in fructan accumulating plants.

5 Sugars as Signals

Small soluble sugars (glucose, fructose, sucrose) can also act as signals. They are now recognized as pivotal integrating regulatory molecules that control gene expression related to plant metabolism, stress resistance, growth and development (Rolland et al. 2006; Smeekens et al. 2010; Cho and Yoo 2011; Li et al. 2011). It is becoming increasingly clear that the hexose/sucrose ratio is an important parameter to adjust plant metabolism (Weber et al. 1995; Xiang et al. 2011). Therefore, the interest in acid and neutral type of invertases and their inhibitors (or binding partners) is steeply increasing (Xiang et al. 2011; Hothorn et al. 2010). Intriguingly, the responses to sugar signals and to oxidative stress are linked. Sugars also affect scores of stress-responsive genes (Bolouri-Moghaddam et al. 2010) but the crosstalk between sugar and ROS signaling pathways needs further exploration.

Moreover, many jasmonate-, ABA-, and stress-inducible genes are coregulated by sugars (Ma et al. 2009).

So far, hexokinase (HXK1) and Snf1-related kinase 1 (SnRK1) have been identified as conserved sugar signaling components controlling energy homeostasis, stress resistance, survival and longevity (Moore et al. 2003; Baena-Gonzalez et al. 2007). Both glucose and HXK (producing glucose 6-phosphate) take a central position in sugar signaling and antioxidant networks. They form an important bridge between sugar metabolism and biosynthesis of AsA and of glycosylated phenolic compounds via NDP-glucose production (Bolouri-Moghaddam et al. 2010). Glucose 6-phosphate dehydrogenase (G6PDH), catalyzing the first reaction in the oxidative pentose phosphate (OPP) pathway, has been postulated to affect the redox equilibrium of the chloroplast as well as the capacity to detoxify ROS (Debnam et al. 2004). Therefore, endogenous sugars feed the OPP and trigger (indirect) ROS scavenging. Moreover, sugar availability can enhance ascorbate biosynthesis (Linster et al. 2008; Bolouri-Moghaddam et al. 2010).

During plant defense reactions, invertase-related sugar signals seem to be very important (Roitsch et al. 2003; Bonfig et al. 2010). For instance, in tomato the LIN6 cell wall invertase is a pivotal enzyme for the integration of metabolic, hormonal and stress signals, regulated by a diurnal rhythm (Proels and Roitsch 2009). Hormone signals (e.g., cytokinines) often influence the expression of extracellular invertase genes (Lara et al. 2004), suggesting that such enzymes act as central modulators of assimilate partitioning, integrating sugar, stress, and hormone signals. Recent structural insights have led to a new point of view that not the invertases as such but rather the invertase/inhibitor complexes should be considered as central modulators (Hothorn et al. 2010). Perhaps, a similar function should be attributed to neutral invertase/PIP5K 9 complexes (Lou et al. 2007). Recent insights demonstrated that sugars can regulate the biosynthesis of auxins (LeClere et al. 2010), placing sugars (and sugar metabolizing enzymes and their complexes) at the heart of the regulatory processes that drive plant growth and development, both under normal conditions and under stress. In conclusion, numerous environmental and endogenous developmental and metabolic cues are integrated by sugar signals, operating in concert with plant-specific hormone signaling and stress-related pathways in a complex network (Bolouri-Moghaddam et al. 2010). It is not clear whether small fructans or RFOs can act as sugar signals too. Kawakami et al. (2008) hypothesized that fructans in transgenic rice could activate other stress signaling pathways leading to chilling tolerance. However, recent insights point to a more direct effect of fructans acting as true ROS scavengers (see next paragraph).

6 Sugars as Antioxidants: A New Concept

A stress-induced disturbance of the redox equilibrium in plant cells requires activation of antioxidant enzymes, primarily superoxide dismutase (SOD). However, a certain time is needed to accomplish additional SOD synthesis. Therefore, low molecular weight antioxidants (AsA, GSH) play a significant role at the initial stage

of oxidative stress. In the last few years, also plant oligosaccharides have been proposed as emerging antioxidants in plants (Nishizawa et al. 2008; Foyer and Shigeoka 2011; Sinkevich et al. 2010; Stoyanova et al. 2011). They might play a role in scavenging hydroxyl and superoxide radicals.

6.1 RFOs and Galactinol as Antioxidants in Arabidopsis

In *in vitro* tests, the hydroxyl radical scavenging capacity of galactinol and raffinose was superior compared to those of typical antioxidants, such as AsA, GSH and citrulline (Nishizawa et al. 2008). The concentrations that reduce hydroxylation of salicylate by 50 % (ID_{50}; the concentration of a compound required to inhibit $^{\bullet}OH$ catalysed hydroxylation of salicylate (or terephthalate) by 50 % of the maximum yield observed in the absence of the compound) of galactinol, raffinose and stachyose were 3.1 ± 0.3, 2.9 ± 0.2 and 2.2 ± 0.1 mM, respectively (Nishizawa et al. 2008). For comparison, the concentrations of galactinol, raffinose and stachyose are 7.4, 0.74 and 3.6 mM in *Arabidopsis* seeds. This suggests that the initial intracellular levels of galactinol and stachyose might be in a good range to protect cellular components from oxidative damage (Nishizawa et al. 2008). Furthermore, raffinose concentrations in chloroplasts of stressed plants are estimated between 0.27 and 1.35 mM, comparable to the levels of AsA and GSH (Nishizawa et al. 2008). Stoyanova et al. (2011) reported an ID_{50} of 0.5152 mM for raffinose (hydroxyl radical) scavenging *in vitro*, suggesting that raffinose could act as a direct scavenger of hydroxyl radicals in chloroplasts too.

As already explained above, additional evidence for a role of galactinol and raffinose as antioxidants in plants was generated by paraquat treatments on plants overexpressing GolS (GolS1, GolS2, GolS4) and RafS. These transgenic plants showed more effective ROS scavenging capacity and oxidative stress tolerance compared to wild-type plants (Nishizawa et al. 2008). It can be speculated that RFOs might be operating as ROS scavengers in chloroplasts which is a major point of ROS production under different stress conditions (Foyer and Shigeoka 2011). The oxidized RFO radicals might be regenerated to RFOs by ascorbic acid (AsA) or other reducing antioxidants.

6.2 Disaccharides and Sugar Alcohols as Antioxidants

In vitro studies further demonstrated that the ID_{50} values of other sugars and sugar alcohols were even better than the ones obtained for raffinose and galactinol. Compared to mannitol, a widely recognized osmoprotectant, sucrose and especially inulin-type fructans show excellent antioxidant properties *in vitro* (Stoyanova et al. 2011). Some plant species such as sugarcane and sugar beet accumulate sucrose to extremely high levels (2 M) in their vacuoles. Therefore, sucrose might be a

good candidate to act as a ROS scavenger in their vacuoles. Of course, such vacuolar antioxidant mechanisms occur in concert with the classic cytosolic antioxidant mechanisms (Van den Ende and Valluru 2009). So, at lower sucrose concentrations, sucrose might preferably act as a signal molecule, while it might become a ROS scavenger at high concentrations (Bolouri-Moghaddam et al. 2010).

Accordingly, transgenic potato plants carrying a yeast invertase gene (B33-inv plants), with decreased sucrose efflux and 20–30 % higher total sugar contents, showed enhanced cold tolerance and lower level of malondialdehyde (MDA; Sinkevich et al. 2010). MDA is an indicator of lipid peroxidation (LPO). The reduced MDA levels suggest that there is less ROS-mediated membrane damage in the transgenic potatoes, despite the higher levels of superoxide found in stressed B33-inv plants. These authors speculated that sugars act as primary antioxidants, and that the generated sugar radicals are reduced by AsA.

A group of sugar alcohols (such as mannitol, inositol, sorbitol) also possesses ROS scavenging capacities (Shen et al. 1997; Stoyanova et al. 2011). Mannitol protects thioredoxin, ferredoxin, GSH and the thiol-regulated enzyme phosphoribulokinase in *Nicotiana tabacum*. Genetically engineered tobacco plants with increased chloroplastic mannitol showed increased tolerance under paraquat treatments. In the same experimental setup, mannitol did not reduce $^{\bullet}OH$ radical production in the chloroplast, but it increased the capacity to scavenge these radicals protecting the cells against oxidative damage (Shen et al. 1997). Furthermore, mannitol accumulation had no harmful effects on these plants. This means that no sugar-mediated negative feedback on photosynthesis was observed, as is the case for metabolizable sugars such as glucose, fructose and sucrose (Bolouri-Moghaddam et al. 2010).

Trehalose also can act as a ROS scavenger *in vitro* (Stoyanova et al. 2011) and it was demonstrated that this sugar acts as a ROS scavenger *in vivo* in yeast (Nery et al. 2008). Exposing yeast to exogenous H_2O_2 leads to trehalose accumulation, which reduces the oxidant-induced modifications of proteins and the levels of lipid peroxidation. Transgenic rice plants accumulating increasing levels of trehalose showed increased tolerance to salt, drought and low-temperature stresses. Moreover, several transgenic lines exhibited sustained plant growth, less photo-oxidative damage and a more favorable mineral balance under stress (Garg et al. 2002).

6.3 Fructans: A Role in Vacuolar Antioxidant Mechanisms?

Fructans as water-soluble vacuolar oligo- and polysaccharides are probably good candidates to act as vacuolar ROS scavengers. Fructans can accumulate to a great extent in plant tissues (up to 20 % on a fresh weight basis; Van Laere and Van den Ende 2002). This kind of fructan levels cannot be solubilized *in vitro*, but apparently they can be kept in a solubilized (gelly-like) state in the vacuole, where they might interact profoundly with the inner side of the tonoplast. Under stress, when the redox equilibrium is disturbed, a spike in ROS occurs. The excess cytoplasmic H_2O_2, derived from ROS produced in chloroplasts or other cell compartments, can

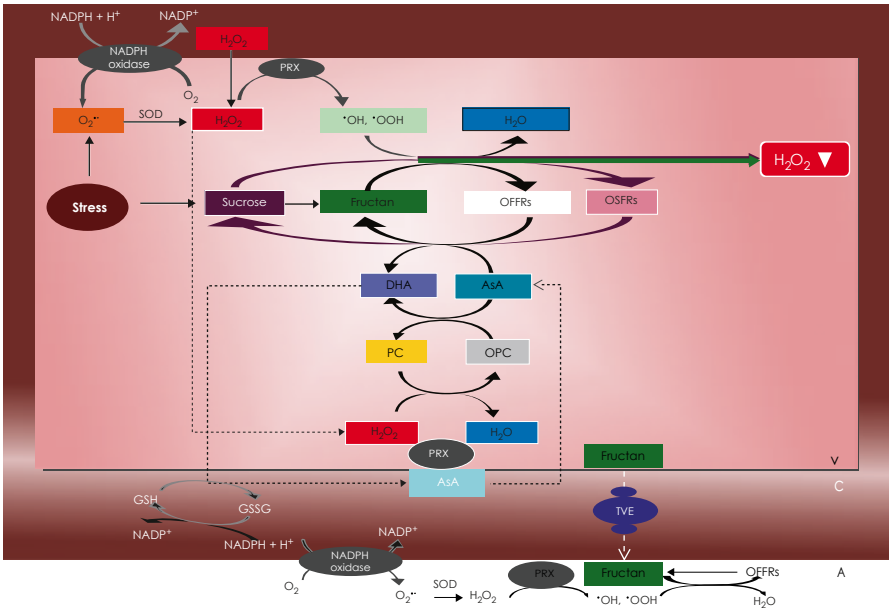

Fig. 13.2 Possible vacuolar scavenging mechanisms of fructans and sucrose in oxidative stress defense.
A apoplast, *AsA* ascorbate, *C* cytoplasm, *DHA* dehydroascorbate, *GSH* reduced glutathione, *GSSG* oxidized glutathione, H_2O_2 hydrogen peroxide, $O_2^{\bullet-}$ superoxide ion, $^{\bullet}OH$ hydroxyl radical, *OFFRs* oxidized fructan-free radicals, *OSFRs* oxidized sucrose-free radicals, *OPC* oxidized phenolic compounds, *PC* phenolic compounds, *PRX* peroxidase, *SOD* superoxide dismutase, *TVE* tonoplast vesicle-derived exocytosis, *V* vacuole

be directed to the vacuole (plant cell "detoxification factory" and "dump site"). H_2O_2 can diffuse through the tonoplast directly and/or through aquaporins. Another possible way to transmit the oxidative stress from the cytosol into the vacuole is through a putative vacuolar NADPH oxidase. Carter et al. (2004) reported this enzyme at the tonoplast but its localization was never confirmed by other studies (Fig. 13.2). This NADPH oxidase may be positioned in the neighbourhood of SOD and tonoplast-bound class III peroxidases (Fig. 13.1) catalyzing the reduction of H_2O_2. They use various substrates as electron donors, such as phenolic compounds, lignin precursors, auxin or secondary metabolites. However, as by-products of the so-called hydroxylic cycle of these peroxidase enzymes, the dangerous $^{\bullet}OH$ and $^{\bullet}OOH$ can be produced. The localization of fructans along the tonoplast make them ideally positioned to stabilize the tonoplast, but also to temporarily scavenge the aggressive $^{\bullet}OH$ and $^{\bullet}OOH$ radicals that are produced in the vicinity of these membranes (Fig. 13.1). The neutralization of these highly toxic radicals by the fructans or other vacuolar sugars/sugar-like compounds results into (less harmful) radicals (Fig. 13.1). It has been proposed that such sugar radicals could be recycled back into sugars with the help of phenolic compounds or anthocyanins with the use of AsA,

GSH and cytosolic NADPH as final reductors (Fig. 13.2). For a long time, it was not clear whether AsA and GSH could cross the tonoplast. However, a recent report suggests that vacuolar accumulation of GSH is part of the oxidative stress response (Queval et al. 2011). In addition, AsA concentration in vacuoles was reported to increase four-fold under high light conditions that may cause oxidative stress (Zechmann et al. 2011). Additionally, oxidized sugar-free radicals may react with phenolic radicals (oxidized phenolic compounds), products of vacuolar peroxidases, to form complex sugar-phenol compounds. In resurrection species, such compounds considerably increase during drying (Moore et al. 2005). Notably, phenolic radicals may also combine to form polymers (Ferreres et al. 2011). Alternatively, they might be recycled, perhaps with assistance of vacuolar AsA and/or GSH (Fig. 13.2). Thus, vacuolar sugars or sugar-like compounds, present in the vicinity of the tonoplast and interacting with this membrane, might fulfill crucial roles in scavenging radicals and thus preventing lipid peroxidation by excess H_2O_2 produced under stress conditions. Perhaps phenolic compounds and fructans (or other vacuolar, sugar-like compounds) might operate in a synergistic way to scavenge excess vacuolar H_2O_2 (Bolouri-Moghaddam et al. 2010). The glucose that is produced by 1-SST during fructan biosynthesis may directly fuel, after retranslocation to the cytoplasm, the biosynthesis of classical antioxidants such as AsA (Bolouri-Moghaddam et al. 2010).

The reactions of fructan-accumulating transgenic plants (see above) fit well with the concept that these sugars can act directly as ROS scavengers. Notably, the dynamics of fructan concentrations in immature wheat kernels showed a close correlation with changes in concentrations of classical antioxidants such as AsA and GSH, suggesting a close cooperation of cytosolic and vacuolar antioxidant mechanisms (De Gara et al. 2003).

In conclusion, various vacuolar compounds and in particular sugars come into the picture as important new players in the defense against oxidative stress. Furthermore, many of these compounds are important food additives or are present in medical extracts. Therefore, the enzymes of sugar and phenol metabolism are interesting targets to improve crop yield, stress tolerance and to delay senescence in crop plants as well as to improve food quality (Bolouri-Moghaddam et al. 2010).

7 Opportunities for Crop Improvement through Sugar Metabolism

Today, mankind is challenged by increasing frequencies of Global Warming-associated abiotic stresses (e.g., drought, heat, and temperature extremes), decreases in arable land and rapidly growing populations with higher living standards. As a consequence, world crop yield needs to be doubled in the next 50 years (Ruan et al. 2010). This should be accomplished without destroying extra forests for arable land. Therefore, mankind needs stress-tolerant crops with higher yields per acre. A considerable part of world crops are so-called “reproductive crops”, relying on

plant's reproductive structures such as grains, fruits, nuts and flowers. For example, in the USA 75 % of its harvested acreage is devoted to reproductive crops. The drastic and fast changes in climate, even for a short period of time, can greatly affect all crops but reproductive crops in particular. Drought and heat stresses during the time of flower development and pollination lead to irreversible damage, leading to fewer grains or fruits. Stresses occurring later in reproduction generally lead to smaller grains or fruits but this can often be reversed when the stress is relieved. However, in a case of fruit abortion the losses are permanent (Ruan et al. 2010).

Wheat is an economically-important crop. Worldwide, terminal drought is causing massive yield losses. Grain yield depends on carbon from two resources: flag leaf photosynthesis and remobilization of water soluble carbohydrates, mainly fructans, from the wheat stems (Yang and Zhang 2006). The production of new photosynthesis products may become limited under drought stress, due to decreases in leaf stomatal conductance and net CO_2 assimilation. Therefore, the contribution of stored carbohydrates may become the predominant source of transported materials (Plaut et al. 2004). Reserve pools have been estimated to contribute up to 10–12 % of the final wheat grain yield. This contribution further increases up to 40 % under drought and heat stress (Davidson and Chevalier 1992). The total amount of wheat stem fructans depends on the expression and activity of FTs (Xue et al. 2008). More importantly, however, efficient fructan mobilization depends on the activity of FEHs (Joudi et al. 2011,) Next to the focus on wheat stem fructans, also the dynamics of fructan pools in the reproductive organs (Ji et al. 2010) and in wheat kernels (Paradiso et al. 2006) deserve further attention for optimizing grain yield under terminal drought.

Besides drought, heat is one of the major types of abiotic stress experienced by most plants in the field. Heat and drought often come together but not always. Compared to drought, much less is known about plant responses under heat. However, among various cellular and metabolic responses, it appears that impairment of carbon metabolism and utilization are central factors causing abnormal development and yield losses under heat stress (Ruan et al. 2010).

Since carbohydrates constitute about 90 % of plant biomass, they form a crucial yield determinant. Sugar metabolic enzymes, particularly those acting on sucrose and starch, play major roles in regulating carbohydrate partitioning, plant development, and crop yield and quality (Ruan et al. 2010), especially under stress. For instance, tomato flowers are sensitive to heat stress. It was suggested that pollen development is strongly affected from heat stress than the female organs (Ruan et al. 2010). The observed decrease in pollen viability is caused by decreasing starch accumulation in developing pollen grains and low total soluble sugar in the anther wall (a similar scenario is observed in maize ovaries under water deficit). It was hypothesized that normal anther development depends on the activity of cell-wall invertases, which seems to be affected under heat stress, perhaps by increasing levels of cell wall invertase inhibitors (Frank et al. 2009). Therefore, downregulation of cell wall invertase inhibitors might be a keen strategy to increase crop yield under stress (Jin et al. 2009). Likewise, derepression of cell wall invertase activity appears to be a crucial event during the initial interactions with pathogens (Bonfig et al.

2010). By contrast, downregulation of invertases or an upregulation of invertase inhibitors proved to be a very efficient strategy to prevent cold-induced sweetening in potato tubers, in order to maintain the quality of chips and French fries, and to avoid browning and the formation of toxic acrylamides during the baking process (Bhaskar et al. 2010).

Cold stress is an important threat for the crops, especially during early growth stages and seed emergence (Zinn et al. 2010; Ohnishi et al. 2010). Rice, one of the major crops, is a good example of a species that is very sensitive to chilling. Expansion of rice cultivation into regions that experience periodic or sustained low temperatures, such as the Hokkaido region of Japan, has increased the risk of crop loss through chilling injury (Kawakami et al. 2008). Under low temperature conditions, rice suffers from leaf turgor losses and prolonged low temperatures result in leaf dehydration (Kawakami et al. 2008). It is a known fact that chilling-sensitive plants experience increased oxidative stress at temperatures not far above 0 °C (Valluru and Van den Ende 2008). Although chilling and frost tolerance are generally considered as complex multigenic treats (Hughes and Dunn 1996), it was rather spectacular and unexpected to see that chilling tolerance could be achieved in rice (Kawakami et al. 2008) by introducing a single gene encoding a 1-SST for fructan biosynthesis, counteracting oxidative stress (see above). However, introducing FTs in non-fructan accumulating crops may not always be that easy. For instance, introducing fructan synthesis in *Arabidopsis* proved to be difficult, only resulting in a minor accumulation of low DP fructans (Valluru and Van den Ende 2008). Perhaps, this can be explained by the high invertase and/or FEH activities in this species. It should be highlighted again that, besides the actual presence of a particular sugar as antioxidant and/or osmoprotectant, the exact subcellular location of such compounds is even more important for their functionality (Iftime et al. 2011).

In conclusion, the sensitivity of crops to harsh climates and soil conditions is a major limitation for worldwide food production. Worldwide, researchers look for new, desirable traits and their genes in extremophiles and resurrection plants, species that can survive extreme stress conditions (Amtmann 2009; Otto et al. 2009). Among many opportunities for crop improvement, the introduction of specific sugars (as antioxidants/osmoprotectants) and the modulation of key enzymes in sugar metabolism (or their inhibitors/partners) are very promising strategies to produce stress-tolerant crops with higher yield and quality.

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Chapter 14
Chromium Toxicity and Tolerance in Crop Plants

Ishrat Khan, Hema Diwan and Altaf Ahmad

1 Introduction

Industrialization and technological advancements, the hallmarks of civilization have been increasing heavy metal releases into the environment, that pose a significant threat to environment and public health because of their toxicity, accumulation in the food chain and persistence in nature. Chromium (Cr) is of particular concern as it, when accumulated at high levels, can generate serious trouble and diseases and, as concentration reaches a saturation point, it can become lethal. Within the environment, chromium is found primarily in two oxidation states: Cr(VI) and Cr(III). These two oxidation states of chromium are drastically different in charge, physicochemical properties as well as chemical and biochemical reactivity. Cr(III) results from the weathering of minerals and is the most stable state of environmental chromium. Cr(VI) in the environment is man-made, the result of contamination by industrial emissions (Bartlett 1991; Kotas Stasicka 2000), and is more toxic (EPAUS 1984). Examples of Cr(III) compounds include chromium acetate, chromium chloride, chromic oxide, and chromium sulfate; examples of Cr(VI) compounds include ammonium chromate, calcium chromate, potassium chromate, potassium dichromate, and sodium chromate. Although chromium contamination can originate from natural sources (e.g., in situ weathering of rock minerals can lead to metal contamination in soils), it mainly comes from several industrial and agricultural activities such as ore refining, electroplating, tanning, phosphate fertilizers and waste disposal on land (Shanker et al. 2005). Chromium(VI) is used on a large scale in many different industries, including metallurgical, electroplating, production of paints and pigments, tanning, wood preservation, chromium chemicals production, and pulp and paper production. Often wastes from such industries

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(e.g., sludge, fly ash, slag, etc.) are used as a fill material at numerous locations to reclaim marshlands, for tank dikes, and for backfill at sites following demolition (Salunkhe 1998). Cr(VI) from the soils reaches into the groundwater. The tanning industry is especially a large contributor of chromium pollution to water resources; Chandra et al. (1997) estimated that in India alone about 2,000–3,200 tonnes of elemental chromium escapes into the environment annually from the tanning industries, with a chromium concentration ranging between 2,000 and 5,000 mg L^{-1} in the effluents compared to the recommended permissible limit of 2 mg L^{-1}. Cr(VI) exerts toxic effects on biological systems. It was found that occupational exposure to hexavalent chromium compounds leads to a variety of clinical problems. Inhalation and retention of Cr(VI)-containing materials can cause perforation of the nasal septum, asthma, bronchitis, pneumonitis, inflammation of the larynx and liver and increased incidence of bronchogenic carcinoma. Skin contact of Cr(VI) compounds can induce skin allergies, dermatitis, dermal necrosis and dermal corrosion (Lee et al. 1989). The toxic properties of chromates arise from the possibility of free diffusion across cell membranes and strong oxidative potential. The toxicological impact of Cr(VI) originates from the action of this form itself as an oxidizing agent, as well as from the formation of free radicals during the reduction of Cr(VI) to Cr(III) occurring inside the cell.

Chromium enters the food chain through consumption of plant material. A high concentration of chromium in plants has been found to be harmful to vegetation. Both metal forms cause serious damage to plant tissues and organs and adversely affect several biological parameters, albeit at different concentrations (Fig. 14.1). In this chapter, concept and mechanism of phytotoxicity and the role of metal-binding proteins, phytochelatins, metallothioneins along with other defense strategies that help in imparting tolerance to plants to withstand chromium stress, has been presented.

2 Uptake and Accumulation of chromium in Plants

Environmental risk associated with increasing chromium concentration into the environment can be accessed through the study of uptake and accumulation of chromium in plants. Interaction of chromium with plants begins with its uptake process. The uptake of chromium, especially in + VI oxidation state, is active and is a metabolically driven process (Aldrich et al. 2003; Diwan et al. 2008). As chromium is a nonessential element to plants, they do not possess specific mechanisms for its uptake. Its uptake is mediated through carriers used for the uptake of essential metals for plant metabolism. In barley plants, chromate influx shows Michaelis-Menten kinetics at an external concentration ranging between 0.52–8.32 μg ml^{-1}, and it is competitively inhibited by sulphate (Shewry and Peterson 1974; Chatterjee and Chatterjee 2000; Cervantes et al. 2001). Smith et al. (1989) reported that Cr(VI) uptake is a metabolically-mediated process via the sulphate pathway. This suggests that chromate enters root cells using the same transport system as sulphate. As both sulphate and chromate appear to be transported by the same transport system (Skeffington et al. 1976), the higher initial uptake of chromate in sulphate-deprived pre-

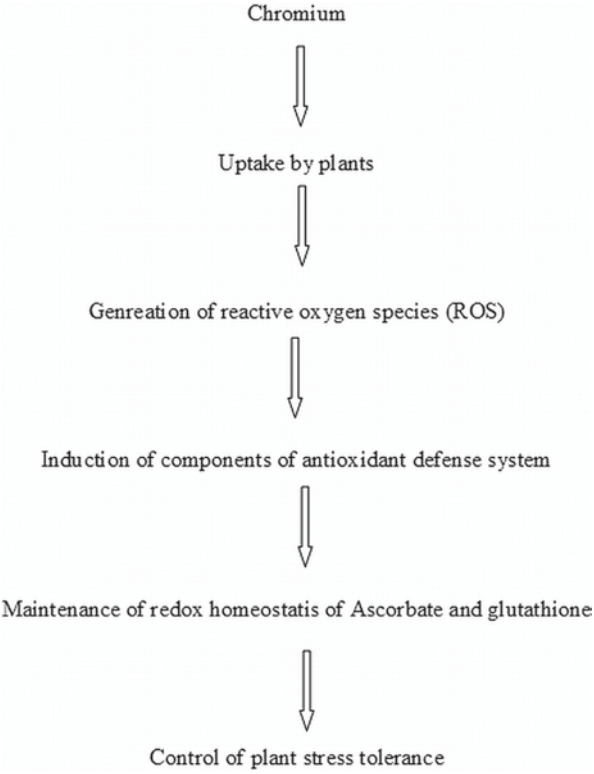

Fig. 14.1 Schematic summary of uptake of major heavy metals by plants, generation of reactive oxygen species, induction of major components of plant antioxidant defense system for the maintenance of ascorbate-glutathione-homoeostasis and the control of plant stress tolerance. *AsA* ascorbate, *GSH* reduced glutathione, *ROS* reactive oxygen

treated plants may be the result of the de-repression of that system. The fact that both anions are transported by the same system may, also explain the increase of chromate uptake by the lack of competition when sulphate is not present (Kleiman and Cogliatti 1997). Iron (Fe), sulphur (S) and phosphorous (P) are also known to compete with chromium for carrier binding (Wallace et al. 1976). Independent uptake mechanisms for Cr(VI) and Cr(III) have been reported in barley. The use of metabolic inhibitors diminished Cr(VI) uptake whereas it did not affect Cr(III) uptake, indicating that Cr(VI) uptake depends on metabolic energy and Cr(III) does not (Skeffington et al. 1976). In contrast, an active uptake of both chromium species, slightly higher for Cr(III) than for Cr(VI), was found in the same crop (Ramachandran et al. 1980).

Uptake of chromium from soil to plant is also a function of soil type and retention time (Stewart et al. 2003). Iron oxides are known to have a high affinity for Cr(III), with increase in pH favouring the sorption. The sorption edge for Cr(III) is highly proportional to the hydrolyzable constant with specific pH. Cr(III) is said to be the most available form in the soil under reducing conditions (Dzomback and Morel 1990). In contrast, the Cr(VI) is very unstable and is easily mobilized in acidic as well as alkaline medium (Banks et al. 2006). Cr(VI) solubility is related to the formation of oxides and hydroxides, chemical secretion in the rhizosphere and immobilization (Ball and Nordstrom 1998). Oxides of aluminum or kaolinite

have been seen to adsorb chromium successfully; the process being pH-dependent (Turan et al. 2007). The Cr(VI) is effectively reduced in acidic soils because acidic conditions enhance the rate of release of iron species from soil minerals for reaction with agricultural species (Fendrof et al. 2004). Early research established that calcium (Ca) stimulates the uptake of sulphate, orthophosphate and rubidium (Leggett and Epstein 1956; Rains et al. 1964). On the other hand, chromium inhibits calcium uptake by plants. Terry (1981) observed that at toxic concentrations of Cr(VI) (>2 mg kg^{-1} Cr), sugar beet plants absorbed very little calcium and were calcium-deficient.

The uptake of chromium results into its accumulation in plant parts, especially in roots (Cary 1982; WHO 1988; Zayed et al. 1998). Among the aerial parts, leaves usually contain more chromium than other parts like seeds. High chromium accumulation in roots might be because of immobilization of chromium in the vacuoles of the root cells. Translocation of chromium in the plant is governed by its translocation potential, which is dependent on chromium forms, underlying chemistry in the plant and chromium complexation with some ligands. The uptake of Cr(III) is higher than that of Cr(VI) (Mishra et al. 1995). This could be due to passive transport of Cr(III) in the plant, dissipating no metabolic energy in this process, and also because Cr(III) has a role of in amino acid and nucleic acid metabolisms (Richard and Bourg 1991). However, restriction in chromium translocation irrespective of the chromium forms, despite the differential accumulation in roots and shoot can be attributed to the non-essential behaviour of the metal with no key role in the plant metabolism. However, in Salsola kali, Cr(VI) was found to move from roots to the aerial parts more easily than Cr(III); the anionic form of Cr(VI) possibly moves fast, whereas Cr(III) can interact with the cell wall easily [14]. Also, Cr(VI) is taken up by plants actively and thus forms a metabolically driven process, whereas Cr(III) is taken up passively and is retained by the cation exchange sites of the cell wall (Marchner 1995; Gardea-Torresday et al. 2005).

Chromium levels in plants growing in 'normal' soils are usually less than 1 mg g^{-1} Cr (DW), rarely exceed 5 mg kg^{-1}, and typically in the order of 0.02–0.2 mg kg^{-1} DW (Kabata-Pendias and Pendias 1992). The lowest chromium concentration in above ground plant tissues is always observed in the fruit, with increases in the stem and the highest in the leaf. Leaves usually contain higher concentrations of chromium than grains (Cary and Kubota 1990). In general, chromium concentrations in shoots of various plants are considered very low and may not meet the nutritional requirements for human diet. This is largely because chromium is a relatively immobile element in both soils and plants and it would appear that this is due to the prevalence of the more insoluble Cr(III) form. Some plant species (especially those growing on serpentine soils), however, can accumulate relatively large amounts of the element in their shoots. These are termed 'Cr accumulators'. Leaf contents in certain accumulator plants, namely *Leptospermum scoparium,* were reported to be as high as 20,000 mg kg^{-1} (Lyon et al. 1969). Peterson (1975) measured in mg kg^{-1} (DW): 48,000 for *Sutera fodina*; 30,000 for *Dicoma niccolifera*; and 2,470 for *Leptospermum scoparium*. In general, plant to soil concentration ratios vary widely with some very low values such as 0.01 (Adriano et al. 1986).

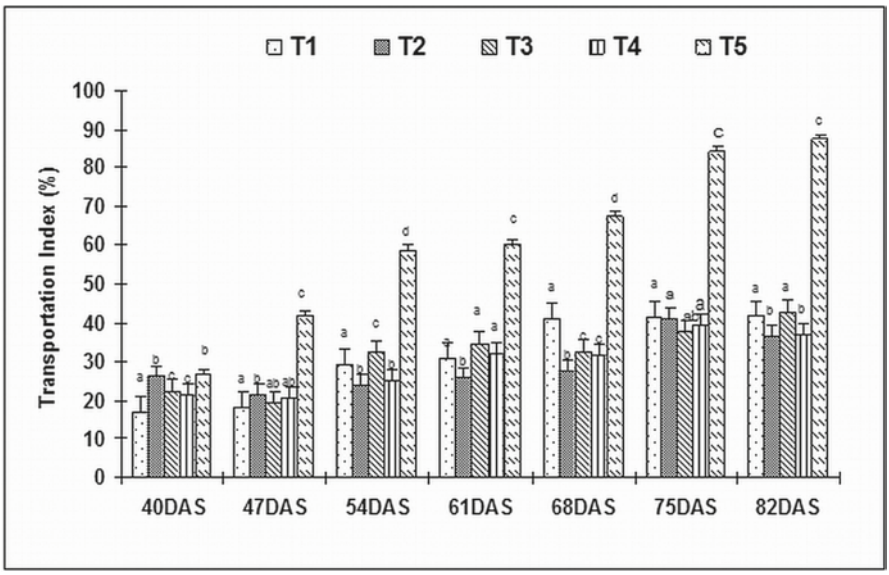

Fig. 14.2 Transportation index of field-grown Indian mustard as influenced by duration and doses of chromium exposure. T0, T1, T2, T3, T4 and T5 are the treatments with 0, 100, 200, 300, 400 and 800 mg Cr kg^{-1} soil, respectively. *DAS* days after sowing. Results are presented as means ± standard error (n = 3). Values followed by same letters are not significantly different at $p < 0.05$

3 Translocation of Chromium in Plants

Once chromium is absorbed by roots from nutrient solution, it is poorly translocated elsewhere and largely retained in the roots itself (Zayed et al. 1998). The transportation index is used to work out the ability of plants to translocate heavy metal from the root to the aerial harvestable plant parts. It varied with duration and doses of chromium exposure (Fig. 14.2). Transportation index of Indian mustard has been worked out at various levels of chromium application. Shoot concentrations of chromium barely exceeded one-hundredth of those in roots, regardless of the chromium species supplied (Parr and Taylor 1980). The restriction in the translocation of both chromium forms in plants to the same degree, despite the differential accumulation in roots and shoots, suggests that conversion of Cr(VI) to Cr(III) is almost certain to occur in roots. Since the predominant species of chromium in roots is Cr(III), very little translocation of chromium to the shoot is expected to occur when plants are supplied with either forms of chromium. Supporting evidence for this hypothesis comes from chromium uptake studies when chromium was supplied in chelated forms. A marked enhancement in the translocation of chromium to plant tops was observed when chromium-EDTA, which is not retained by ion exchange, was supplied as compared to the ionic forms of chromium (Athalye et al. 1995). In addition, Skeffington et al. (1976) illustrated that Cr(III) and Cr(VI) enter the vascular tissue with difficulty; however, once in the xylem, chromium moves more readily. Even

though the tendency to retain chromium in the roots seems to be common to all plant species studied thus far by various researchers, there are quantitative differences among plant species in this regard. Leafy vegetables that tend to accumulate iron (e.g., spinach, turnip leaves) appeared to be the most effective in translocating chromium to the plant top. The leafy vegetables that do not accumulate relatively high concentrations of iron in their leaves (e.g., lettuce, cabbage) are substantially less effective in translocating chromium to their leaves (Zayed et al. 1998; Cary et al. 1977).

4 Chromium Toxicity to Plants

Once entered into the plant, chromium leads to changes in the growth and development pattern of the plant. The level at which chromium is phytotoxic depends on several factors, including experimental conditions, plant species, soil characteristics, and chromium species. Chromium phytotoxicity occurs at lower concentrations when chromium is supplied in hydroponic culture relative to chromium supplied in soil. For example, Turner and Rust (1971) observed that the initial symptoms of chromium toxicity on plants occurred with the addition of as little as 0.5 mg kg^{-1} chromium to the nutrient culture and as much as 60 mg kg^{-1} to the soil culture. There is higher toxicity of chromium in solution culture because the chromium supply is soluble and is thus available for plant uptake, whereas in soil major portions of chromium become unavailable due to adsorption, reduction, and precipitation processes. Similarly, chromium is more toxic to plants growing on sandy soils compared with peat soils due to the lower reducing and adsorbing capacity of sandy soils (Mishra et al. 1995). Visual symptoms of chromium toxicity in plants are stunted growth, a poorly-developed root system, curled and discolored leaves (Pratt 1966), leaf chlorosis, narrow leaves (Hunter and Vergnano 1953), chlorotic bands on cereals (Kabata-Pendias and Pendias 1992), yield reduction (Parr and Taylor 1980), and some plants may exhibit brownish-red leaves containing small necrotic areas or purpling of basal tissues (Adriano 1986; Hunter and Vergnano 1953). Immediate wilting and plant death has also been reported as a result of exposure to very high levels of chromium (Parr and Taylor 1980) (Fig. 14.3).

4.1 Cr(VI) is More Toxic than Cr(III)

Chromium toxicity in plants is highly dependent on the chemical species of the element. Cr(VI) has been reported to be more toxic to plants than Cr(III) in barley and rape seed (Hauschild 1993), wheat and buckwheat (Kleiman and Cogliatti 1998). Higher toxicity of Cr(VI) than Cr(III) has been explained by various hypotheses. In one hypothesis, it has been proposed that at natural pH levels, Cr(VI), being water soluble and of smaller size than the hydrated Cr(III) ion, readily penetrates cell

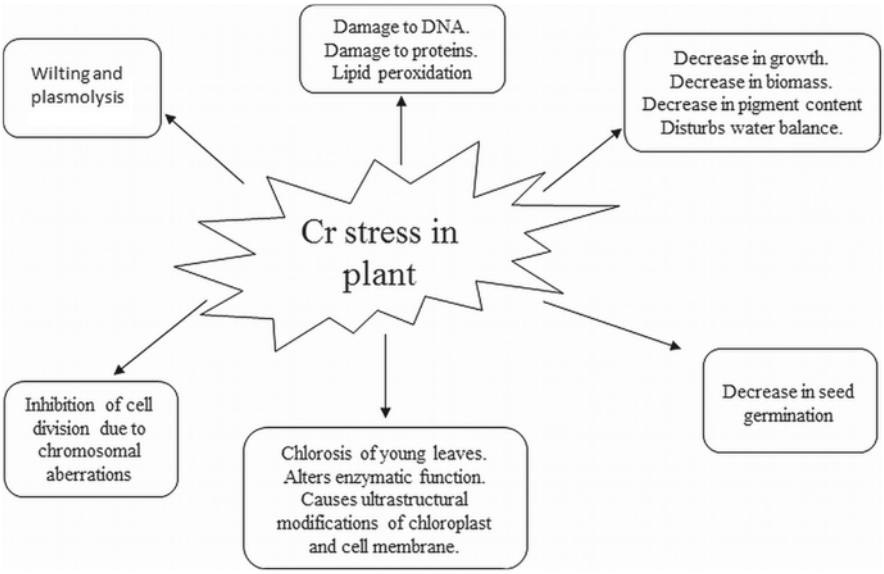

Fig. 14.3 Chromium toxicity to plants

walls and exhibits its toxic behaviour (Mishra et al. 1995). The hydrated Cr(III) cation does not pass through the cell membrane, even at low pH (Cary et al. 1977). The more toxic nature of Cr(VI) may also be explained by its ability, being a strong oxidizer, to cause oxidative damage to the cells. Oxidative damage caused by Cr(VI) to outer root cells of bean plants is evident from electron microscopy studies (Vazquez et al. 1987). This may cause malfunctions in the uptake of mineral nutrients and water leading to mineral nutrition deficiency (particularly chlorosis) of the rest of the plant and eventually death (Vazquez et al. 1987). Hauschild (1993) hypothesized that due to strong oxidative damage of Cr(VI) to root cells, their efficient selective mechanisms for control of inorganic uptake into the root may be destroyed, permitting large amounts of Cr(VI) to enter the root passively. This may explain the higher Cr(VI) uptake compared to Cr(III) and the apparent breakthrough of chromium to the plant tops. If Cr(VI) is transported in the xylem in the same form, then oxidative damage to leaf cells and the consequent chlorotic symptoms are to be expected (Hauschild 1993). Chromium toxicity symptoms have been correlated with elevated levels (10-fold higher than the control) of putrescine in leaves (Hauschild 1993). Putrescine belongs to the group of aliphatic di- and poly-amines that act as antiethylene compounds preventing senescence (Flores et al. 1989). It is well documented that putrescine levels increase in plants in response to various environmental stresses (Hauschild 1993). Based on these facts, Hauschild (1993) proposed a common mechanism of toxicity for the two chromium species as follows: Chromium exposure causes root stress which results in, (a) induction of putrescine and other stress compounds (e.g., chitinases); (b) reduced root growth, leading to reduced shoot growth, water content, and leaf chlorosis (i.e., root-induced iron deficiency);

and (c) increased uptake of Cr(VI) leading to increased chromium levels in leaves, which in turn results in oxidative stress and chlorosis of leaves (i.e., leaf-induced iron deficiency).

4.2 Chromium Toxicity to Growth and Development of Plants

Growth of root and shoot has been traditionally considered as a valuable trait for scoring heavy metal tolerance in plants. For instance, this trait is widely used to select plant varieties for tolerance to aluminum, a metal that strongly limits agricultural production in acidic soils. Root growth arrest caused by chromium can be considered as a toxicity symptom (Diwan et al. 2010a). Roots serve as an interface between the soil and plants. Being in direct contact with heavy metals (HM), roots play a dominant role in preventing plants from adverse HM effects. Chromium in the soil affects root growth adversely, depending on its concentration and the plant species. Root growth was completely inhibited in *Vigna radiata* and *V. sinensis* with chromium treatments ranging from 40 to 160 ppm (Jamal et al. 2006). In *Salvia,* root growth inhibition was evident at 10 mg L^{-1}; not only the main root elongation but the lateral root development was also affected. Speciation of chromium also has a bearing on root growth (Corradi et al. 1993). Reductions in root growth in *Vigna radiata* cv CO4, as determined by dry weight of roots, were more pronounced with Cr(VI) than with Cr(III) (Shanker et al. 2004). The inhibitory effect of chromium in roots could be due to the accumulation of high chromium concentrations in roots, and non-existence of any defined translocation mechanism for chromium thereby enhancing its sequestration in the tissue and thus inhibiting the root development. The other reasons for reduced root growth may include inhibition of cell division, root elongation or cell cycle extension in the roots. The direct contact of roots with metals results in a collapse of roots and their inability to absorb water from the media (Barcelo et al. 1986; Sanita di toppi et al. 2002). Chromium affects shoot development adversely by inhibiting growth of stem axis. Reduced plant height was reported in pea, tomato, cauliflower, maize and green gram plants under chromium stress (Hunter and Vergnano 1953; Shanker et al. 2004). Application of Cr(III) (20 mg L^{-1}) was inhibitive for *Salsola kali* but ineffective for *Oryza sativa* (Mishra et al. 1997). The reduction observed in shoot length and biomass accumulation could be due to impaired root growth leading to lesser uptake of essential nutrients and water and the consequent impact on the cellular metabolism of shoot.

4.3 Chromium Toxicity to Mineral Nutrition of Plants

Chromium, due to its structural similarities with some essential elements, can affect mineral nutrition of plants in a complex way. Fernandes et al. (2002) and Moreira et al. (2005) have reported that the decrease uptake of these elements in

chromium-stressed plants could have been due to the inhibition of the activity of the plasma membrane H^+-ATPase. The decrease in ATPase activity causes a decrease in proton extrusion. This in turn causes a decrease in the transport activities of the root plasma membranes and reduces the uptake of some nutrient elements. Moreover, chromium interfered with the mechanism controlling intracellular pH. This hypothesis was supported by the fact that chromium could be reduced in the cells thereby utilizing the protons (Zaccheo et al. 1982). For the chromium action in the translocation of mineral nutrients, it is known that Phosphorous and chromium are competitive for surface root sites and iron is also known to be in competition with chromium for transport binding, inducing iron deficiency (Chatterjee and Chatterjee 2000). Chromium causes severe decrease in calcium concentration in the leaves even though calcium is necessary for the development of the cell wall and the maintenance of membrane structure (Marschner 1999) of the leaves. Chatterjee and Chatterjee (2000) and Sinha et al. (2006) have reported that *Brassica oleracea* and *Vigna radiata* plants stressed with chromium presented manganese (Mn) deficiencies. However, other studies showed that Cr(VI) supply increased manganese content in *Citrullus vulgaris* (Dube et al. 2003). Chromium by inducing competitive phenomena in root assimilation and translocation in plants with other mineral elements, may therefore indirectly affect water status and plant growth.

Chromium interacts with Nitrogen (N), phosphorous, potassium (K), aluminum, chloride (Cl) and zinc (Zn), thereby influencing the plant metabolism. It simultaneously affects the content of many minerals, thereby leading to severe mineral imbalance in the plant. Inhibition in the uptake of potassium was observed owing to plant treatment with Cr(VI), which was dependent on both the incubation period and the concentration of chromium applied (Zaccheo et al. 1982). Similarly, chromium-induced reductions in the Nitrogen, Magnesium, phosphorous and potassium contents and increases in the aluminum, iron and zinc contents are on record (Gardea-Torresday et al. 2005; Davies et al. 2001; Vernay et al. 2007). Calcium uptake can be restricted by chromium treatment (Gardea-Torresday et al. 2005). Since phosphorus and chromium are competitive inhibitors, they share an antagonistic relationship with each other, resulting in a lower phosphorus level, the effect being more prominent in leaves and roots in comparison to stem. Chromium also enjoys structural similarity with other essential elements like iron and sulphur, thus interfering with the uptake of these elements. The reductions in the nutrient contents like Nitrogen, phosphorous and potassium, could be due to the reduced root growth and impaired absorption of these minerals.

4.4 Chromium Toxicity to Carbon Assimilation of Plants

Carbon assimilation of plant is impaired by chromium toxicity in terms of CO_2 fixation, electron transport, photophosphorylation and enzyme activities (Vernay et al. 2007). In presence of higher chromium concentrations, *L. perenne* plants present a decrease of calcium content in leaves of about 13-fold compared to control plants.

This decrease in calcium content may decline water oxidation at the level of the water splitting system at the oxidising side of photosystem II (PSII) and decrease the electron flow needed for photosynthesis (Barry et al. 2005). Calcium is an essential cofactor for O_2 evolutions and has been shown to play a vital role in stabilizing the protein structure that ligates manganese and plays role in H_2O oxidation (Vander Meulen et al. 2004). It is known that the high depletion of calcium content in PSII reduces proper photochemical function preventing oxygen evolution. PSII is believed to play a key role in the responses of leaf photosynthesis to environmental perturbations. Several heavy metals such as copper, cadmium, zinc, chromium, have been shown to have their primary targets in the PSII complex (Giardi et al. 2001). Cr(VI) is a strong oxidant with a high redox potential of 1.38 eV. This may cause serious oxidative damage to the photosynthetic apparatus reflecting the results obtained in this study with *L. perenne*. In our studies, we have observed that chromium caused a slight decrease in the maximal photochemical efficiency of PSII (Fv/Fm) in *L. perenne* plants at 500 μM chromium. On the contrary, a significant increase in Fo value was observed from a 100 μM chromium stress. The Fo level is affected by environmental stresses that cause structural alterations in the pigment–protein complexes of PSII or when the transfer from antennae to reaction centres is impeded. These changes affect the ability of the photon harvesting assemblages to trap photons and transfer the energy to the acceptor of PSII (Ouzounidou 1993). In contrast to Fo, Fm is achieved when QA or the plastoquinone pool is fully reduced by electrons. The decline in Fm, observed from 250 μM chromium and 45 days of exposure would suggest a change in the ultrastructure of the thylakoid membrane and indicates irreversible or slowly reversible damage to the photosynthesis system which is termed ‘‘photoinhibition’’, and the target is mostly localized in PSII. Furthermore, it has been reported that increasing Fo and decreasing Fm observed in response to chromium stresses, from 250 μM chromium, can be attributed to the separation of LHC II from the PSII complex, inactivation of PSII reaction centre, and perturbation of electron flow within the PQ pool (Yamane et al. 1997). Up to 100 μM chromium-treated plants’ NPQ coefficients values are higher than in control plants without changes in qP values. This relationship indicates that non-radiative dissipative mechanisms are involved in dissipation of the excess excitation energy. There is general agreement that photoinhibition is primarily caused by an inactivation of the electron transport system in thylakoids. The dominant effect seems to be an alteration of the reaction centers of PSII leading to a decreased photochemical efficiency. This photoprotection mechanism is enhanced under stress conditions when an excess of reducing power (NADPH) is generated at the electron transport chain. The significant changes in PSII photochemistry in the light adapted leaves, such as the decreased qP and F_0 v=F_{0m}, as well as the increased of NPQ, can be seen as the regulatory response to down-regulate the quantum yield of PSII electron transport PSII ¼ F_0 v=F_{0m}xqPÞ (Genty et al. 1990), that would match with the high decrease of CO_2 assimilation rate. It is suggested that the decay in PSII of the chromium-stressed plants may be a mechanism to down-regulate the photosynthetic electron transport so that production of ATP and NADPH would be in equilibrium with the decreased CO_2 assimilation capacity in stressed plants. The

fraction of PSII reaction centers that is opened, as indicated by the decrease of qP, is reduced in plants cultivated with nutrient solutions added with 250 μM chromium. From 170 μg g^{-1} DW, the chromium content in leaves of *L. perenne* induced a small decrease in Fv/Fm, and a decrease of qP, indicating that chromium decreased the capacity of reoxidizing QA during actinic illumination, and increased the capacity pressure on PSII. This suggests a decrease in the fraction of open PSII becoming unable to carry out stable charge separation (Krause and Weis 1991). The decrease in qP from 250 μM chromium in the treated plants indicates that the down-regulation of PSII electron transport by increased NPQ was not sufficient to match the decreased demand for electron through ATP and NADPH consumption. Altered PSII centers still act as efficient excitation energy traps, but convert the trapped energy to heat (Genty et al. 1989). Altered PSII centers can be detected by an increase in Fo, as recorded in chromium-treated plants. The increase in NPQ of chromium-treated plants may indicate a control mechanism in the thylakoid membrane that adjusts thermal dissipation of excitation energy in excess of that required for carbon metabolism. It has been shown in many studies that an increase in the thermal dissipation in the PSII antennae competes with the excitation energy transfer from the PSII antennae to PSII reaction centers, thus resulting in a decrease in the efficiency of excitation energy captured by open PSII reaction centers ðF 0 v=F 0 mÞ (Demmig-Adams et al. 1996). Bishnoi et al. (1993) observed that the effect of chromium is rather more important on the PSI than on the PSII activity in isolated chloroplasts of pea. An inhibition of the electron transport processes and a shunt of electrons from the electron-donating side of PSI to Cr(VI) is a potent explanation for the chromium-induced decrease of photosynthetic rate. The electrons produced by the photochemical process were not necessarily used for carbon fixation as evidenced by the low photosynthetic rate of the chromium-stressed plants. Due to the known oxidative potential of Cr(VI), it is possible that alternative sinks for electrons could have been enhanced by reduction of molecular oxygen (part of Mehler reaction) which in part explains the oxidative stress brought about by Cr(VI) (Shanker et al. 2005). The overall effect of chromium ions on photosynthesis and excitation energy transfer could also be due to Cr(VI)-induced abnormalities in the chloroplast ultrastructure like a slight lamellar system with widely spaced thylakoid and a few grana (Rocchetta et al. 2006). Chromium is known to inhibit photosynthesis and the photosystem II (PSII) is known as the main target for this negative action (Davies et al. 2002). However, the relationship between chromium and the primary reactions of photosynthesis is not well described.

4.5 Chromium Toxicity to Chlorophyll Biosynthesis of Plants

Decreases in total chlorophyll content have been well documented under chromium stress (Panda and Choudhury 2005). This decrease suggests that the chlorophyll synthesizing system and chlorophyllase activity are affected by the exposure to high chromium concentrations (Van Assche and Clijsters 1990). Iron depletion or sub-

stitution of magnesium by chromium may be a contributing damage mechanism. Chromium possesses the capacity to degrade d-aminolevulinic acid dehydratase, an important enzyme involved in chlorophyll biosynthesis, thereby affecting the d–aminolevulinic acid (ALA) utilization resulting in the buildup of ALA and finally reducing the level of chlorophyll (Vajpayee et al. 2001). Chromium, mostly in its hexavalent form, can replace magnesium ions from the active sites of many enzymes. d-aminolevulinic acid dehydratase is a metalloenzyme and its activity in plants is dependent on the availability of magnesium (Ilag et al. 1994). Moreover, Cr(VI) also causes a iron deficiency in stressed plants of *L. perenne*, leading to an interruption in chlorophyll biosynthesis at the level of the oxidation step from coporphyrinogen (Barcelo et al. 1986). In this study, the chlorophyll a/b ratio decreased when the plants were exposed to an excess of chromium, which may have been due to a faster breakdown or decreased synthesis of chlorophyll a compared to chlorophyll b, although chlorophyll b also decreased. These effects were reported by many researchers (Vajpayee et al. 2001; Appenroth et al. 2003). The decrease in the chlorophyll a/b ratio by chromium could induce a reduction in the size of the peripheral part of the antenna complex (Shanker et al. 2005). This decrease in the chlorophyll b could be due to the destabilization and degradation of the protein of the peripheral part. The observed reduction of photosynthetic pigment concentrations in chromium-stressed *L. perenne* and *Salvinia* plants involves a reduction in the light harvesting capacity of the plants as observed in plants treated with Cr(VI) at wavelengths ranging between 400–450 and 650–700 nm (Nichols et al. 2000). Synthesis of d-ALA is the first identified step in tetrapyrrole biosynthesis, leading to the formation of heme, chlorophyll, billins, vitamin b and specialized products (Garnick and Sassa 1971). ALA synthetic ability plays a key role in the regulation of chlorophyll biosynthesis in higher plants (Beale 1978). However, Naito et al. (1980) suggested that chlorophyll biosynthesis might be regulated not only by ALA synthetic ability but also by d-aminolevulinic acid dehydratase (ALAD) activity. Altered ALAD activity concomitant with reduced chlorophyll content has been reported in many terrestrial plants exposed to various concentrations of lead, cadmium and mercury (Prasad and Prasad 1987).

4.6 Chromium Toxicity to Nitrogen Assimilationin Plants

Nitrogen is not only a constituent of protein but also a core element of a number of biomolecules such as nucleic acids, purines, pyrimidines, porphyrins, coenzymes and other derivatives. Nitrate reductase (NR), an important enzyme of nitrate assimilation pathway, is a rate-limiting enzyme as it catalyzes the first step of nitrate assimilation (Kandlbinder et al. 2000). So, NR activity can be seen as a stress index for plants grown in soils exposed to abiotic stress (Caravaca et al. 2003; Caravaca et al. 2005). A decrease in NR activity is witnessed with exposure of plants to chromium. Inhibition of NR activity has been observed in *Polytricum commune* at chromium concentration of 1, 10, and 100 mM after 24 h; the activity dropped by about

17, 31, and 45 % respectively, as compared with the control (Panda and Choudhury 2005). In *Nymphea alba,* decline of NR activity was positively correlated with chromium concentration in the medium and with the exposure duration. The maximum inhibition was recorded at 200 μM chromium treatment. The NR activity possibly declines owing to the ability of chromium to affect the SH group of the NR enzyme (Panda and Choudhury 2005). NR activity also depends on active photosynthesis or the production of photosynthate and requires the photosynthetically generated reductant (NADP) and energy (Vijayraghavan et al. 1982; Raghuram and Sopory 1995; Ahmad and Abdin 1999). Reduced protein content under the influence of chromium treatments may be correlated with the decline in NR activity (Panda and Choudhury 2005; Vajpayee et al. 2001; Rai et al. 1992).

4.7 Mechanism of Chromium Toxicity to Plants

Chromium exerts its toxic effects on plants in a variety of ways. Cr(VI) is able to pass the membrane, penetrate the cytoplasm and react with the intracellular material (Gikas and Romanos 2006). Negatively charged hexavalent chromium ion complexes can easily cross cellular membranes by means of sulfate ionic channels, and then undergo immediate reduction reactions leading to the formation of various reactive intermediates. These intermediates are themselves harmful to cell organelles, proteins and nucleic acids (Kaszycki et al. 2005). As the chromium concentration in plants increases, it adversely affects several biological parameters. Consequently, there is loss of vegetation (Dube et al. 2003). Due to high oxidation power of chromium, membrane damage has been observed. Moreover, changes in the redox status of the plant may also trigger chromium action. The redox imbalance may be caused by the depletion of oxygen by root and microbial respiration in the water-saturated soils (Cohen et al. 1998). This, in turn, increases the overall accessibility of the contaminant to the plant.

One of the common responses to a wide range of abiotic stresses is the generation of reactive oxygen species (ROS). These ROS are produced in cells as an intermediate product during the reduction of O_2 to H_2O. Chromium is a toxic heavy metal that can generate ROS like H_2O_2, O_2^-, OH^- which cause oxidative damage to plants (Diwan et al. 2010a; Panda and Patra 2000; Dixit et al. 2002; Panda and Khan 2003; Choudhury and Panda 2005; Diwan et al. 2010b). Its presence causes oxidative damage to the biomolecules such as lipids, proteins and nucleic acids (Kanazawa et al. 2000). In the plants, metal-induced lipid peroxidation has been reported (De Vos et al. 1991), which profoundly alters the structure of membranes and consequently modifies their enzymatic and transport activities. Lipid peroxidation is considered to be an indication of oxidative damage by which the integrity and functionality of the membrane is lost. Malondialdehyde (MDA) is the cytotoxic product of lipid peroxidation and an indicator of free radical production and consequent tissue damage (Ohkawa et al. 1979). The enhanced production of O_2– anions and hydroxyl (·OH) radicals has been demonstrated to be a cause of chromium-

induced oxidative stress, DNA damage, and apoptotic cell death in cultured human chronic myelogenous leukaemic K562 cells and promyelocytic leukaemic HL-60 cells (Bagchi and Bagchi 2000). Chromium reactivity is apparent from its interaction in cell-free systems with glutathione, NADH and hydrogen peroxide to form hydroxyl radicals (Aiyar et al. 1991). The participation of chromium in redox reactions is, however, not clearly understood (Strlic et al. 2003), but one study has demonstrated its participation in redox or Fenton reactions just as copper or iron (Shi and Dalal 1989). Production of H_2O_2, OH^- and O_2^- under chromium stress has been demonstrated in many plants, generating oxidative stress leading to damage of DNA, proteins and pigments as well as initiating lipid peroxidation (Panda and Patra 2000; Choudhury and Panda 2005).

5 Tolerance Mechanism of Plant against Chromium

Plants are known to generate a host of defense responses to cope with the oxidative stress and develop tolerance to metal exposure. Tolerance can be defined as the ability of plants to cope with stresses which they are exposed to and which are lethal or inhibitory to non-tolerant individuals. Plants have several lines of defense that help in protecting them from metal toxicity.

5.1 Induction of Metal Homeostasis Network System

The first protective barrier against heavy metalis of a highly non-specific nature. The cation exchange capacity (CEC) of the cell walls and the presence of root exudates and root-tip mucilage enable the roots to moderate the heavy metal concentration reaching the cytoplasm (Banks et al. 2006). However, filtering capacity in plants against chromium is extremely limited and does not prevent chromium from reaching the interior of the cell, satisfactorily. A complex metal homeostasis network system has evolved in plants to regulate heavy metal uptake and distribution, thus protecting the metabolic process. Several strategies/mechanisms have been proposed to describe how plants tolerate heavy metals in the soil/growing media environment. These include exclusion, restricting heavy metal uptake, sequestrating and compartmentalizing metal in organs and organelles. The root exudates are very important agents that form complexes with trace metals and affect their redox behaviour (Caltado et al. 1988). Root exudates containing organic acids can form complexes with chromium compounds, making them available for plant uptake (Bartlett and James 1988). Studies on the role of organic acids in chromium toxicity in *Lycopersicon esculentum* showed that in the presence of organic acids like carboxylic acid and amino acids, chromium uptake in roots is enhanced (Srivastava et al. 1999a). However, of these types of organic acids, amino acids have been found to be less effective in mobilizing chromium (Srivastava et al. 1999b).

Organic acids like citric acid, aspartic acid and oxalic acid can convert inorganic chromium to organically bound chromium, making it soluble for a longer period of time and thereby available to plants (James and Bartlett 1983). Whether organic acids can play significant role in chromium detoxification is still not completely understood. It is interesting to note that recent studies have shown that soluble metal ions can activate preexisting signaling pathways in the cell that can cause the cell to respond to what it thinks are physiological signals (Ye and Shi 2001). In many cases with a toxic metal ion, these signals are not physiological since they may be multi-component. Hexavalent chromium has been shown to also affect cell signaling by activating NFB, API, HIF-1, and VEGF (Ding and Shi 2002). However, it is not clear whether these effects occur without its entry into cells.

5.2 Induction of Chelating and Sequestering Agents

Chelation and sequestration of metal ions is another defense line operative within plants. This is done by a particular class of metal binding ligands denominated metallothioneins (MTs) and phytochelatins (PCs) (Cobbett 2000; Cobbett and Goldsbrough 2002; Schmidt 2003; Quartacci et al. 2005; Quartacci et al. 2006; Diwan et al. 2010c). The role of MTs and PCs in chromium detoxifictation in plants has not been studied as thoroughly as for other heavy metals. However, it has been reported that the production of ROS as a result of chromium exposure triggers signals to induce mRNA transcription (Shanker et al. 2004). Thus, MTs may have a role in chromium detoxification in plants possibly through binding to chromium ions and making them non-toxic. MTs are cysteine-rich polypeptides encoded by a family of genes. MTs have a possible role in chromium detoxification in plants and it has been reported for sorghum that MT-like proteins are expressed under chromium stress (Shanker et al. 2004). These are products of mRNA translation and are characterized as cysteine-rich metal-binding proteins (Kagi 1991). A study of Cr(VI) effects on the *MT3* gene expression using chromium tolerant and susceptible varieties revealed a high intensity band matching the gene of interest in the tolerant variety compared to the susceptible one (Shanker et al. 2004). This suggests that under chromium stress, there could be high transcription rates of MTs, particularly in the tolerant variety (Shanker et al. 2004). In a study on maize, an induction of MTs synthesis was detected starting from 2 ppm potassium dichromate. However, a consistent induction was observed starting from 100 ppm chromium. At the concentration of 300 ppm, the MTs content was seen to get doubled the value observed at 100 ppm. The highest concentration of MTs was obtained at 600 ppm of potassium dichromate. It is suggested that the production of ROS and H_2O_2 as a result of chromium exposure triggers signals to induce MT mRNA transcription (Shanker et al. 2004). Thus, MTs may have a very important role in chromium detoxification in plants, possibly by binding chromium ions and making them non-toxic. However, the role of MTs in chromium detoxification in plants is not well understood nor thoroughly studied, so their role in this respect still remains a challenge for the future.

Like MTs, PCs are also involved in chelating activity. They are a family of enzymatically synthesized cysteine-rich peptides. PCs are small metal binding peptides with the structure (γ-Glu-Cys)n-Gly, where value of 'n' varies from 2 to 11, induced by various metals. PCs are synthesized from GSH by the action of enzyme γ-glutamylcysteine dipeptidyl transpeptidase, trivially named as phytochelatin synthase. The enzyme is constitutively expressed but may be regulated at transcriptional and translational levels by metals and metalloids. Nutrient culture studies on *P. vulgaris* have depicted a marked enhancement in uptake, and translocation of chelated ^{51}Cr in chromium chelated by DTPA was most effectively translocated followed by ^{51}Cr-EDTA and ^{51}Cr-EDDHA (Athalye et al. 1995). Similarly, significant increases in chromium accumulation from Cr(III)-treated maize plants in the presence of increasing concentrations of organic acid have been observed (Srivastava et al. 1999a). Shahandeh and Hossner (2000) have reported a high increase in chromium uptake aided by organic acids. Srivastava et al. (1999b) found that increasing concentrations of organic acids resulted in increased uptake of chromium without affecting the distribution in plant parts. Induction of PCs against accumulated chromium has been reported in root and leaves of *B. juncea* and *Vigna radiata* (Diwan et al. 2010c). The induction of PCs in leaves was lesser than those observed in roots. This may be due to binding of metal to other ligands (Salt et al. 1995) or to cell wall (Vecchia et al. 2005) or due to binding with GSH. In *V. radiata*, there was lesser induction of PCs at 200 μM chromium than at 50 and 100 μM chromium treatments. The decrease in the PCs at higher concentration may be due to its transport to shoot or due to the fact that PCs might have got degraded due to excessive chromium accumulation (Diwan et al. 2010c). 2DE analysis performed on controls (water) and samples exposed to 100 and 300 ppm chromium showed a consistent growth inhibition (30 % and 50 % of IG values at 100 and 300 ppm of potassium dichromate, respectively) and metabolic changes (metallothionein synthesis and chromium uptake) were observed. A total of 22 proteins showed significant and reproducible up-regulation in the 100 as well as 300 ppm potassium dichromate treated samples (Labra et al. 2006).

5.3 Induction of Antioxidant Defense System

Chromium-induced production of ROS in plants is regulated by ROS-scavenging system of the cell (Fig. 14.4), based on the ascorbate cycle and the scavenging enzymes, comprising enzymes like superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), glutathione reductase (GR), and small antioxidant molecules like ascorbic acid, cysteine, glutathione, tocopherol, caretenoids, hydroquinones and polyamine. The enzymatic parameters, namely SOD and CAT are involved in the transformation of superoxides and peroxides to non-toxic species (Caravaca et al. 2005). Other enzymes, namely, ascorbate peroxidase and glutathione reductase are involved in the ascorbate glutathione pathway. The reaction of ascorbic acid with H_2O_2 can occur directly, or it can be catalysed by APX, oxidizing

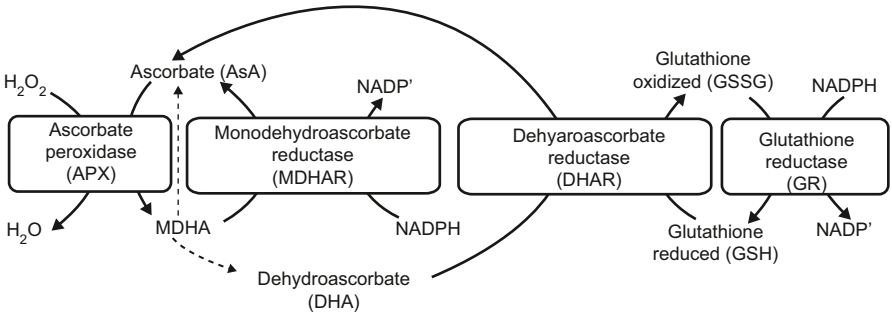

Fig. 14.4 The ascorbate-glutathione (Asc-GSH) cycle or Asada-Halliwell pathway. H_2O_2 is removed by APX and Asc is regenerated by the Asc-GSH cycle, involving MDHAR, DHAR and GR. Asc is first oxidized to MDHA. If MDHA is not rapidly reduced again to Asc by MDHAR, it will spontaneously disintegrate into Asc and DHA. DHAR recycles Asc from DHA using GSH that is regenerated through the action of GR in a NADPH-dependent reaction. The dotted line indicates non-enzymatic reactions

the ascorbic acid (AA) to monodehydroascorbate (MDHA), which is then reduced by NADPH in a reaction catalysed by monodehydroascorbate reductase (MDHAR). MDHA also undergoes spontaneous disproportionation reaction to AA and dehydroascorbate (DHA). The latter is reduced to AA by GSH in a reaction catalyzed by dehydroascorbate reductase (DHAR), producing glutathione disulphide (GSSG), which in turn is reduced by glutathione reductase (GR) to GSH in the presence of NADPH (Clijsters et al. 1999). Inadequate regulation of ROS generation potentially leads to oxidative damage. The first line of defense against ROS-mediated toxicity is achieved by SOD that catalyzes the dismutation of superoxide radicals to H_2O_2 and O_2. Enhanced SOD activity in both the plants suggested that chromium caused oxidative stress (Diwan et al. 2008; Diwan et al. 2010a; Jabeen et al. 2010). SOD, the first enzyme in detoxifying process, converts O_2^- radicals to H_2O_2. Chromium mediated enhancement in activity of SOD has been observed in several studies, which may be due to either direct effect of this metal on the SOD gene or to an indirect effect mediated via an increase in the level of O_2^- radicals (Chongpraditnum et al. 1992). Enhanced SOD activity has been associated with stress tolerance in plants because it neutralizes the reactivity of O_2^-, which is overproduced under oxidative stress. It has been well documented that SOD activity has a protective role in heavy metal plants. At higher levels of chromium, however, the increase in SOD activity was not comparable to that evident at lower chromium levels as there might be inactivation of the enzyme by H_2O_2 (Yamaguchi et al. 1995). On the other hand, the up-regulation in the SOD activity in chromium-sensitive plants in response to chromium stress was not strong enough to detoxify the superoxide radicals completely, thus reflecting lesser tolerance towards chromium stress, which also indicated that O_2 scavenging function of SOD was impaired with duration and levels of chromium treatments (Diwan et al. 2010a; Labra et al. 2006; Zhang et al. 2005; Zhang et al. 2007). Peroxidases are known to play a significant role under oxidative stress conditions and it has been shown that peroxidase activity can be

used as a potential biomarker for sub-lethal metal toxicity in examined plant species (Radotic et al. 2000). In plants, the detoxification of H_2O_2 has been known to be an important function of the peroxidases that use ascorbate as the hydrogen donor (Hegedus et al. 2001). The induction of APX activity in plants has also been reported under chromium stress (Diwan et al. 2010a; Hegedus et al. 2001). Among H_2O_2 destroying enzymes, APX activity was found to increase in roots and leaves of the chromium treated plants (Shah et al. 2001). The increase in the activity of APX levels could be the consequence of either the microenvironment or the tissue specific gene expression in the treated plants (Hegedus et al. 2001). Increase in APX activity suggested a role of APX in the detoxification of H_2O_2 and its up-regulation under chromium-induced oxidative stress as established earlier with reference to many other heavy metals (Diwan et al. 2008; Qureshi et al. 2005; Israr et al. 2006; Khan et al. 2009). Importance of APX as a limiting factor of defense against photo-oxidative stress has been confirmed in transgenic tobacco plants (Rizhsky et al. 2002; Yabuta et al. 2002).

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Chapter 15
Boron Toxicity and Tolerance in Crop Plants

Robert J. Reid

1 Introduction

Boron (B) is an essential plant nutrient required in relatively small quantities for normal plant growth. In many respects, boron is the most difficult of plant nutrients to manage, principally due to the fact that it is the only essential nutrient that normally exists as a neutral solute, boric acid. Combined with its small size, this lack of charge allows it to pass easily through membranes and as a result, its distribution within the plant can be hard to control. Both boron deficiency and boron toxicity are common in agricultural crops. The deficiency can be easily corrected by its fertilisation, but toxicity is much more difficult to manage. This review examines the role of boron in plants, possible targets for toxicity, and the mechanisms by which some plants have developed tolerance to excess boron.

2 Functions of Boron in Plants

The essentiality of boron for plant growth was established by Warington in 1923, but it took many decades to understand which processes depended on boron. The first clue came from the discovery that boron was strongly bound in plant cell walls (Tanaka 1967) and that the boron content was well correlated with the amount of pectic polysaccharide in cell walls (Matoh 1997, 2000). From these latter studies, it is now well established that boron plays an important structural role as a component of the rhamnogalacturonan II (RGII) complex that links cell wall polysaccharides.

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Other potential roles for this plant nutrient have been suggested based on the binding to cellular components of boronic acids which compete with boric acid for binding. Bassil et al. (2004) found that boronic acids interfered with cytoskeletal elements and disrupted cell to cell wall adhesion in cultured cells. Wimmer et al. (2009) identified various boron-binding proteins from microsomes by boronate affinity chromatography and proposed that boron interacts with the sugar moiety of membrane glycolipids, thereby increasing membrane stability (Wimmer et al. 2009).

3 Boron in Soils

Naturally, boron-toxic soils appear in pockets around the world and are much less common than boron-deficient soils. Low boron soils are loosely considered as those with less than about 10 mg kg^{-1} and high boron soils are those with more than this value up to very high levels of 100 mg kg^{-1} (Power and Woods 1997). However, prediction of toxicity to plants based on soil boron levels can be problematic. Mertens et al. (2011) found that the boron concentration required to inhibit root growth of barley by 10 % varied from 5–52 mg B kg^{-1} depending on the soil type. A large proportion of this variability could be explained by differences in soil moisture content.

The boron concentration of soils is mainly known for areas in which agriculture is practiced, because of the effects on crop yields. Regions with high soil boron include Israel (Ravikovitch et al. 1961), Turkey (Avci and Akar 2005), Syria (Ryan et al. 1998), Malaysia (Shorrocks 1964), the southwest of the USA (Ashworth et al. 1985; Chesworth 1991), and large tracts of southern Australia (Cartwright et al. 1986).

Naturally, high concentrations of boron are commonly found in soils derived from marine sediments (Erd 1980) but in many areas, it is high boron concentrations in irrigation water that create toxicity problems.

Boric acid has a pK around 9.2 and therefore exists in soils mainly as the neutral boric acid. In high pH soils, a proportion of boric acid will be present as the borate anion which causes it to be adsorbed to soil particles (Goldberg 1997). In soil of neutral or acidic pH, the lack of charge predisposes boric acid to leaching by rainfall or irrigation, and high concentrations can therefore be found in the subsoil but not in the topsoil.This situation favours the early growth of plants but restricts exploration by roots deeper into the profile where boron concentrations are higher. If the pattern of rainfall during the growing season is regular, and moisture is retained in the topsoil, then plants can avoid toxicity by exploiting nutrients in the topsoil. However, in many areas, most of the rainfall occurs in winter and early spring but can be highly variable late in the growing season. This can have negative impacts if the topsoil moisture is depleted and roots are forced to explore the high boron subsoils for water. Under low rainfall conditions, the lack of leaching allows boron to remain in the topsoil, which presents challenges for seed germination and establishment, unless the species has developed tolerance mechanisms to combat boron toxicity.

4 Boron Toxicity

4.1 Symptoms

Visual symptoms of B toxicity include inhibition of root and shoot growth, and chlorosis and necrosis of shoots (Lovatt and Bates 1984; Nable et al. 1990a). The underlying cause of these developmental changes may be linked to the disruption of a range of physiological processes, including inhibition of photosynthesis, lower stomatal conductance (Lovatt and Bates 1984), increased peroxidation of lipids, and alterations in enzymes within antioxidation pathways, increased membrane leakiness (Karabal et al. 2003), and reduced proton extrusion from roots (Roldán et al. 1992). Increased deposition of suberin and lignin has also been reported (Ghanati et al. 2002).

Toxicity symptoms are generally correlated with the accumulation of high concentrations of boron in shoots, which is related to the soil boron concentrations and the length of exposure. Early observations by Oertli and Kohl (1961) of 29 plant species, including grasses, citrus, vegetables and flower crops established that in general, chlorosis of the leaves occurred at approximately 1,000 mg kg^{-1} DW and necrosis between 1,500–2,000 mg kg^{-1} DW. The pattern of necrosis was correlated with venation, such that symptoms developed first at the ends of the veins. From this, it was concluded that excess boron remained in the xylem and therefore accumulated where the xylem vessels terminated. This means for grasses, which have parallel venation, toxicity will first be observed at the leaf tip, whereas for dicots, which generally have reticulate venation, toxicity is observed around the leaf margins.

4.2 Causes

The actual cause of boron toxicity still remains a mystery. Compared to other essential plant nutrients, boron is relatively unreactive, which in theory should limit the possible targets for toxicity. Complexation with boron is mainly restricted to those compounds possessing two hydroxyls in the cis-conformation, known as cis-diols. The most stable complexes occur with cis-diols attached to a furanoid ring Hunt (2002). The only certain role for boron in plants is as a component of the rhamnogalacturonan II (RGII) complex in cell walls where boron is bound to the cis hydroxyl groups of the furanoid ring of the sugar apiose (Matoh 1997, 2000). Plant boron requirements closely reflect cell wall content of 2-keto-3-deoxysugars, which includes apiose (Matoh and Kobayashi 1998). This largely explains the lower requirement for boron in monocots compared to dicots. However, there is no strong evidence of disruption of cell wall structures under high boron conditions.

The other possible target for boron complexation is the sugar ribose which occurs in a number of key metabolites such as ATP, NADH, NADPH and in nucleic acids.

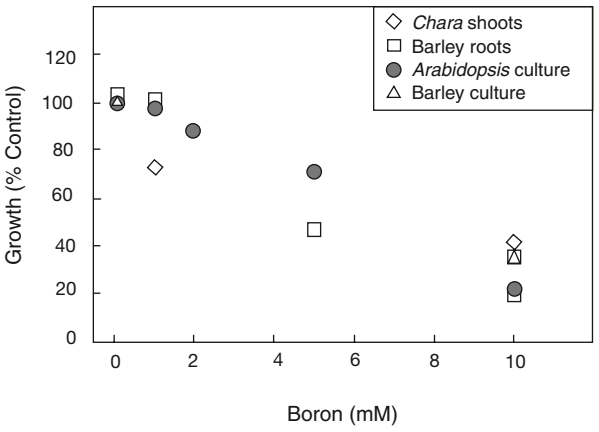

Fig. 15.1 Similarity in intracellular boron concentrations required to inhibit growth in a range of cell types. (Reprinted from Reid et al. (2004) with permission)

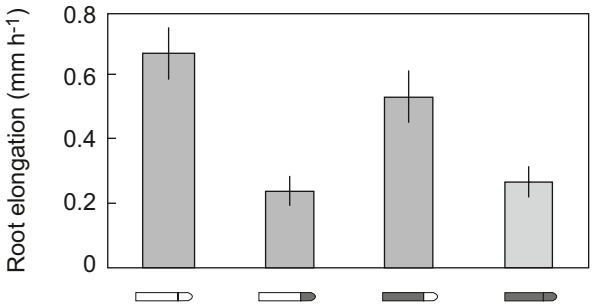

Fig. 15.2 Spatial sensitivity of growth of wheat roots to 10 mM boron applied either to the mature section of the root or to the apical 5 mm. Shading indicates regions where boron was applied. (Reprinted from Reid et al. (2004) with permission)

Binding to the former three inhibits energy metabolism while binding to the latter affects a number of developmental processes related to gene expression and protein synthesis. Reid et al. (2004) examined the effect of increasing boron concentrations on a range of cellular activities, and compared responses to tissue concentrations at which toxicity symptoms were observed. Using barley seedlings, they found that quite high concentrations (ca. 50 mM) were required to significantly inhibit respiration, photosynthesis, protein synthesis and selected enzymes of energy metabolism (malate dehyrogenase, isocitrate dehydrogenase) and acid phosphatase. This contrasted with the threshold concentration for visible toxicity symptoms to appear in leaves of around 20 mM, which was still an order of magnitude higher than the boron concentration at which inhibition of root growth occurred. Measurements of growth of the giant alga *Chara,*and suspension cultures of *Arabidopsis* and barley all showed a similar threshold for inhibition of growth of around 2 mM (Fig. 15.1). It was further established that inhibition of root growth only occurred if high concentrations were applied to the tip; no inhibition was observed if the chemical was applied to the mature sections of the root (Fig. 15.2) (Reid et al. 2004).

The difference in sensitivity between mature and meristematic tissue suggested that toxicity might be related either to cell expansion or cell division, possibly by

binding to ribose for which boron has a high affinity. The latter hypothesis is supported by observations of a reduction in the mitotic index in *Viciafaba* root tips in the range of 1–10 mM B (Liu et al. 2000). DNA contains deoxyribose which lacks the necessary cis-diol groups to bind boron, so inhibition of DNA replication does not seem likely. Although RNA does contain ribose, one of the hydroxyl groups is involved in linking the nucleotide bases so that it no longer presents a cis-diol for complexation. However, both of these hydroxyl groups of ribose are exposed at the 3' end of RNA molecules, and potentially more frequently during the processing of mRNA. In plants and animals, RNA undergoes extensive splicing, during which the 3'ribose would be briefly exposed to boron. Shomron and Ast (2003) have demonstrated in vitro that B can indeed inhibit one of the steps in splicing of mRNA. Perhaps more importantly, both hydroxyl groups are also exposed at the 3' end of tRNA molecules which could potentially interfere with translation of proteins because one of these hydroxyls is the target for attachment of the amino acid by amino acyl tRNAsynthetases.

5 Boron Tolerance

5.1 Early Observations

It is well known that different species and different cultivars of the same species have different abilities to grow on soil high in boron. Screening studies such as those conducted in southern Australia by Nable, Paull and their colleagues (Nable 1988; Paull et al. 1988) identified cultivars of wheat and barley with significant tolerance to boron. Similar studies by Kaur et al. (2006) subsequently identified tolerant cultivars of *Brassica rapa*. The common feature of tolerant cultivars was that the boron concentrations in their tissues were lower than in sensitive cultivars. From this, it was hypothesised that the tolerance trait was associated with an ability to restrict boron uptake from the soil into the roots, thereby reducing transfer to the shoot.

Further research using the tolerant and sensitive cultivars led to the identification of chromosome regions in wheat, barley and rape seed that were associated with tolerance to high boron concentrations (Jefferies et al. 1999, 2000; Kaur et al. 2006, 2008).

5.2 Tolerance Mechanisms

The observation that boron tolerant cultivars had lower concentrations of the chemical in their roots and shoots implied some form of control over net boron uptake from the soil. This could arise by two separate mechanisms; either by restricting entry into the root or by efflux of boron from the root. A problem in deciding

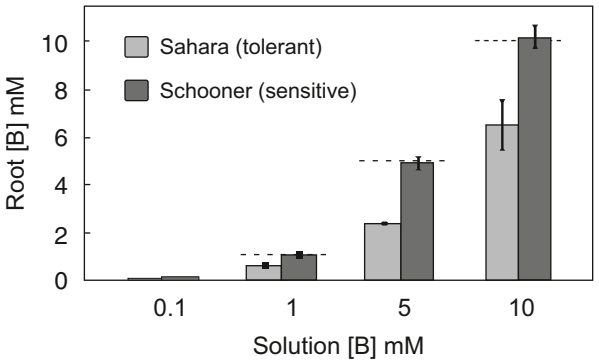

Fig. 15.3 Boron-tolerant cultivars of barley are able to maintain root boron concentrations below that of the external solution, whereas in sensitive cultivars, the concentrations in the root and in the external solution are the same. The dashed lines indicate the equivalent external concentration. (RJ Reid unpublished data)

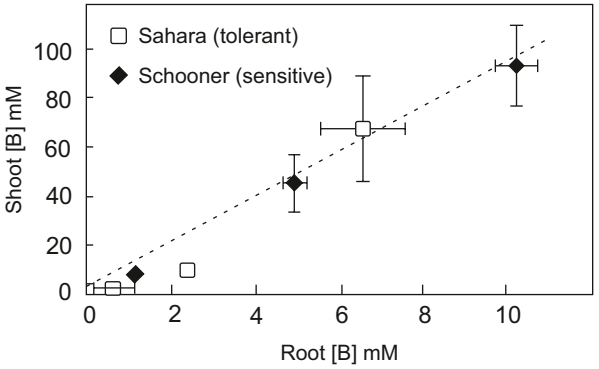

Fig. 15.4 The transfer of boron from the root to the shoot is approximately linearly related to the root concentration, and is similar for boron-sensitive and boron-tolerant cultivars. (RJ Reid unpublished data)

between these two alternatives was that little was known about the processes by which boron crossed biological membranes. Calculations by Raven (1980) based on ether-water partition coefficients suggested that membrane permeability to boric acid should be high, a prediction that was supported by direct studies by Dordas and Brown (2000) on artificial lipid bilayers. If this was the case, then boric acid should equilibrate rapidly between root tissue and the surrounding medium. Hayes and Reid (2004) established that this did in fact happen in sensitive cultivars; root B concentrations mirrored those in the external solution (Fig. 15.3). However, in tolerant cultivars, the root concentration was lower than that in the external solution. Despite the lower intracellular boron concentrations, the rates of boron influx and efflux were similar in tolerant cultivars to rates in sensitive cultivars (Hayes and Reid 2004), indicating that the differences in root boron concentrations were not related to differences in membrane permeability.

Experiments with barley showed that boron concentrations in shoots were linearly related to boron concentrations in roots, both in boron-sensitive and boron-tolerant cultivars (Fig. 15.4). What this means in terms of tolerance is that the principal factor determining shoot boron concentration is the root concentration, and if this can be lowered, then it will have a direct effect on shoot concentrations, and consequently

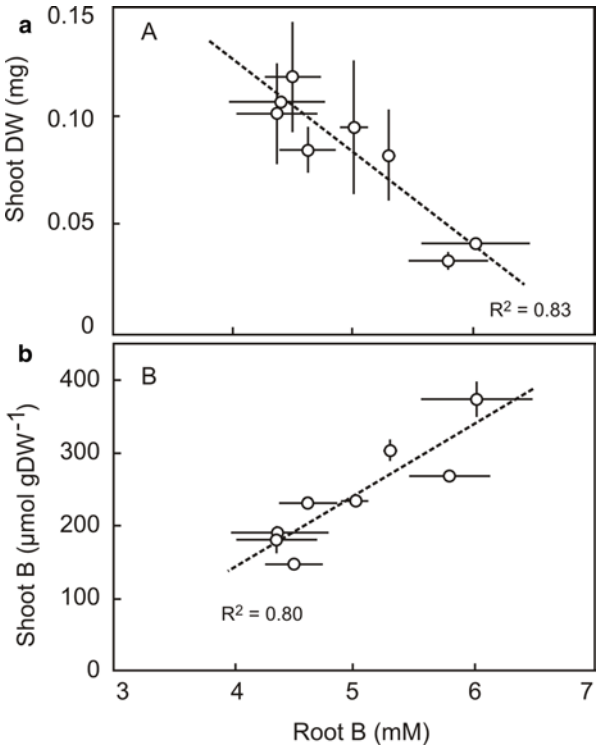

Fig. 15.5 Correlation between root boron concentration and shoot DW (**a**) and shoot boron concentration (**b**) in eight cultivars of wheat grown in solution containing 5 mM boron. (Reprinted from Reid (2007) with permission)

toxicity. An experiment with eight varieties of wheat all grown at the same solution, boron concentration illustrates this relationship (Fig. 15.5). In these varieties, the root boron concentrations ranged from 4–6 mM. Both shoot boron concentration and shoot dry weight were linearly related to the root boron concentration.

From this research, it became clear that tolerant cultivars were able to efflux boron from roots but how this was achieved at the molecular level was not known. This led to a search for genes-encoding membrane transporters capable of pumping boron out of cells, as described in the next section.

Although reduced tissue boron concentration as a result of efflux pumping seems to be a common feature of tolerant cultivars, there is also evidence in support of two other mechanisms. Choi et al. (2007) reported morphological changes in the root tip of tolerant species following exposure to high boron concentrations, which were associated with increased concentrations of reducing sugar. It was thought that these changes allowed osmotic balance to be maintained so that root elongation could continue.

The second mechanism relates to a transcription factor that in rice makes plants sensitive to high boron concentrations. Ochiai et al. (2008) investigated the reasons behind the greater boron tolerance of japonica rice varieties in comparison to indica varieties and located a QTL associated with tolerance. Map-based cloning eventu-

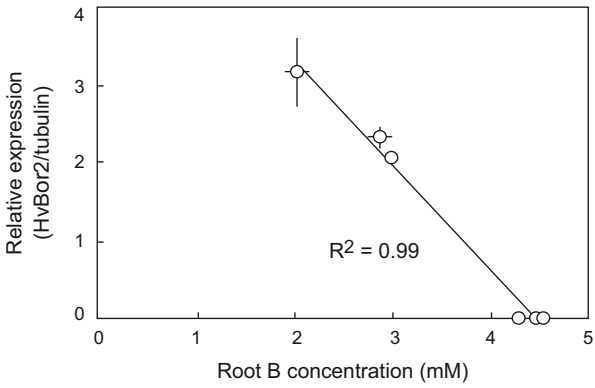

Fig. 15.6 Relative expression of *HvBor2* as a function of root boron concentration in barley cultivars grown in a nutrient solution containing 5 mM boron. (Reprinted from Reid (2007) with permission)

ally led to the identification of a NAC-like transcription factor with a single nucleotide polymorphism between the sensitive and tolerant varieties (Ochiai et al. 2011). The deletion of the single nucleotide appeared to confer tolerance by disruption of the gene in the tolerant cultivars. Hence, the functional gene, which was named *BET1* (Boron Excess Tolerant 1), should best be described as a boron-sensitive gene rather than a tolerance gene. Suppression of *BET1* expression by RNAi increased tolerance to boron (Ochiai et al. 2011). Since these changes in tolerance occurred in the absence of differences in root or shoot boron concentrations (Ochiai et al. 2008), this mechanism must be independent of boron efflux.

5.3 Boron Efflux Transporters

The identification of boron transporter genes involved in boron tolerance was greatly assisted by the discovery of a boron efflux transporter in *Arabidopsis* that pumped boron into the root xylem under deficiency conditions (At*Bor1*) (Takano et al. 2002). However, under high boron conditions, the transporter was found to be degraded (Takano et al. 2005), which eliminated any role in boron tolerance. Four homologues of At*Bor1* were found in rice. Reid (2007) used primers prepared from sequences of these rice genes to probe expression of related genes in sensitive and tolerant wheat. From these experiments, a gene with high similarity to Os*Bor2* was sequenced and named Ta*Bor2*. A gene with 90 % similarity to Ta*Bor2 at* the amino acid level was subsequently identified in barley (Hv*Bor2*). Expression of both of these genes was shown to be high in tolerant cultivars and low in sensitive cultivars, with expression negatively correlated with root boron concentrations (Fig. 15.6) (Reid 2007). Sutton et al. (2007) using positional cloning methods also reported a gene from barley with the same sequence as Hv*Bor2* which they named Bot1. In the same year, Miwa et al. (2007) showed that overexpression of At*Bor4* in the distal regions of *Arabidopsis* roots resulted in tolerance to high boron concentrations.

5.4 The Role of Aquaporins

These discoveries emphasised the importance of boron efflux transporters as a major tolerance mechanism across a diversity of plant types. The ability of effluxers to maintain reduced intracellular boron concentrations is also dependent on the rate at which boron can enter the cell across the plasma membrane. Fitzpatrick and Reid (2009) provided evidence that possibly 50 % of boron influx was mediated by aquaglyceroporins, the remainder presumably entering via direct diffusion through the lipid bilayer. Theoretically, closure of these channels under boron toxicity conditions could greatly increase the effectiveness of the efflux transporters. However, although they were able to show that two PIP1 type aquaporins were capable of transporting boron in yeast, Fitzpatrick and Reid (2009) could not detect any change in expression of the genes for these transporters between high and low boron conditions in barley. Schnurbusch et al. (2010) showed that boron could also enter cells through NIP2, 1, an aquaglyeroporin from the nodulin-26-like intrinsic protein (NIP) subfamily, and that the expression of the gene for this transporter was lower in a tolerant barley cultivar compared to a sensitive cultivar. From this, they proposed that this could confer an extra level of tolerance to that provided by the boron efflux transporters.

5.5 Leaf Tolerance

Reduced boron accumulation via efflux pumping from the root back into the external medium is easy to comprehend. Less intuitive is tolerance conferred by the same transporters operating in the shoot. By careful dissection of necrotic lesions on leaves of barley and wheat, Reid and Fitzpatrick (2009) were able to show that in tolerant cultivars, death of leaf cells occurred at higher tissue boron concentrations than in sensitive cultivars. To explain the higher expression of *Bor2* genes in leaves of tolerant cultivars, they proposed that toxicity was reduced by pumping of boron from the sensitive cytoplasmic compartment into the cell walls where it was much less toxic. Thus for the same total leaf concentration, much less of the boron would be exposed to metabolic processes within the cell in tolerant cultivars. Reid and Fitzpatrick (2009) provided evidence in support of this hypothesis by showing that boron was much more rapidly eluted from leaves of tolerant cultivars, consistent with a larger fraction of boron being located outside of the cell.

The leaf elution experiments conducted by Reid and Fitzpatrick (2009) highlighted the ease with which tissue boron could be solubilised, and caused them to revisit the observations by Nable et al. (1990b) on the lack of consistency of boron concentrations required to cause toxicity in the field compared to the glasshouse. Nable et al. (1990b) suggested that the lower concentration observed in the field was due to leaching of boron from leaves by rain, but were not able to demonstrate this under controlled conditions. Reid and Fitzpatrick (2009) simulated seasonal

rainfall during the early stages of growth of barley plants by spraying the leaves at regular intervals.They were able to show that more than 50 % of shoot boron could be washed out in this way, resulting in substantial improvements in growth of not only shoots but also roots.

6 Boron-Salinity Interactions

A confounding factor in assessing boron toxicity is the co-occurrence with salinity toxicity. Saline soils often contain high levels of both salts and boron (Nable et al. 1997) while in many areas of the world, saline groundwater used for irrigation also contains high levels of boron. Increasingly, recycled wastewater is being used for irrigation and this can represent an additional source of both boron and salinity (Feigin et al. 1991; Tsadilas 1997). Although both salts and boron can be leached from the root zone in areas receiving high rainfall, in semi-arid regions they tend to remain in the topsoil (Keren and Bingham 1985).

Where two toxicity stresses are present simultaneously, the effect of one toxicity can intensify the other, ameliorate the other, or simply be additive. There is no clear consensus of which of these processes applies in the case of boron and salinity since studies done under different conditions or with different crops tend to yield conflicting conclusions (Bingham et al. 1987; Mikkelsen 1988; Grattan et al. 1997; Shani and Hanks 1993; Holloway and Alston 1992; Grieve and Poss 2000; Ben Gal and Shani 2002; Alpaslan and Gunes 2001; Ferreyra et al. 1997; Yermiyahu et al. 2008).

Much of the research on boron tolerance mechanisms is targeted at improving crop productivity in high boron soils.Cultivars with high levels of expression of boron-efflux transporter genes showed improved growth and yield at high boron levels in solution culture, or in glasshouse trials with boron uniformly distributed through soil and with adequate watering and low salinity. However, field trials in southern Australia have been disappointing, generally showing little or no improvement (Emebiri et al. 2009; McDonald et al. 2009). In reality, high boron soils invariably contain other abiotic stress factors such as high salinity or variable moisture (Fig. 15.7) and the inability of the plant to deal with these stresses may outweigh any advantage gained by tolerance to high boron (Nuttall et al. 2006). Furthermore, where these stresses are heterogeneous in soil, avoidance by plasticity of root growth may be more important than tolerance (Choi et al. 2006).

7 Concluding Remarks

The past two decades have seen some important discoveries concerning the role of boron in plants and the consequences of having too little or too much of it. Despite considerable effort, there is still much that we do not really understand about boron, most significantly, the chemistry of its toxicity. Even its essential role in plant

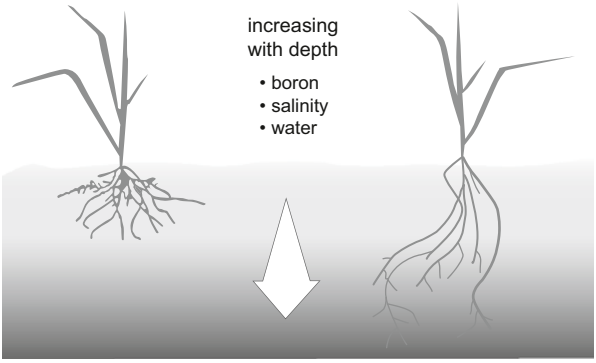

Fig. 15.7 Most soils containing high concentrations of boron are also affected by salinity and low rainfall. Boron and sodium concentrations usually increase down the soil profile which has an inhibitory effect on roots trying to access subsoil moisture. The effect of cellular boron tolerance mechanisms on the pattern of development of roots early in the growing season, may have positive or negative impacts on late season growth and yield. (Reprinted from Reid (2009) with permission)

growth is not well understood, and we can only confidently say that it is important in stabilising cell walls. On a more positive note, the past decade has provided us with some excellent insights into the functioning of boron transporters and their importance in avoidance of boron deficiency and toxicity. This is likely to be a fertile area for further research. The recent discovery of a boron tolerance mechanism not related to boron transport suggests that internal tolerance does exist and might be exploited.The quest for crop plants that can tolerate high levels of soil boron will no doubt continue but has been made more difficult by the need to consider the effect of associated stresses such as drought and salinity.

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Chapter 16
Arsenic Toxicity in Crop Plants: Approaches for Stress Resistance

Dhammaprakash Pandahri Wankhede, Meetu Gupta and Alok Krishna Sinha

1 Introduction

Among the heavy metals (HM), arsenic is one of the major environmental health concerns for human (Nriagu 2002). For plants, arsenic is a nonessential element and is toxic to them. Regions of South and Southeast Asia along with South America are worst affected by arsenic pollution. Anthropogenic activities like use of arsenic-based herbicide, insecticides, mining activities and also irrigation with arsenic contaminated ground water has added to arsenic pollution in the soil.

Arsenic is readily taken up by crop plants, enters food chain and causes food safety problem. Rice plants in particular are shown to be an efficient accumulator of arsenic. Arsenic is also found in rice grain at higher concentrations that pose serious health threats to the people dependent on rice as their staple food (Williams et al. 2007; Zhu et al. 2008). Inorganic arsenic mostly exists in arsenate or arsenite forms. In some regions, arsenic is also found in methylated form. Phosphate transporter recognizes arsenate while arsenite is taken up as a silicon (Si) transporter. Arsenate interferes in the processes like ATP synthesis and oxidative phosphorylation, arsenite on the other hand binds with sulphhydryl group and interferes in general protein synthesis.

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There has been substantial progress in our understanding of how arsenic is taken up by the plants and how it is being metabolized inside the plants (Zhao et al. 2009, 2006; Mendoza-Cózatl et al. 2011). These works have helped in devising strategies to counter arsenic stress. Discovery of brake fern (*Pteris vittata*) as hyper accumulator of arsenic by Ma et al. (2001) is one of the significant achievement in this regard. After this discovery, 12 species of fern have been identified as hyper accumulator of arsenic (Zhao et al. 2009). Among the crop plants, interestingly rice (*Oryza sativa*) is much more efficient in arsenic accumulation compared to wheat and barley (Williams et al. 2007; Su et al. 2010). The reason for rice being efficient arsenic accumulator is the bioavailability of arsenic since rice grows under anaerobic condition and that the highly efficient silicon pathway in rice also helps in uptake and transport of arsenic.

In this chapter, we will focus on different mechanisms involved in arsenic uptake, transport and its metabolism in the plants. Finally, the different approaches being used to develop stress resistance against arsenic in plants will be discussed.

2 Arsenic Toxicity in Plants

The nature of arsenic toxicity varies with different arsenic species. Since arsenate is an analogue of phosphate, it interferes with essential cellular processes such as phosphorylation and ATP synthesis, whereas arsenite, the reduced form of arsenate, binds with vicinal sulphyhydryl groups of proteins resulting in alteration in their structure and catalytic properties, and thus deleterious effects on protein functioning (Hughes 2002). The toxic effects of arsenate are largely attributed to arsenite since the former is radially reduced to the latter (Hughes 2002). Arsenate stress results in generation of reactive oxygen species (ROS) in different plants viz., rice, maize and *Holcus lanatus* and thus induces oxidative stress such as lipid peroxidation (Rao et al. 2011; Ahsan et al. 2008; Hartly-Whitakar et al. 2001; Mylona et al. 1998; Requejo and Tena 2005). Activity of several antioxidant enzymes like superoxide dismutase, catalase, etc., and mRNA transcripts of genes encoding these enzymes are also up-regulated in response to arsenic stress (Mylona et al. 1998; Requejo and Tena 2005). In maize, these responses are found to be tissue as well as developmental stage specific. Depletion of cellular reduced GSH is considered to be the cause of arsenic-induced oxidative stress (Mylona et al. 1998). The toxicity level of arsenic in shoots varies depending upon plant species, whether it is hyperaccumulator or nonaccumulator. As hyperaccumulator *P. vittata* withstands 5,000–10,000 mg/kg^{-1} of arsenic in frond tissue without any detectable effects of toxicity, whereas non-hyperaccumulator plants show toxicity effects even with arsenic concentration ranging from 1100 mg/kg^{-1} (Kabata-Pendias and Pendias 1992; Lombi et al. 2002; Tu and Ma 2002). Rice seedlings grown in hydroponics with medium containing arsenic concentration beyond 10 μM show roots as well as shoots growth retardation accompanied by decreased photosynthetic yields with other toxic effects

(Rao et al. 2011). The toxic effects of heavy metals are speculated to be due to direct interaction of arsenic with proteins and/or as deleterious effects of ROS. As also causes cell death in plants on higher concentrations (Rao et al. 2011; Requejo and Tena 2005).

3 Arsenic Uptake and Transport in Plants

Arsenic is known to form organic and inorganic complexes in environment (Zhao et al. 2010), which are absorbed by the plants in three different forms, namely arsenate, arsenite and methylated arsenic. While arsenate is the main species in aerobic soil, arsenite is predominant under anerobic conditions. Both these species are interconvertible depending on the redox potential. Methylated species, which exist as monomethylatsonic acid (MMA) and dimethylarsinic acid (DMA) are usually minor arsenic species in the environment (Francesconi and Kuehnelt 2002).

The aerobic soil species arsenate is recognized by phosphate transporter. This has been demonstrated by several physiological and electrophysiological experiments, where these transporters have shown higher affinity for phosphate than for arsenate (Asher and Reay 1979; Ullrich-Eberius et al. 1989; Meharg et al. 1994). The uptake of one molecule each of arsenate and phosphate involves co-transport with at least two molecules of proton (Ullrich-Eberius et al. 1989). Several phosphate transporters are reported and characterized in plants with Phosphate Transporter 1 (Pht 1) family having more than 100 members (Rausch and Bucher 2002; Bucher 2007). Most of these phosphate transporters are expressed strongly in roots and are responsible for the uptake of phosphate from the soil. Interestingly, double mutant of phosphate transporters, Pht1;1 and Pht1;4 (*Pht1;1Δ4Δ*) in *Arabidopsis thaliana* were found to be resistant to arsenate compared to the wild type. This suggested that the arsenate is taken up by Pht1;1 and Pht1;4 phosphate transporter (Shin et al. 2004). The finding of Pht1;1 transporter assisting the uptake of arsenate was further supported by González et al. 2005, who reported that *A. thaliana* mutant defective in phosphate transport traffic facilitator 1 (PHF1), a trafficking protein of Pht1;1, also makes plants resistant to arsenate. Similarly, Pht1;3 transporter is also reported in *A. thaliana* as arsenate transporter (Catarecha et al. 2007).

The information about the uptake of arsenite has been deduced largely from the studies in microbial system. It has been reported that in *E. coli*, yeast and even in humans, arsenite can be taken up by aquaglycoporins, a subfamily of the aquaporins superfamily having larger pores (Bhattacharjee and Rosen 2007). However, an alternative mechanism has also been predicted in yeast, whereas deletion of hexosepermease gene results in 80 % inhibition in arsenite uptake (Liu et al. 2004). Recent research has shown growing evidence of involvement of nodulin 26-like intrinsic proteins (NIPs), a subfamily of plant aquaporins family to be involved in arsenite transport. Many genes from *Arabidopsis* (*AtNIP5;1 andAtNIP6;1*), rice

(*OsNIP2;1* and *OsNIP3;2*), lotus (*LjNIP5;1* and *LjNIP6;1*) have been identified as potential NIPs involved in arsenite transport by complementing in yeast system (Bienert et al. 2008). Isayenkov and Maathuis (2008) predicted the role *AtNIP7;1* also in arsenite transport. Interestingly, Ma et al. (2008) identified in rice OsNIP2;1 (also named as Lsi1 because of its role silicon (Si) transport) as major pathway during the entry of arsenite in rice roots. This data postulated that arsenite and silicon transport follow the same route in rice. Apart from OsNIP2;1, three other NIP proteins in rice, OsNIP1;1, OsNIP2;2 and OsNIp3;1 were also shown in arsenite transport in an heterologous system. In addition to Lsi1, a different protein Lsi2 was shown to mediate arsenite efflux in the direction of xylem (Ma et al. 2008).

The mechanism or the uptake of methylated form of arsenic species is largely unknown at present. The efficiency of taking methylated forms like MMA and DMA is much lower compared to arsenite and arsenate forms (Raab et al. 2007a). The concentration dependent uptake of MMA in rice roots can be described by Michaelis-Menten kinetics whereas DMA uptake did not follow Michaelis-Menten kinetic (Abedin et al. 2002). Interestingly, in *Zea mays* DMA uptake followed the Michaelis-Menten kinetic (Abbas et al. 2008).

Arsenic species once taken up by roots are transported to different parts of the plants through xylem stream. However, the rate of transport of arsenic species is always much slower than that of phosphorus species (Raab et al. 2007a). This inefficient uptake of arsenic is generally determined by looking at shoot:root ratio of arsenic accumulation. There are several studies in plants where the shoot:root ratio of arsenic uptake was determined. The most significant of these studies was one performed by Raab et al. (2007a) where 46 different species were analyzed. They proposed the ratio between 0.01 and 0.9, with the median at 0.09. Interestingly DMA, which is very poorly taken up by the roots is efficiently translocated to shoots from the roots. The ratio of the DMA transport varied from 0.02–9.8 with a median at 0.8 (Raab et al. 2007a). The main limitation in transport of arsenic species is because of the rapid reduction of arsenate species into arsenite species in the roots. These arsenite following complexation with thiols gets sequestered in the root vacuoles. This was shown by silencing *Arabidopsis arsenate reductase* (*AtACR2*) by RNAi resulting in more accumulation of arsenic in shoot (Dhankher et al. 2006). However a T-DNA insertion line of AtACR2 showed the opposite effect, where the mutant was found to accumulate less arsenic in shoots (Bleeker et al. 2006). Several studies confirmed that arsenite is the main form found in the xylem sap, even when the arsenate was fed to the plants (Zhao et al. 2009). DMA was rarely found in the xylem sap (Mihucz et al. 2005; Xu et al. 2007). As compared to xylem, little is known about the transport of arsenic species by phloem. Other heavy metals like cadmium (Cd) can make complexes with phytochelatine (Cd-PC) and glutathione synthatase (Cd-GS) and are known to be transported through phloem (Mendoza-Cózatl et al. 2008). However, existence of arsenic species in As-C and As-GS forms is not known. Further, As-PC and As-GS are unstable at pH of more than 7.5 and hence their stability in phloem sap, which is slightly alkaline, is also a question. A simplified schematic diagram of arsenic uptake and metabolism is shown in Fig. 16.1.

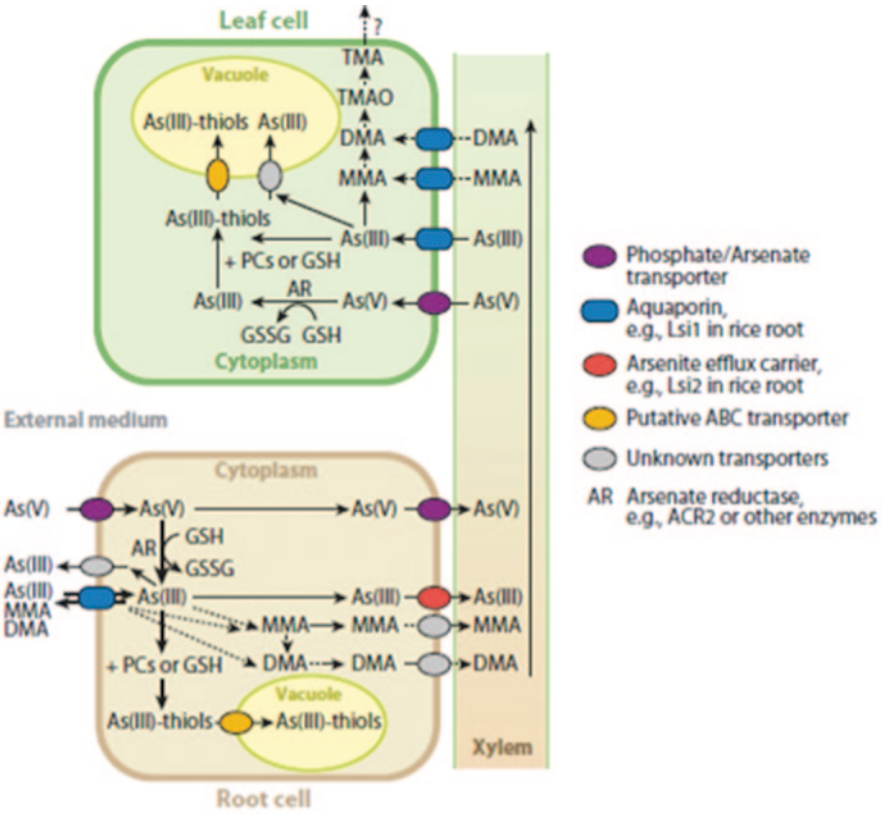

Fig. 16.1 Arsenic uptake and metabolism and detoxification in plants. The thickness of arrow lines is indicative of the relative flux. Transporters for arsenic uptake into leaf cells are assumed to be similar to those in roots, but there is little knowledge of their identities. (Source: Zhao et al. 2010

4 Arsenic Metabolism

Studies involving different plant species have shown the predominance of arsenite in plant tissues accounting > 90 % of total arsenic, which indicates that once arsenate finds its way in plants, it is reduced to arsenite (Zhao et al. 2009). Further, plant tissue extracts show arsenate reduction activity (Duan et al. 2007). In agreement with this, plant homologues of yeast *Acr2p* have been isolated in *Arabidopsis* (Dhankher et al. 2006), *H. lanatus* (Bleeker et al. 2006), *O. sativa* (Duan et al. 2007), and *P. vittata* (Ellis et al. 2006). Interestingly, plant ACR2 but not yeast Acr2P and PvACR2 show additional tyrosine phosphatase activity that may have a role in cell cycle regulation (Ellis et al. 2006; Landrieu et al. 2004a; Landrieu et al. 2004b). Plants with ACR2 proteins expressed in a heterologous system, also show reduction of arsenate in-vitro using GSH and glutathione as reductants. However,

in-planta function of ACR2 warrants further investigations since T-DNA insertion (knock-out) lines and RNAi (knock-down) lines show no deviation from wild type phenotype under normal conditions (Dhankher et al. 2006; Bleeker et al. 2006). Additionally, ACR2 knock-out mutants show dominance of arsenite suggesting functional redundancy, presence of other arsenate reductase, non-enzymatic reduction and/or existence of other enzymes in reduction of arsenate in plants (Zhao et al. 2009, 2010). A triosephosphate isomerase (TPI), an enzyme involved in glycolysis and isolated from *P. vittata*. *PvTPI,* is shown to confer arsenate resistance in *E. coli* strain lacking *ArsC*. However, in-planta function of PvTPI in arsenate reduction is not yet known (Rathinasabapathi et al. 2006).

In addition to arsenite and arsenate, plants also show presence of methylated arsenic species in the form of MMA, DMA trimethylarsinine oxide (TMAO) (Francesconi and Kuehnelt 2002; Meharg and Hartley-Whitaker 2002). Plants grown in hydroponics with no methylated arsenic species have shown presence of DMA and/or MMA in tissues and xylem sap albeit at very low concentrations (Raab et al. 2007a; Xu et al. 2007; Quaghebeur and Rengel 2003). Different rice genotypes also show various levels of inorganic arsenic (As_i) and DMA in grains (Liu et al. 2006). Plants starved with nitrogen and phosphorus also show significant proportion of methylation (Nissen and Benson 1982). These studies indicate existence of in-planta biomethylation activity. Arsenic-methylation activity of leaf but not root extract of *Agrostis capillaris* has been demonstrated in an in vitro assay with ^{3}H-labelled S-adenosyl-L-methionine (SAM) as the methyl donor (Wu et al. 2002). The activity is induced upon pre-exposure of plants to arsenic with MMA and DMA as methylation product. However, arsenic methylation pathways and enzymes are largely unknown at present. Possibly in plants, arsenic methylation follows the Challenger pathway which has been well studied in fungi and bacteria (Zhao et al. 2009; Bentley and Chasteen 2002).

In soil bacterium *Rhodopseudomonas palustris*, arsenic methytransferases (ArsM) have been identified and genes encoding the same are cloned (Qin et al. 2006). Similarly in an algae, *Cyanidioschyson* sp., living in arsenic rich environment, ArsM have been identified which are able to methylate arsenite sequentially to mono-di- and trimethyl arsenic with final product, a volatile TMA gas (Qin et al. 2009). Interestingly, rice genome possesses methyltransferase genes as that of microbes (Norton et al. 2008a), however role of these genes in arsenic methylation remains to be investigated. Furthermore, it is not known whether plants can generate and volatilize TMA as seen in microorganisms.

5 Arsenic Detoxification

Arsenic once taken up by plants needs to be detoxified to avoid detrimental effects on cellular processes. Detoxification of arsenic in plants occurs through complexation, vacuolar compartmentalization and efflux of arsenite to the external environment.

In plants, arsenic induces phytochelatin (PC) biosynthesis (Qin et al. 2009; Sneller et al. 1999). Arsenite has high affinity to the sulphhydryl (-SH) groups of peptides viz. GSH and PCs. GSH and arsenite also form $(GS)_3$-arsenite complex in vitro (Delnomdedieu et al. 1994). The toxicity of arsenite is also due to binding of arsenite to the –SH groups of proteins with deleterious effect on their structure and activity (Meharg and Hartley-Whitaker 2002). Dimethylarsinothioyl glutathione (DMAS-GS) complex have also been identified from *Brassica oleracea*, a sulphur rich plant (Raab et al. 2007b). Furthermore, blocking of PC synthesis with $_L$-buthionine-sulfoxime results in hypersensitivity to arsenic (Bleeker et al. 2006; Schat et al. 2002; Schmöger et al. 2000). More profound evidence comes from *Arabidopsis* mutant *cad1–3* (cadmium sensitive), a PC deficient mutant, which showed 10–20 times more sensitivity to arsenate compared to wild type plants (Ha et al. 1999). Further, overexpression of bacterial γ-glutamylcystein synthase gene (γ-*ECS*), *Arabidopsis* PC synthase (*AtPCS1*) also led to more arsenic-tolerant phenotype to transgenics (Dhankher et al. 2002; Li et al. 2004). These studies provide unequivocal evidence that thiols and PCs play significant role in arsenic detoxification in nonaccumulator plants. Interestingly, hyper accumulator, *P. vittata* and *P. cretica* do not follow PC-based mechanism to detoxify arsenic (Zhao et al. 2009).

Like other heavy metal (Cd) and its complex with proteins localized in vacuoles (Vögeli-Lange and Wagner 1990), it is believed that PC-arsenite complexes are also stored in vacuoles, where acidic pH (~5.5) helps in maintaining stability of complexes (Zhao et al. 2009). The yeast vacuolar transporter Ycf1p (yeast cadmium factor 1p), a member of ATP binding cassette (ABC) superfamily confers arsenite resistance by transporting the glutathione-*S*-conjugated arsenite into vacuole (Ghosh et al. 1999). The ABC proteins are also likely transport PC-arsenite complexes; however such proteins have not yet been identified (Zhao et al. 2010). In support of the ABC mediated transport, it has been demonstrated that As(III)-$(GS)_3$ complex was transported into the tonoplast vesicles prepared from *H. lanatus* in a MgATP-dependent manner (Bleeker et al. 2006). Inorganic arsenic species is also known to be stored in vacuoles in *P. vittata* (Lombi et al. 2002). Owing to the possible large concentration gradient from the cytoplasm to the vacuole, transport of arsenite across the tonoplast probably involves an energy-dependent active mechanism. A transporter(s) responsible for arsenite uptake into the vacuoles is not yet known but may be the key determinant of the hypertolerance phenotype in *P. vittata* and other hyperaccumulator plants (Zhao et al. 2010).

6 Strategies for Stress Resistance

There are three broad objectives before plant scientists with respect to arsenic-plant interaction, enhancing tolerance of crop plants to withstand high arsenic concentrations in soils, reducing accumulation of arsenic in edible plant part so as to reduce arsenic toxicity for humans; and development of plants with increasing arsenic

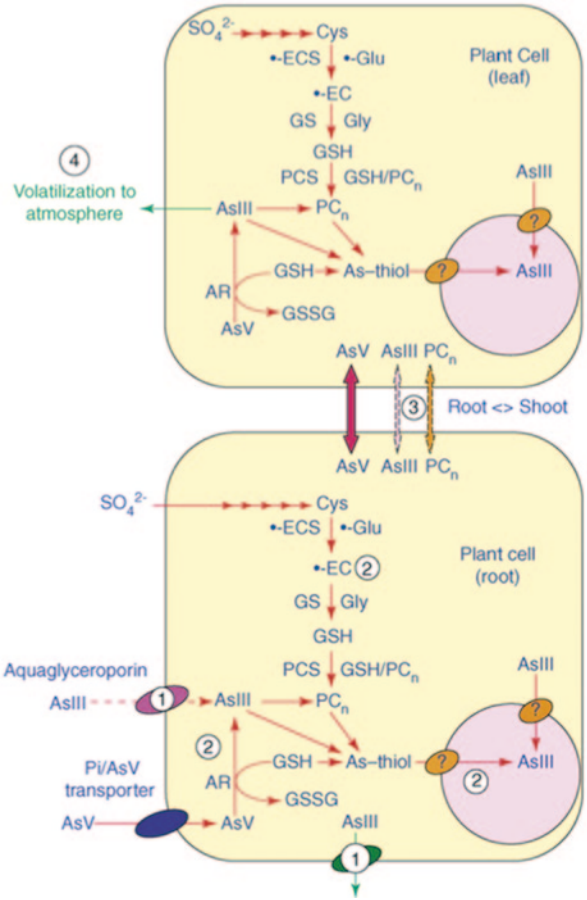

Fig. 16.2 Strategies to develop crops with reduced arsenic contents: *1* Overall reduction in arsenic load can be promoted through lowering arsenite uptake at the root–soil boundary and/or by extrusion of arsenite through carriers and pumps by using genes from bacterial sources. Lowering arsenate influx is more problematic because it negatively affects phosphate uptake *2* Localizing arsenic concentrations in root tissues by root-specific upregulation of arsenate reductase, PC biosynthesis and vacuolar sequestration would safeguard cytoplasmic metabolism but might create increased sulphur demand for the synthesis of thiol compounds. Thus, sulphur assimilation pathways will also need to be enhanced *3* Exclusion from shoot tissue and above ground edible parts could be achieved by reducing the root-to-shoot translocation of arsenite by suppression of the arsenite transporters *4* Volatilization of arsenic as trimethylarsine from leaf cells using bacterial genes would reduce shoot arsenic levels. Target processes that need to be increased in capacity are depicted using bold lines, those that need repression are shown using dotted lines. (Source: Tripathi et al. TRENDS in Biotechnology-(65))

uptake keeping in view phytoremediation. In addition to agronomic practices that deal with soil, water management and fertilization to minimize arsenic stress, crop improvement practices can be grouped into classical/molecular breeding and genetic engineering approaches (see Fig. 16.2).

7 Development of Elite Cultivars Through Plant Breeding

A significant genetic variation in rice has been reported, especially among rice cultivars from Bangladesh with regard to arsenic accumulations. Further, a number of local cultivars with low grain arsenic concentrations have been identified (Norton et al. 2009). These variations may form basis for selection as well as improvement of rice cultivars with minimum arsenic accumulations through breeding practices. Additionally, the 'red bran' character was found to be associated with arsenic tolerance (Norton et al. 2009), which may aid in selection of arsenic-tolerant genotypes provided that there is a strong linkage between the two traits.

Genetic mapping of arsenic tolerance has also been reported in rice (Dasgupta et al. 2004) which revealed a segregation of a single gene which was termed *AsTol* and mapped to a rice chromosome 6. However, further report showed presence of three-gene model of arsenic tolerance (Norton et al. 2008b). From the position of these three major genes, it may be possible to produce a list of candidate genes, and, by integrating microarray analysis (Norton et al. 2008b), it was possible to narrow these lists down further. Additionally, quantitative trait loci (QTL) have been proposed for arsenic tolerance in rice (Zhang et al. 2008).

There has been a negative correlation observed between arsenic accumulation in straw and grain and root porosity and oxygen release likely through iron plaque formation, arsenite oxidation and arsenate retention on the iron plaques. Selection of genotypes with profound oxygen release characteristic may also help to reduce arsenic concentrations in grains (Zhao et al. 2010; Mei et al. 2009). This information may form a basis to initiate breeding programs for development of cultivars with less arsenic and arsenic stress tolerance.

8 Genetic Engineering

Increase in synthesis of PC and GSH through overexpression of genes involved in their biosynthesis has been used to enhance arsenic tolerance in plants (Pickering et al. 2006). Overexpression of *AtPCS1* led to increase in arsenic resistance and greater biomass than that of wild type plants upon arsenic treatment. However, these transgenic plants showed cadmium hypersensitivity (Li et al. 2004). Chloroplast targeting *AtPCS1* made transgenic plants sensitized to arsenic whereas cytosolic targeting of the same resulted in conferring tolerance to plants (Li et al. 2004). These results suggest that mere increase in PCS may not be sufficient for tolerance in plants rather the unwanted phenotype of overexpression was attributed partly due to limiting metabolites such as cysteine and glutamylcystein and GSH which are essential for the biosynthesis of PCs (Picault et al. 2006). Overexpression of other components of GSH biosynthesis such as γ-glutamylcystein synthase (γ-*EC*) and glutathione synthase (*GS*) may prove to be a successful approach in enhancing biosynthesis of PCs (Tripathi et al. 2007). Enhanced production of γ-EC, GSH and

PCs have been observed upon exposure to arsenic in *Arabidopsis* overexpressing *γ-ECS* conferring significant resistance to arsenic (Li et al. 2005, 2006). These studies suggest that multigenic approach might lead to higher tolerance in plants against arsenic stress (Cherian and Oliveira 2005). Overexpression of bacterial arsenic reductase gene in leaves (*arsC*) under control of light inducible RuBisco promoter and constitutive actin promoter driven *γECS* in roots as well as shoots have markedly increased tolerance and accumulation of arsenic in shoots (Dhankher et al. 2002). The leaf-specific expression of *arsC* possibly involved in arsenate reduction, in spite of high endogenous activity of arsenate reduction in the wild type plants, whereas *γ–ECS* overexpression might boost the biosynthesis of thiol rich peptides for arsenite complexation. These results imply that enhanced shoot tolerance has the effect of driving more arsenic accumulation in shoots. In future, it may be possible to engineer high-biomass plants for arsenic phytoextraction using genes from *P. vittata*, specifically those responsible for efficient xylem loading of arsenic and detoxification in fronds, although the molecular mechanisms for arsenic hyperaccumulation are obscure at present (Zhao et al. 2010). Another strategy could be an increased biosynthesis of PCs in roots so that the As-PC complexes are sequestered in vacuoles in roots thus restricting translocation of arsenic to shoots (Zhao et al. 2009, 2010).

Since arsenate is taken up by plant roots via phosphate transporters and different phosphate transporters may vary in their affinity for arsenate, it may be possible to identify variant of phosphate transporters which are more discriminatory against arsenic. Similarly, variants of NIP aquaporins or Lsi2-like carrier proteins may also help in reducing arsenic in shoots, thus increasing tolerance against arsenic materials (Zhao et al. 2010).

Arsenic specific methyltransferase, if identified, may be a good target for manipulation since methylated species of arsenic are less toxic than the inorganic form. *arsM* genes of microbial or algal origin may also be used for overexpression to achieve the conversion of inorganic to methylated or even volatilizable species of arsenic (Qin et al. 2006, 2009). Members miotgen activated protein kinase (MAPK) cascade (OsMPK3, OsMPK4 and OsMKK4 in rice) which are activated upon arsenic stress (Rao et al. 2011) might be an additional target provided that their in-planta role in arsenite stress tolerance is established. Acitivities of MAPK were also shown to be upregulated upon arsenic stimulation in *Brassica juncea* (Gupta et al. 2009).

9 Future Perspective

Our knowledge of precise mechanism of tolerance in case of hyperaccumulator plants is very limited. Similarly, genes and enzymes involved in arsenic metabolism in nonaccumulator plants need further elucidation. Further research on metabolism, root-to-shoot translocation and sequestration of arsenic would be needed. In plant arsenate reduction which involves multiple pathways and enzymes, only one enzyme has been identified until now (Zhao et al. 2009). Since plants and seeds

represent a major source of metal intake for human and livestock, an indepth understanding of these processes could help to ensure the accumulation of essential nutrient metals and avoid entry of toxic metals in the food supply (Mendoza-Cózatl et al. 2011). Furthermore, it would be interesting to know whether plants methylate arsenic and if so what are the genes involved in the process (Zhao et al. 2010).

Advances in the analytical techniques for arsenicspeciation, their uptake and transport have significantly increased our understanding of plant arsenic metabolism. These analytical tools, which in combination with structural and functional genomics approaches provide ample opportunities for unraveling the mechanisms of arsenic transport, metabolism, and regulation, may translate in generation of plants with least arsenic accumulation and tolerance against arsenic stress.

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Chapter 17
Mechanism of Cadmium Toxicity and Tolerance in Crop Plants

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1 Introduction

Environmental pollution by heavy metals is a serious problem worldwide, which causes considerable loss to agricultural productivity. Over the last few decades, there is a dramatic, troublesome increase in heavy metal contamination of soil, water and air, globally. Heavy metals are ascribed to transition metals with atomic mass of over 20 and having a specific gravity of above 5 g cm^{-3} or more. Every year, India loses hundreds of millions of rupees from reductions in crop productivity (Mahajan and Tuteja 2005). According to Bray et al. (2000), the relative decreases in potential maximum yields associated with abiotic stress factors, vary between 5482 %. Although heavy metals occur naturally in soil as rare elements, burning of fossil fuels, mining and smelting of metalliferous ores, electroplating, gas exhaust, energy and fuel production, fertilizer and pesticide application, use of sludge or municipal compost, pesticides, fertilizers, and emissions from municipal waste incinerators and car exhausts all contribute to their spread in the environment (Alkorta et al. 2004; Wei and Zhou 2008). Major heavy metal pollutants include cadmium (Cd), chromium (Cr), copper (Cu), lead (Pb), Arsenic (As), mercury (Hg), nickel (Ni),

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silver (Ag), selenium (Se) and zinc (Zn) (Long et al. 2002). Contamination of arable soil with Cd is one of the most serious agricultural problems in the world, and therefore it is considered to be one of the most toxic elements to plants, animals and human beings. Due to its high solubility in water, it is promptly taken up by plants and this represents the main entry pathway into the food chain (Metwally et al. 2005; Farinati et al. 2010).

The agricultural soil may have toxic levels of heavy metals due to various anthropogenic activities (Xie et al. 2006; Alkorta et al. 2004; Verma et al. 2007; Wei and Zhou 2008). It has been estimated that the heavy metal concentration in soil typically ranges from less than one to as high as 100,000 mg kg^{-1}. Kamnev and Van der Lelie (2000) reported that ~9.9–45.0 tons of Cd is discharged into the soil every year globally, which is one of the most highly toxic environmental pollutant in the atmosphere, soil and water, and in excessive amounts can cause serious problems to all organisms (Benavides et al. 2005). Even at low concentration, significant reduction in plant growth and biomass has been reported in the literature (Sanita di Toppi and Gabbrielli 1999; Sandalio et al. 2001; Dominguez et al. 2003; Drazkiewicz and Baszynski 2005; Gianazza et al. 2007; Singh et al. 2008). The regulatory limit of Cd in agricultural soil is 100 mg kg^{-1} soil (Salt et al. 1995) but this threshold is continuously exceeding because of various anthropogenic activities. Cd is toxic for most of the plants at concentrations greater than 5–10 μg Cd g^{-1} leaf dry weight (White and Brown 2010), except for Cd-hyperaccumulators, which can tolerate Cd concentrations of 100 μg Cd g^{-1} leaf dry weight (Verbruggen et al. 2009). Furthermore, significantly higher levels of Cd in the agricultural soil lead to the degradation of soil quality, loss of crop productivity as well as poor quality of agricultural products (Long et al. 2002), which pose significant hazards to human, animal, and ecosystem health (Blaylock and Huang 2000).

Cadmium has no biological function and is not even essential for plant growth. Being water soluble, it can be easily absorbed in tissues and can cause various phytotoxic visible symptoms, which include leaf chlorosis, root putrescence, growth inhibition which ultimately cause plant death (Skrzyska-Polit et al. 2010; Valentoviová et al. 2010). Cadmium causes inhibition of shoot and root growth (Schutzendubel et al. 2001), disorganization of the grana structures and reduction in the biosynthesis of chlorophyll (Somashekaraiah et al. 1992; Siedlecka and Krupa 1996). It also interferes with photosynthesis, respiration and water relations (Perfus-Barbeoch et al. 2002; Balakhnina et al. 2005; Mobin and Khan 2007; Singh et al. 2008; Gill et al. 2011). Moreover, it can also inhibit the activity of several groups of enzymes, such as those of the photosynthetic Calvin cycle (Sandalio et al. 2001), carbohydrate metabolism (Sanita di Toppi and Gabbrielli 1999; Verma and Dubey 2003) and phosphorus metabolism (Shah and Dubey 1998; Sharma and Dubey 2007). Cadmium is known to cause the generation of reactive oxygen species (ROS), which leads to oxidative stress in plant tissues (Skorzynska-Polit et al. 2003/2004; Romero-Puertas et al. 2004; Mobin and Khan 2007; Gill and Tuteja 2010). The presence of Cd-lead high concentration of ROS causes oxidative damage to photosynthetic pigments, bio-molecules such as lipids, proteins and nucleic acids, leakage of electrolytes via lipid peroxidation resulting in dramatic reductions

in growth and productivity, and eventually cause the death of plants (Gill and Tuteja 2010). ROS are efficiently scavenged by enzymatic (SOD, CAT, APX and GR) and non-enzymatic antioxidants such as ascorbate (AsA) and glutathione (GSH), which protect plants against oxidative damage (Mittler et al. 2004; Gill and Tuteja 2010). The enzymes SOD and CAT are involved in the detoxification of $O_2^{\cdot -}$ and H_2O_2, respectively, thereby preventing the formation of $OH^{\cdot}$ radicals, whereas, APX and GR as well as ascorbate (AsA) and glutathione (GSH) are important components of the ascorbate-glutathione cycle -(AsA-GSH cycle) responsible for the removal of H_2O_2 in different cellular compartments (Gill and Tuteja 2010). In the present chapter, we will attempt to understand the response of crop plants to Cd toxicity and mechanism of Cd tolerance.

2 Uptake, Transport, Accumulation and Localization of Cd in Crop Plants

Cadmium (Cd) is a widely spread heavy metal, which causes serious environmental and human health problems due to its high mobility in the soil-root-shoot (grain) system (Vázquez et al. 2006). Cadmium can get absorbed into the plant root system through some other nutrient metabolic pathways such as zinc, iron, and calcium (Pence et al. 2000; Cosio et al. 2004; Vert et al. 2002; Nakanishi et al. 2006) after it reaches root cell membranes via the apoplast, including cell wall continuum and intercellular space. Plants show a differing metal distribution and accumulation pattern among different parts. Most of the Cd that enters the plant system, accumulates in the roots and only a small portion is translocated to the parts above the ground (Patel et al. 2005; Kovacik et al. 2006; Ekmekci et al. 2008; Liu et al. 2007; Singh et al. 2008; Gill et al. 2011). The actual accumulation of Cd in plant parts depends on the plant species and soil properties (Fediuc and Erdei 2002; Arao et al. 2003). Substantial variability among 99 *Pisum sativum* genotypes in tolerance to Cd and uptake of different heavy metals was reported (Belimov et al. 2003). Ten times higher Cd accumulation in the roots than in the parts above the ground was reported in *Hordeum vulgare* plants (Vassilev et al. 1998). Kovacik et al. (2006) reported that Cd accumulation was seven- (60 μM Cd) to eleven- (120 μM Cd) fold higher in the roots than in the leaves of *Matricaria chamomilla*, whereas only 6 % of Cd was accumulated in the leaves of *Crotalaria juncea* compared to roots with 2 mM $CdCl_2$ (Pereira et al. 2002). Cadmium was accumulated 1826 times more in the roots of *Allium sativum* than the control with the application of 10-2 M Cd and very low amount was transported to the bulbs and shoots (Jiang et al. 2001). Zhang et al. (2000) reported significant difference among *Triticum aestivum* genotypes in shoot Cd concentration. Significant differences in Cd accumulation and tolerance were found in *Sedum alfredii* populations (Deng et al. 2007). The Milyang 23 rice accumulated 10–15 % of the total soil Cd in its shoot (Murakami et al. 2007). Djebali et al. (2008) reported that the roots of Cd treated Solanum lycopersicum plants accumulated four to five-fold Cd as compared to mature leaves. Liu et al. (2007)

suggested that the root tissue was a barrier to Cd uptake and translocation within the rice plants. The uptake of Cd by plants varies not only among plant species but also among cultivars (Metwally et al. 2005; Grant et al. 2008). Li et al. (1997) reported significant variation in the grain Cd level of *Helianthus annuus*, *Triticum aestivum* and *Linum usitatissimum*. In Glycine max plants, about 98 % of the accumulated Cd is strongly retained by roots and only 2 % is transported to the shoot system (Cataldo et al. 1983). Rice plants absorb Cd from the rooting medium against the concentration gradient and the localization of absorbed Cd in rice is greater in roots than in shoots (Shah and Dubey 1997). The absorption of Cd by green microalgae, *Chlorella vulgaris*, *Ankistrodesmus braunii* and *Eremosphera viridis* shows that Cd is mainly absorbed in the cell wall. In *Eichhornia crassipes*, Cd was found to accumulate throughout the roots (Hosayama et al. 1994). Ammar et al. (2007) reported that Cd was found to be mainly accumulated in roots, but a severe inhibition of biomass production occurred in *Lycopersicon esculentum* leaves, even at its low concentration (1.0 μM).

Higher plants can uptake metals from the atmosphere through shoots and leaves, entering via roots and rhizomes from the soil (Lyubenova and Schröder 2010). Toxicity of metals within the plant occurs when metals move from soil to plant roots and get further transported and stored in various sites in the plant (Verma and Dubey 2003). The transfer of HMs from soils to plants depends primarily on the total amount of potentially available or the bioavailability of the metal (quantity factor), the activity as well as the ionic ratios of elements in soil solution (intensity factor), and rate of element transfer from solid to liquid phases and to plant roots (reaction kinetics) (Brümmer et al. 1986).

Most of the Cd accumulates in the vacuole within a cell. The fact that Cd is found in golgi apparatus and endoplasmic reticulum is apparently related to metal secretion through the cell surface and into the vacuole. A small quantity of Cd reaches nuclei, chloroplast, and mitochondria and exerts toxic effects on these organelles (Miller et al. 1973; Malik et al. 1992a, b). Localization of Cd appears to be maximum in roots than in other parts of the plant (Hart et al. 1998).

3 Cadmium Toxicity to Crop Plants

The most characteristic symptoms of Cd stress are brown and short roots, chlorosis, fewer tillers, senescence and reduced plant growth and biomass (Wu et al. 2003; Cosio et al. 2006). In *Elodea canadensis*, a thinner stem, less expanded leaves with partial bleaching of green tissues and 40 % internode shortening were observed in response to Cd treatments when compared with control plants (Vecchia et al. 2005). Roots of *Pisum sativum* plants were more sensitive to Cd toxicity than shoot (Metwally et al. 2005). Leaf expansion and root growth were inhibited significantly at high Cd concentrations in *Sedum alfredii*, and Cd was suggested to suppress cell expansion and induced senescence (Zhou and Qiu 2005). Ekmekci et al. (2008) reported that increasing Cd concentration significantly reduced the leaf and root

dry weight of *Zea mays* cultivars. Ghnaya et al. (2005) reported that Cd severely inhibited *Mesembryanthemum crystallinum* growth even at low concentration. Wahid et al. (2007) observed increased shoot Cd accumulation and leaf chlorosis with a concomitant reduction in shoot dry weight, leaf area, relative growth rate, net assimilation rate and relative leaf expansion rate in *Vigna radiata* seedlings under Cd stress. Kachout et al. (2009) showed that exposure of plants to different levels of metal (Cu, Ni, Pb, Zn) reduced the DM production and height of shoots in *Atriplex hortensis* and *A. rosea*. The decrease in root growth caused by toxicity of metals was more severe than the decrease in shoot growth. Atriplex plants exhibited gradual decline in height when exposed to following metal (a 4-week exposure of *A. hortensis* to 25, 50, 75, and 100 % contaminated soil). Kuriakose and Prasad (2008) reported that at concentrations above 3.0 mM Cd, seed germination of *Sorghum bicolor* was adversely affected with a complete cessation of seedling growth. Skorzynska-Polit and Baszynski (1997) reported that Cd-induced necrosis of leaf tissues might be the reason of Cd mobilization and its transport to plant parts above the ground. Cadmium has been shown to affect the photosynthetic functions through interacting with photosynthetic apparatus at various levels of organization and architecture, viz., accumulation of metal in leaf (main photosynthetic organ), partitioning in leaf tissues like stomata, mesophyll and bundle sheath cells, interaction with cytosolic enzymes and alteration of the functions of chloroplast membranes. Anjum et al. (2011) reported that mung been (*Vigna radiata*) seedlings treated with Cd (25, 50, and 100 mg $CdCl_2$ kg^{-1} soil) caused significant decrease in the dry weight and leaf area, photosynthetic parameters (net photosynthetic rate and chlorophyll content). The increasing level of Cd in soil resulted in a gradual decrease in plant dry weight, leaf area, photosynthesis, and chlorophyll content in both *V. radiata* genotypes. At 100 mg Cd kg^{-1} soil, the plant dry weight, leaf area, photosynthesis and chlorophyll content were reduced by 59.8, 39.8, 30.8, and 40.0 %, respectively in Cd-susceptible cv. PS 16 as compared to the control, but in Cd-tolerant cv. Pusa 9531, decreases in these parameters were 26.2, 23.0, 27.5, and 36.3 %, respectively. Farouk et al. (2011) observed that Cd at 100 and 150 mg kg^{-1} soil decreased the length, fresh and dry weights of shoot and root systems as well as leaf number per plant, significantly. Chlorophyll, total sugars, nitrogen, phosphorus, potassium, relative water content, water deficit percentage and soluble proteins as well as total amino acid contents were also decreased. John et al. reported that *Brassica juncea* plant exhibited a decline in growth, chlorophyll content and carotenoids with Cd and Pb but Cd was found to be more detrimental than Pb treatment in it. The protein content at the flowering stage decreased by 95 % due to Cd (900 μM) and by 44 % due to b (1500 μM). Cd has been shown to be the most effective inhibitor of photosynthetic activity (Bazzaz et al. 1974; Huang et al. 1974), particularly the oxygen-evolving reactions of photosystem II (Bazzaz and Govindjee 1974; Baszynski et al. 1980; Atal et al. 1991). With only a small amount of Cd in chloroplasts, many direct and indirect effects are observed, resulting in strong inhibition of photosynthesis. Most researchers connect the reduction of chlorophyll (Chl) in Cd-treated plants with inhibition of its biosynthesis. Stobart et al. (1985) established that Cd inhibited chlorophyll biosynthesis at two levels —in the synthesis of 5-aminolaevulinic acid

(ALA) and in the formation of photoactive protochlorophyllide reductase complex. Horvath et al. (1996) reported that the photoconversion of protochlorophyllide was not inhibited, but Cd disturbed Chl molecules' integration in stable complexes. On the other hand, Greger and Lindberg (1986) suggested that the lower Chl concentrations in plants were a result of the deficiency of Mg and Fe in the leaves of Cd-treated sugar beet plants. Rai et al. (2005) and Singh et al. (2008) reported decreased Cd and Chl content in *Phyllanthus amarus* and *Vigna mungo* plants with increasing Cd concentration, respectively. Ekmekci et al. (2008) reported that the increase in Cd concentration caused loss of Car in *Zea mays* cultivars. Collins et al. also reported decreased concentration of Car in *Arabidopsis* plants. Earlier investigations have demonstrated a marked reduction in the rate of photosynthesis by Cd in different plant species (Wojcik and Tukendorf 2005; Mobin and Khan 2007). It has been reported that Cd affects photosynthesis at various facets, such as Chl metabolism (Padmaja et al. 1990), functioning of photochemical reactions (Skorzynska and Baszynski 1995) and the activities of the Calvin cycle enzymes (Krupa 1999). Cadmium-induced reduction in the activity of ribulose 1,5 bisphosphate carboxylase (Rubisco) has been reported in *Hordeum vulgare* (Vassilev et al. 2005), *Cajanus cajan* (Sheoran et al. 1990a, b), *Triticum aestivum* (Malik et al. 1992a), *Pisum sativum* seedlings (Chugh and Sawhney 1999), *Zea mays* (Krantev et al. 2008) and *Brassica juncea* (Mobin and Khan 2007). Wahid et al. (2007) reported that Cd induced reduction in transpiration rate, stomatal conductance and net photosynthesis due to reduced CO_2 fixation by Rubisco in *Vigna radiata* plants. Stomatal closure to minimize water loss has been identified as an early event in plant response to Cd-induced water deficiency leading to limitations in carbon uptake by leaves (Barcelo et al. 1986a, b; Chaves 1991; Poschenreider et al. 1989). In addition, conductance and index of stomata, transpiration and net CO_2 uptake are greatly reduced with elevated Cd levels in the growth media (Bindhu and Bera 2001; Balakhnina et al. 2005). Cd interacts with the water balance (Costa and Morel 1994) and damages the photosystem apparatus, particularly in the photosystems I and II (Siedlecka and Krupa 1996). Mobin and Khan (2007) found that the Cd-induced decrease in net photosynthetic rate in non-tolerant cultivar (RH30) of *Brassica juncea* was accompanied by an increased transpiration rate and stomatal conductance but in tolerant cultivar (Varuna), it remained unaltered.

Photosynthesizing plants are naturally prone to oxidative stress because they have an array of photosensitizing pigments. These pigments produce and consume oxygen which can easily donate electrons to form ROS. It is reported that 1 % of the oxygen consumed by the plants is diverted to produce activated oxygen species like hydroxyl radical (OH), singlet oxygen ($^1O_2$) and superoxide radicals ($O_2^{\cdot -}$). Free radicals and other derivatives of oxygen are inevitable byproducts of biological redox reactions. Their production is considered to be a universal and common feature of living world under natural conditions as a byproduct of respiration and photosynthesis during electron transport systems of mitochondria and chloroplast. Their concentration increases under unfavorable conditions. Intracellular structures like membranes and biomolecules like proteins, enzymes, lipids and DNA have a high degree of organization that is at the risk of being destructed by these oxidative

radicals. The chlorophyll pigments associated with the electron transport system are the primary source of $^1O_2$. It may also arise as a byproduct of lipoxygenase activity and is highly destructive, reacting with most biological molecules at near diffusion-controlled rates. Superoxides, produced by the transport of electron to oxygen, are not compatible with metabolism and are required to be eliminated by the antioxidative defense system while recycling of phosphoglycolate to phosphoglycerate (re-enter the Bassam-Calvin cycle) results in a considerable loss of assimilated carbon.

Cadmium has been found to induce oxidative stress in plants (Liu et al. 2007; Djebali et al. 2008; Gill and Tuteja 2010; Gill et al. 2011), but in contrast with other heavy metals, such as Cu, it does not seem to act directly on the production of ROS through Fenton type reactions (Salin 1988). cadmium-exposed plants adopt the process of avoidance of the production of ROS as the first line of defense against oxidative stress. Once formed, ROS must be detoxified as efficiently as possible to minimize eventual damage. Thus, the detoxification mechanisms constitute the second line of defense against the detrimental effects of ROS (Moller 2001). In fact, compounds having the property of quenching the ROS without undergoing conversion to a destructive radical can be described as 'antioxidant'. Antioxidant enzymes are considered as those that either catalyse such reactions, or are involved in the direct processing of ROS (Medici et al. 2004). Hence, antioxidants (enzymatic and non-enzymatic) function to interrupt the cascades of uncontrolled oxidation (Noctor and Foyer 1998). Though the expression for antioxidant enzymes is altered under stress conditions, their upregulation has a key role in combating the abiotic stress-induced oxidative stress. However, the level of upregulation is subject to type and magnitude of the stress. Superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APOX), glutathione reductase (GR), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), guaiacol peroxidase (GOPX) and glutathione S-transferase (GST) showed great variations in their activities depending on the Cd concentration and the plant species used.

Plants exposed to heavy metal stress exhibited an increase in lipid peroxidation due to the generation of free radicals (Vanaja et al. 2000). Cadmium notably increased the accumulation of lipid peroxides in *Pisum sativum* (Metwally et al. 2005), *Oryza sativa* (Ahsan et al. 2007), *Helianthus annuus* (Groppa et al. 2001), *Arabidopsis* seedlings (Cho and Seo 2004), *Brassica juncea* (Mobin and Khan 2007), *Glycine max* (Noriega et al. 2007), *Lycopersicon esculentum* (Ammar et al. 2007), *Brassica napus* (Filek et al. 2008), *Vigna mungo* (Singh et al. 2008) and *Lepidium sativum* (Gill et al. 2011). The accumulation of H_2O_2 after Cd exposure has been detected in the leaf of different plant species such as *Pisum sativum* (Romero-Puertas et al. 2004), *Arabidopsis thaliana* (Cho and Seo 2005), *Brassica juncea* (Mobin and Khan 2007) and *Vigna mungo* (Singh et al. 2008). Balestrasse et al. (2006) also reported that Cd produced increased concentrations and in situ accumulation of H_2O_2 and $O_2^{\cdot -}$ in soybean leaves. Guo et al. (2007) reported that exposure to 50 mM Cd significantly increased the H_2O_2 content in the roots of *Oryza sativa*. It has also been reported that Cd increased the accumulation of H_2O_2 in soybean root tips (Yang et al. 2007).

4 Tolerance Mechanism of Plant against Cd

Plants have evolved a complex array of mechanisms to maintain optimal metal levels and avoid the detrimental effects of excessively high concentrations (Clemens 2001). When these homeostatic mechanisms are overwhelmed, plants suffer metal-induced damage and pro-oxidant conditions within cells. However, higher plants are very well equipped with antioxidant mechanisms (Mittler et al. 2004; Gill and Tuteja 2010). Plant cells display an antioxidant network, including numerous soluble and membrane compounds, particularly in mitochondria and in chloroplasts where respiratory and photosynthetic electron transfer chainstake place, respectively. Antioxidant enzymes are considered as those that either catalyze such reactions, or are involved in the direct processing of ROS (Gill and Tuteja 2010). Plants possess very efficient enzymatic (SOD; CAT; APX; GR; MDHAR; DHAR; GOPX and GST) and non-enzymatic (AsA; GSH; phenolic compounds, alkaloids, non-protein amino acids and α-tocopherols) antioxidant defense systems.

Ascorbate peroxidase (EC 1.11.1.11) is a heme protein, and its primary function is the rapid removal of H_2O_2 at the site of generation (Asada 1992). APX isozymes are distributed in at least four distinct cell compartments, the stroma (sAPX), thylakoid membrane (tAPX), the mitochondria (mAPX), and the cytosol (cAPX) (Ishikawa et al. 1998; Asada 1992). The various isoforms of APX respond differentially to metabolic and environmental signals (Kuboi et al. 1987). Thylakoid membrane-bound APX is a limiting factor of antioxidant systems under photoxidative stress in chloroplasts and the enhanced tAPX activity maintains the redox status of ascorbate under stress conditions (Yabuta et al. 2002). Chloroplasts contain APX in two isoforms, thylakoid-bound and soluble stromal enzymes. At least one-half of the chloroplastic APX is tAPX, but the ratio of tAPX/sAPX varies according to the plant species, possibly, leaf age, but the biosynthetic ratio of the two APXs is controlled by alternative splicing (Asada 2006). The tAPX binds with the stroma thylakoids where the PSI complex is located, while sAPX is thought to be localized in the stroma (Asada 2006). Plants also contain the cytosolic isoforms of APX (cAPX), which has a different amino acid sequence in comparison to chloroplastic APXs, but participate in the scavenging of H_2O_2 in compartments other than chloroplasts. The cAPX is a homodimer and its electron donor is not so specific for ascorbate, unlike tAPX and sAPX (Asada 2006).

Ascorbate peroxidase has an important role in the scavenging of H_2O_2 under stressed conditions but its activity depends on the Cd concentration applied. Increased leaf APX activity under Cd stress has been reported in *Ceratophyllum demersum* (Arvind and Prasad 2003), *Brassica juncea* (Mobin and Khan 2007), *Pisum sativum* (Romero-Puertas et al. 1999), *Phaseolus aureus* (Shaw 1995), *Phaseolus vulgaris* (Chaoui et al. 1997b), *Zea mays* (Krantev et al. 2008), *Triticum aestivum* (Khan et al. 2007), *Vigna mungo* (Singh et al. 2008) and *Brassica campestris* (Anjum et al. 2008), however, in *Hordeum vulgare* roots the APX activity was reduced at high concentration of Cd (Hegedus et al. 2001). Balestrasse et al. (2001) reported that low Cd levels led to an increased APX activity in *Glycine max* roots and nod-

ules, but the activity decreased with high Cd concentration. The lower APX activity was also noted in *Cucumis sativus* chloroplasts with increasing Cd concentration (Zhang et al. 2003), whereas Cd-induced inhibition of APX activity was also observed in clonal, hydroponically grown *Populus* × *Canescens* (Schutzendubel et al. 2002) and *Helianthus annuus* plants (Gallego et al. 1996a). APX activity in *Ceratophyllum demersum* showed a very high increase in Cd + Zn treated plants as compared to Cd or Zn alone, indicating efficient antioxidant and ROS scavenging activities by Zn against Cd-induced free radicals and oxidative stress (Aravind and Prasad 2003). Khan et al. (2007) also reported increased APX activity in *Triticum aestivum* plants treated with Cd under low Zn levels.

Catalase (EC 1.11.1.6) is a heme-containing enzyme that catalyzes the dismutation of hydrogen peroxide into water and oxygen (Frugoli et al. 1996). Present in all aerobic eukaryotes, this is important in the removal of hydrogen peroxide generated in peroxisomes (microbodies) by oxidases involved in β-oxidation of fatty acids, the glyoxylate cycle (photorespiration) and purine catabolism. CAT is one of the first enzymes isolated in a highly purified state. The isozymes of catalase have been studied extensively in higher plants (Polidoros and Scandalios 1999). Scandalios et al. (2000) characterized three genetically-distinct CAT isozymes in maize plants. All forms of the enzyme are tetramers in excess of 220,000 molecular weight. Multiple forms of catalase have been described in many plants. Maize has three isoforms termed as *CAT 1*, *CAT 2* and *CAT 3*, which are found on separate chromosomes and are differentially expressed and independently regulated (Scandalios 1990). *CAT 1* and *CAT 2* are localised in peroxisomes and the cytosol, whereas CAT 3 is mitochondrial. Plants contain multiple CAT isozymes, e.g., 2 in *Hordeum vulgare* (Azevedo et al. 1998), 4 in *Helianthus annuus* cotyledons (Azpilicueta et al. 2007) and as many as 12 isozymes in mustard (Frugoli et al. 1996). CAT isozymes have been shown to be regulated temporally and spatially and may respond differentially to light (Willekens et al. 1994, Skadsen et al. 1995).

The variable response of CAT activity has been observed under Cd stress. CAT activity declined in *Helianthus annuus* leaves (Gallego et al. 1996b), *Phaseolus vulgaris* (Chaoui et al. 1997b), *Phaseolus aureus* (Shaw 1995), *Pisum sativum* (Dalurzo et al. 1997), *Lemna minor* (Mohan and Hossetti 1997), *Amaranthus lividus* (Bhattacharjee 1998), *Glycine max* roots (Balestrasse et al. 2001), *Phragmites australis* (Iannelli et al. 2002), *Capsicum annuum* (Leon et al. 2002) and *Arabidopsis thaliana* (Cho and Seo 2005) under Cd stress conditions. A significant decline in CAT activity was reported after 50 μM Cd applications for 48 hr in the roots and shoots of *Bacopa monnieri* (Singh et al. 2006). However, CAT activity increased in *Agropyronrepens* (Brej 1998), *Helianthus annus* (Gallego et al. 1999), *Glycine max* nodules (Balestrasse et al. 2001), *Oryza sativa* leaves (Hsu and Kao 2004), in tolerant varieties of *Solanum tuberosum* (Stroinski and Kozlowska 1997), in roots of *Raphanus sativus* seedlings (Vitoria et al. 2001), *Brassica juncea* (Mobin and Khan 2007), *Triticum aestivum* (Khan et al. 2007), *Vigna mungo* roots (Singh et al. 2008) and *Cicer arietinum* (Hasan et al. 2008). Azpilicueta et al. (2007) reported that incubation of *Helianthus annuus* leaf discs with 300 and 500 μM $CdCl_2$ under light conditions increased *CATA3* transcript level but this transcript was not induced by Cd

in etiolated plants. Moreover, in roots of the transgenic CAT-deficient tobacco lines (*CAT 1AS*), the DNA damage induced by Cd was higher than in wild type tobacco (*SR 1*) roots (Gichner et al. 2004). Furthermore, CAT activity remained unaltered under Cd stress in *Glycine max* leaves (Ferreira et al. 2002).

Glutathione reductase (EC 1.6.4.2) is a flavo-protein oxidoreductase, found in both prokaryotes and eukaryotes (Mullineaux and Rausch 2005; Romero-puertas et al. 2006). This enzyme was first reported in eukaryotes and yeast (Meldrum and Tarr 1935) as well as in plants (Conn and Vennesland 1951; Mapson and Goddord 1951). Glutathione reductase maintains the balance between reduced glutathione (GSH) and ascorbate pools, which in turn maintain cellular redox state (Lascano et al. 1999 2001; Reddy and Raghavendra 2006; Romero-Puertas et al. 2006; Ansel et al. 2006; Chalapathi Rao and Reddy 2008). The enzyme protein, although synthesized in the cytoplasm, can be targeted to both chloroplast and mitochondria (Mullineaux and Rausch 2005). In higher plants, GR is involved in defense against oxidative stress, whereas GSH plays an important role within the cell system, which includes participation in the ascorbate-glutathione cycle, maintenance of the sulphhydryl (-SH) group and a substrate for glutathione-S-transferases (Noctor et al. 2002; Reddy and Raghavendra 2006). GR and GSH play a crucial role in determining the tolerance of a plant during environmental stresses (Chalapathi Rao and Reddy 2008). In almost all the biological functions, GSH is oxidized to GSSG which should be converted back to GSH in plant cell to perform normal physiological functions. Hence, rapid recycling of GSH is more essential rather than synthesis of GSH, which is a highly regulated and ATP requiring process. GR activity increases as part of the defense against Cd-exposure, which is dose-dependent and variable over time (Fornazier et al. 2002a). The GR activity increased in the presence of Cd in *Phaseolus vulgaris* (Chaoui et al. 1997a), *Solanum tuberosum* (Stroinski and Zielezinska 1997), *Raphanus sativus* (Vitoria et al. 2001), *Crotolaria juncea* (Pereira et al. 2002), *Glycine max* (Ferreira et al. 2002), *Saccharum officinarum* (Fornazier et al. 2002b), *Capsicum annuum* (Leon et al. 2002), *Arabidopsis thaliana* (Skorzynska et al. 2003/2004), *Vigna mungo* (Singh et al. 2008), *Triticum aestivum* (Khan et al. 2007) and *Brassica juncea* (Mobin and Khan 2007). In *Raphanus sativus,* it exhibited very little variation in the roots and leaves of control plants, indicating a direct correlation with Cd accumulation (Vitoria et al. 2001). In *Pisum sativum*, GR activity was enhanced more with 40 μM than with 4 μM Cd (Dixit et al. 2001). However, a decrease in GR activity after application of Cd has also been reported for a few plant species such as *Helianthus annuus* (Gallego et al. 1996a, b), *Pisum sativum* (Dalurzo et al. 1997) and *Solanum tuberosum* (Stroinski and Kozlowska 1997).

Glutathione S-transferases (EC 2.5.1.18) catalyze the conjugation of tripeptide GSH into a variety of hydrophobic, electrophylic and cytotoxic substrates (Marrs 1996). Noctor et al. (2002) observed that GSTs can remove genotoxic or cytotoxic compounds that have potential to damage or react with genetic material (DNAs and RNAs) and protein. In fact, glutathione-S-transferases can reduce peroxides with the help of GSH and produce scavengers of cytotoxic and genotoxic compounds. An increased GST activity was found in leaves and roots of Cd-exposed *Pisum sa-*

tivum plants by Dixit et al. (2001) and in roots of *Oryza sativa* and *Phragmites australis* plants (Moons 2003; Iannelli et al. 2002). H_2O_2 can be metabolized by another plant peroxidase-scavenging enzyme called glutathione peroxidase (EC 1.11.1.9) (Noctor et al. 2002). Millar et al. (2003) identified a family of seven related proteins in cytosol, chloroplast, mitochondria and endoplasmic reticulum, named AtGPX1-AtGPX7 in *Arabidopsis*. Stress increases GPX activity in cultivars of *Capsicum annuum* plants (Leon et al. 2002) but decreases in roots and causes no significant change in the leaves of Cd-exposed *Pisum sativum* plants (Dixit et al. 2001).

Superoxide dismutase (EC 1.15.1.1) was first isolated by Mann and Kleilin (1938) and was thought to be a copper-storage protein. Subsequently, it was identified by different names, erythrocuprein, indophenol oxidase, and tetrazolium oxidase until its catalytic function was discovered by McCord and Fridovich (1969). SOD catalyzes the disproportionate superoxide $O_2^{\cdot -}$ to hydrogen peroxide and molecular oxygen. It removes $O_2^{\cdot -}$ and hence decreases the risk of hydroxyl radical formation from $O_2^{\cdot -}$ via the metal catalyzed Haber-Weiss-type reaction. There are three distinct types of SOD isozymes, classified on the basis of the metal cofactor the copper/zinc (Cu/Zn-SOD), the manganese (Mn-SOD) and the iron (Fe-SOD), that have been reported in various plant species (Bannister et al. 1987; Alscher et al. 2002). These isozymes can be separated by native polyacrylamide gel electrophoresis. Their activity is detected by negative staining and identified on the basis of their sensitivity to KCN and H_2O_2. The Mn-SOD is resistant to both inhibitors, Cu/Zn-SOD is sensitive to both inhibitors, whereas Fe-SOD is resistant to KCN and sensitive to H_2O_2. The subcellular distribution of these isozymes is also distinctive. The Mn-SOD is found in the mitochondria of eukaryotic cells and in peroxisomes (del Rio et al. 2003); some Cu/Zn-SOD isozymes are found in the cytosolic fractions, and also in chloroplasts of higher plants (del Rio et al. 2002). The Fe-SOD isozymes, often not detected in plants (Ferreira et al. 2002) are usually associated with the chloroplast compartment when present (Bowler et al. 1992; Alscher et al. 2002). The prokaryotic Mn-SOD and Fe-SOD, and the eukaryotic Cu/Zn-SOD enzymes are dimers, whereas Mn-SOD of mitochondria is tetramers. Peroxisomes and glyoxysomes of watermelons (*Citrillus vulgaris*) have been shown to contain both Cu/Zn- and Mn-SOD activity, but there are no reports of extracellular SOD enzymes in plants. All forms of SOD are nuclear-encoded and targeted to their respective subcellular compartments by an amino terminal targeting sequence. Several forms of SOD have been cloned from a variety of plants (Bowler et al. 1992). The response of SOD to heavy metal stress varies considerably depending upon plant species, stage of the plant development, metal in the experiment and the exposure time. SOD activity in leaves exhibited increases in activity in response to Cd, whereas there was no significant variation in its activity in roots (Vitoria et al. 2001). Activity staining for SOD in *Glycine max* revealed seven isozymes in leaves and eight in roots, corresponding to Mn-SOD and Cu/Zn SOD isozymes. Although a clear effect of Cd on plant growth was observed, the activities of the SOD isozymes were unaltered (Ferreira et al. 2002). In *Saccharum officinarum* seedlings, several isozymes have been observed, but growth in the presence of Cd did not result in any significant alteration in SOD activity (Fornazier et al. 2002). In pea plants, a

strong reduction in chloroplastic and cytosolic Cu/Zn SODs by Cd was reported and to a lesser extent for Fe-SOD, while Mn-SOD was affected only by the highest Cd concentration tested. This showed that Mn-SOD was the isozyme more resistant to Cd (Sandalio et al. 2001). In pea leaf peroxisomes, the Mn-SOD activity did not change in response to Cd treatment (Romero-Puertas et al. 1999). On the contrary, increases in total SOD activity were detected following the application of Cd in *Pisum sativum* (Dalurzo et al. 1997), *Solanum tuberosum* (Stroinski and Kozlowska 1997), *Hordeum vulgare* (Guo et al. 2004), *Arabidopsis thaliana* (Skorzynska-Polit et al. 2003/2004), *Oryza sativa* (Hsu and Kao 2004), *Triticum aestivum* (Khan et al. 2007), *Zea mays* (Krantev et al. 2008), *Brassica juncea* (Mobin and Khan 2007), *Vigna mungo* (Singh et al. 2008), *Cicer arietinum* (Hasan et al. 2008) and hyperaccumulator plants of the genus *Alyssum* (Schickler and Caspi 1999). SOD activity remained unaltered in *Helianthus annuus* (Gallego et al. 1996b, 1999) and declined in *Amaranthus lividus* (Bhattacharjee 1998), *Phragmites australis* (Iannelli et al. 2002), *Capsicum annuum* plants (Leon et al. 2002), *Glycine max* (Noriega et al. 2007) under Cd stress. In clonal, hydroponically grown poplar plants (*Populus* × *Canescens*, a hybrid of *Populus termula* × *Populus alba*) (Schutzendubel et al. 2002) and *Arabidopsis lividus* (Bhattacharjee 1998) exposed to Cd resulted in inhibition of SOD activity. Romero-Puertas et al. (2004) studied the involvement of H_2O_2 and $O_2^{\cdot -}$ in the signaling events that lead to the variation of the transcript levels of Cu/Zn-SOD in *Pisum sativum* plants under Cd stress.

Monodehydroascorbate reductase (EC 1.6.5.4) is a flavin adenin dinucleotide (FAD) enzyme that is present as chloroplastic and cytosolic isozymes which share similar properties. MDHAR exhibits a high specificity for monodehydro ascorbate (MDHA) as the electron acceptor, preferring NADH rather than NADPH as the electron donor. Asada (1999) studied the multi-step reduction of FAD in detail. The first step is the reduction of the enzyme-FAD to form a charge transfer complex. The reduced enzyme donates electrons successively to MDHA, producing two molecules of ascorbate via a semiquinone form [E-FAD-NADP(P)$^+$]. The disproportionation by photoreduced ferrodoxin (redFd) in the thylakoids is of great importance. Since redFd (reduced ferrodoxin) can reduce MDHA more effectively than $NADP^+$; MDHAR cannot participate in the reduction of MDHA in the thylakoidal scavenging system. Therefore, MDHAR would function at a site where NAD(P)H is available, but redFd is not. MDHAR is also located in peroxisomes and mitochondria accompanying APX. MDHAR could remove and scavenge H_2O_2 in these organelles similar to that in chloroplasts, which has escaped from breakdown by peroxisomal CAT (del Rio et al. 2002). Schutzendubel et al. (2001) have noted enhanced MDHAR activity in Cd-exposed *Pinus sylvestris* and a declined MDHAR activity in Cd-exposed poplar hybrids (*Populus*× *canescens*).

The tripeptide (γ-GluCysGly) glutathione (GSH) plays a central role in several physiological processes, including regulation of sulfate transport, signal transduction, conjugation of metabolites, detoxification of xenobiotics (Xiang et al. 2001) and the expression of stress-responsive genes (Mullineaux and Rausch 2005). The reduced form of glutathione, GSH, is an abundant compound in plant tissue that exists interchangeably with the oxidized form, GSSG. GSH is abundant (3–10 mM)

in cytoplasm, nuclei and mitochondria and is the major soluble antioxidant in these cell compartments. GSH has been associated with several growth and development related events in plants, including cell differentiation, cell death and senescence, pathogen resistance and enzymatic regulation (Ogawa 2005; Rausch and Wachter 2005) and its content is affected by S nutrition (Blake-Kalff et al. 2000). GSH is the major reservoir of non-protein sulphur. It is the major redox buffer in most aerobic cells, and plays an important role in physiological functions, including redox regulation, conjugation of metabolites, detoxification of xenobiotics and homeostasis and cellular signaling that triggers adaptive responses (Noctor et al. 2002; Kopriva and Koprivova 2005). It also plays an indirect role in protecting membranes by maintaining α-tocopherol and zeaxanthin in the reduced state. It can also function directly as a free radical scavenger by reacting with superoxide, singlet oxygen and hydroxyl radicals. GSH protects proteins against denaturation caused by the oxidation of protein thiol groups under stress. In addition, GSH is a substrate for glutathione peroxidase (GPX) and glutathione-S-transferases (GST), which are also involved in the removal of ROS (Noctor et al. 2002). GSH is a precursor of PCs, which are crucial in controlling cellular heavy metal concentrations. GSH and its oxidized form, GSSG maintains a redox balance in the cellular compartments. This property of glutathione is of great biological importance since it allows fine-tuning of the cellular redox environment under normal conditions and upon onset of stress, and provides the basis for GSH stress signaling. A central nucleophilic cysteine (Cys) residue is responsible for higher reductive potential of GSH. It scavenges cytotoxic H_2O_2, and reacts non-enzymatically with other ROS, i.e., $O_2^{\cdot -}$, $OH^{\cdot}$ and $^1O_2$ (Larson 1988). The central role of GSH in the antioxidative defense system is due to its ability to regenerate another water soluble antioxidant, ascorbate, in ascorbate-glutathione cycle (Foyer and Halliwell 1976; Noctor and Foyer 1998). The role of GSH in the antioxidant defense system provides a strong basis for its use as a stress marker. However, the concentration of cellular GSH has a major effect on its antioxidant function and it varies considerably under Cd stress. Furthermore, strong evidence has indicated that an elevated GSH concentration is correlated with the ability of plants to withstand metal-induced oxidative stress (Freeman et al. 2004). Xiang et al. (2001) observed that plants with low levels of glutathione were highly sensitive to even low levels of Cd^{2+} due to limited PC synthesis. The increased demand for GSH can be met by the activation of pathways involved in sulphur assimilation and cysteine biosynthesis. Its concentration is controlled by a complex homeostatic mechanism where the availability of sulphur seems to be required (May et al. 1998). Manipulation of GSH biosynthesis increases resistance to oxidative stress (Youssefian et al. 2001; Sirko et al. 2004). It has been observed that upon Cd exposure, one of the main responses observed was the induction of genes involved in sulphur assimilation–reduction and glutathione metabolism in the roots of *Arabidopsis* (Herbette et al. 2006).

Feedback inhibition of γ-glutamylcysteine synthase (γ-ECS) by GSH has been considered as a fundamental central point for GSH synthesis. In vitro studies with the enzymes from tobacco and parsley cells showed that the plant γ-ECS was inhibited by GSH (Noctor and Foyer 1998). Oxidation of GSH to GSSG decreases

GSH levels and allows increased γ-ECS activity under stressed conditions (Noctor and Foyer 1998).

Environmental stresses trigger an increase in ROS levels in plants and the response of glutathione can be crucial for adaptive responses. Antioxidant activity in the leaves and chloroplast of *Phragmites australis* Trin. (cav.) ex Steudel was associated with a large pool of GSH, protecting the activity of many photosynthetic enzymes against the thiophilic bursting of Cd exerting a direct important protective role in the presence of Cd (Pietrini et al. 2003). Increased concentration of GSH has been observed with the increasing Cd concentration in *Pisum sativum* (Gupta et al. 2002), romaine lettuce (Maier et al. 2003), *Phragmites australis* (Pietrini et al. 2003), *Brassica juncea* (Qadir et al. 2004), *Pisum sativum* (Metwally et al. 2005), *Sedum alfredii* (Sun et al. 2007), *Oryza sativa* (Hassan et al. 2008). However, decay in GSH content in *Glycine max* roots (Balestrasse et al. 2001), *Helianthus annuus* leaves (Gallego et al. 1996b), *Zea mays* seedlings (Rauser 1990), *Pisum sativum* (Ruegsegger et al. 1990), *Pinus sylvestris* roots (Schutzendubel et al. 2001), *Cucumis sativus* chloroplast (Zhang et al. 2003), *Populus* × *Canescens* roots (Schutzendubel et al. 2002) and *Oryza sativa* leaves (Hsu and Kao 2004) has been reported under Cd stress. Furthermore, unaltered GSH content was observed in the nodules of *Glycine max* (Balestrasse et al. 2001). Cadmium-induced depletion of GSH has been mainly attributed to phytochelatin synthesis (Grill et al. 1985). PC-heavy metal complexes have been reported to be accumulated in the vacuole of tobacco leaves and in *Avena sativa*. These complexes have been shown to be transported across the tonoplast (Salt and Rauser 1995). The decline in the levels of GSH might also be attributed to an increased utilization for ascorbate synthesis or for a direct interaction with Cd (Pietrini et al. 2003). The variety of response to Cd-induced oxidative stress is probably related not only to the levels of Cd supplied, but also to the plant species, the age of the plant and duration of the treatment.

All plants can synthesize ascorbate, which can accumulate to millimolar concentrations in both photosynthetic and non-photosynthetic tissues (Foyer et al. 1983). Ascorbate is one of the most powerful antioxidants (Noctor and Foyer 1998; Smirnoff et al. 2001), which reacts directly with hydroxyl radicals, superoxide and singlet oxygen, and reduces H_2O_2 to water *via* ascorbate peroxide reaction (Noctor and Foyer 1998). Ascorbate also acts as an electron donor in the regeneration of α-tocopherol. Under physiological conditions, it exists mostly in reduced form in leaves and chloroplast and its intracellular concentration can build up to millimolar range (*viz*. 20 mM in the cytosol and 20–300 mM in the chloroplast stroma) (Foyer and Lelandais 1996). The ability to donate electrons in a wide range of enzymatic and non-enzymatic reactions makes ascorbate the main ROS-detoxifying compound in the aqueous phase. In addition to the importance of ascorbate in the ascorbate-glutathione cycle, it plays a role in preserving the activities of enzymes that contain prosthetic transition metal ions (Noctor and Foyer 1998). The ascorbate redox system consists of L-ascorbic acid, MDHA and DHA. Both oxidized forms of ascorbate are relatively unstable in aqueous environments while DHA can be chemically reduced by GSH to ascorbate (Foyer and Halliwell 1976). Evidence to support the actual role of DHAR, GSH and GR in maintaining the foliar

ascorbate pool has been observed in transformed plants overexpressing GR (Foyer et al. 1995). *Nicotiana tabacum* and *Populus* × *Canescens* plants have higher foliar ascorbate contents and improved tolerance to oxidative stress (Aono et al. 1993; Foyer et al. 1995). Demirevska-Kepova et al. (2006) reported that the content of oxidized ascorbate increased during Cd exposure in *Hordeum vulgare* plants. A decrease in the ascorbate content in the roots and nodules of *Glycine max* under Cd stress has been observed (Balestrasse et al. 2001). Cadmium also decreases ascorbate content in *Cucumis sativus* chloroplast and in the leaves of *Arabidopsis thaliana*, *Pisum sativum* and *Brassica campestris* (Zhang et al. 2003; Skorzynska-Polit et al. 2003/04; Romero-Puertas et al.2007; Anjum et al. 2008), respectively, whereas it remained unaffected in *Populus* × *Canescens* roots (Schutzendubel et al. 2002).

5 Conclusion

Literature is full with the reports to counteract the inhibitory effects of Cd in crop plants but we still need sound information to understand the plant responses to Cd toxicity at genomic level to know more about the structural and functional alterations under Cd stress in crop plants. Cadmium toxicity in plants is observed at whole plant as well as at cellular and molecular levels, the important of which include perturbation of metabolic pathways such as photosynthesis, energy transduction, protein synthesis and nutritional disorders, etc. Plants adopt various mechanisms to counteract the inhibitory effects of Cd toxicity by synthesizing metal binding peptides and/or altering the activity of the components of antioxidant machinery. Strategies should aim at manipulating steps of antioxidant defense pathways, thiol production by overexpression of the enzymes of the pathway. Genetic engineering of PC biosynthesis pathway can also be a target to overcome the Cd toxicity as well as the signal pathways through which Cd toxicity leads to gene regulation, are also important to look into.

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